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About the Cover:

The intertwining mechanical gears symbolize a close relationship between neuron and glia during the development of the nervous system, whereas different model organisms employed are illustrated in the vicinity. This special issue presents a collection of articles that discuss molecules, signaling pathways, and mechanisms that mediate neurodevelopment, giving insight on their roles during destabilizing state such as neurodegeneration.

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·**Editorial**·

Neurodevelopment and degeneration

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Over the past decades, continuous effort has been made to resolve unsettled matters in the emerging scene of neuronal development. Despite the rapid progress, neuroscientists remain fascinated by the intricate networks of signaling pathways and molecules conserved among species. Fundamental questions such as progenitor cell specification, compartmentalized cellular tactics, and the identification of factors involved in orchestrating the progression of development are being actively pursued. Furthermore, reversing the effects of development, neurodegeneration symbolizes an opposite force in limiting growth and has been linked to a variety of diseases. To date, a tremendous amount of work from the research community has conceptualized the central image of how nervous systems develop and are equipped to modulate animal functions, albeit with mysterious gaps in the picture that remain to be filled.

In this issue, we have compiled a set of articles that cover topics ranging from neural stem cell (NSC) specification and differential axon/dendrite growth, to signaling molecules that participate in neuronal morphogenesis, synaptic refinement, and behavioral function. Our issue aims to discuss recent progress in the fundamental principles regulating neurodevelopment and degeneration, with the exploitation of model organisms such as *Drosophila* and *Caenorhabditis elegans*. Below, I highlight these contributions in subcategories, to provide our readers with a comprehensive overview.

Basic Unit Assembly

As the recognized building block of the nervous system, the neuron is compartmentalized into axons and dendrites that mediate information processing. Another major cell type, glia, though they do not exhibit major polarized features, play significant roles in regulating neuronal development and function. The origin of these cells and how they are generated from NSCs have long been an active area of research. Song *et al.* describe the development of adult hippocampal NSCs and their progeny[1]. These NSCs exhibit distinct features during different developmental stages. Understanding the mechanism of their specification, development, and regulation provides cues to their therapeutic potential in regard to neuronal regeneration and degeneration. In addition, activity-dependent mechanisms and the required elements from the niche microenvironment for regulating the development of these NSCs are illustrated[1]. Regulatory factors such as neurotransmitters, morphogens, and transcription factors are thoroughly discussed[1].

Once specified and polarized, neuronal compartments such as axons and dendrites begin to undergo differentially-regulated growth. Ye *et al.*, using both mammals and flies as models, discuss the underlying mechanisms regulating axon and dendrite growth[2]. Two models, dedicated and bimodal mechanisms, have been proposed and are reviewed. Whereas the dedicated regulators like BMP7 or Rac1 affect only the growth of either axons or dendrites, bimodal regulators like Sema3A execute binary functions that promote axon or dendrite growth while inhibiting the other[2]. Accompanying growth, differentiated axons exhibit guidance properties that Liu *et al.* review in great detail[3]. A subject of interest for many years, axon guidance is crucial for neuronal function and the microtubule dynamics at the growth cone of axons has been considered as a classical paradigm for understanding cell motility and dynamics[3].

In addition to neurons, a plethora of evidence has suggested that glia play pivotal roles during

neurodevelopment, synaptic function, and plasticity. Ho *et al.* explore glial functions in *Drosophila*, summarizing recent research advances in this particular field[4].

At the Synapse

The proper organization and assembly of a functional synapse is important for neuronal function throughout developmental stages. Wang *et al.* provide experimental evidence that surface-located GluN2A-containing NMDARs, but not those containing GluN2B, cluster at the synaptic site and the clustering is mediated by the carboxyl-terminus[5]. Furthermore, GluN2A-containing NMDARs preferentially associate with PSD-95, suggesting a pivotal role for the synaptic localization of NMDARs during neuronal development and function[5].

Throughout development, synapses undergo dynamic changes including growth-opposing actions like elimination in order to become truly functional. Caspases, initially recognized for their apoptotic roles during neurodegeneration, have emerged as important regulators of synaptic refinement and elimination. In the Perspective, Luo *et al.* discuss the new theme regarding the functions of caspase-3 in mammalian neuromuscular junction and the central nervous system[6]. In both cases, caspase-3 participates in synaptic refinement *via* controlling the ACh cluster dispersion or AMPA receptor internalization, leading to spine elimination[6].

Drug-induced animal behavior has been frequently used to investigate the mechanism that directly links to a synaptic component, providing further implications on how a synapse is developed and structured for function. Li *et al.* thoroughly review the utility of the animal model *C. elegans* to study the underlying mechanism of alcohol addiction[7]. A nice summary of synaptic factors such as BK channels, receptors and neurotransmitters, and the lipid microenvironment is provided in this review and the correlation between drug-induced behavior and synaptic development is also discussed[7].

Epigenetic and Transcriptional Regulation

Identifying the factors involved in neurodevelopment and neurodegeneration has been a continuous task in the field. Cheng *et al.* summarize the function of one such factor, methyl-CpG-binding protein 2 (MeCP2), during neurodevelopment[8]. A classic methylated-DNA binding protein, MeCP2 represses transcription, participates in nuclear microRNA processing, and has been implicated in various neurodevelopmental disorders such as Rett syndrome and autism spectrum disorder[8]. Post-translational modifications of MeCP2 itself have also provided extra layers of complexity in regulating MeCP2 function during neurodevelopment[8].

Doxakis then discusses a set of RNA-binding proteins (RBPs) essential for brain development and function[9]. RBPs participate in the mechanism of pre-mRNA splicing to produce diversity, local mRNA translation to provide control over protein expression, and fine-tuning mRNA translation by alternative polyadenylation[9]. Intriguingly, RBPs have been implicated in a number of neurodegenerative diseases, based upon studies of their localization patterns and mutagenic analysis.

Neurodegeneration: Action and Response

Nervous systems evolve self-defense mechanisms to protect the integrity of the cellular environment to act and function properly. To face dangerous situations such as neurodegeneration, explicit control over networks of proteins is required for strategic planning and problem-solving. Sealing of the axolemmal membrane upon mechanical trauma serves as one good example. Shi *et al.* provide a thorough review on the membrane-sealing mechanisms upon injury, providing insights into how neuronal membranes react to injury and self-heal[10]. In this article, two types of models of membrane integrity are discussed: the line tension and the membrane tension mechanisms. In a different scenario, Hsueh *et al.* discuss how neurons execute the innate immunity program mediated by the Toll-like receptors and their adaptor proteins like Sarm1 upon foreign pathogenic attack[11]. These signaling molecules regulate neuronal morphology and function in the absence of an immune challenge, and also play significant roles during neurodegeneration.

This special issue on “Neurodevelopment and Degeneration” presents a collection of articles that cover the signaling mechanisms for cell specification, differential growth, and information delivery *via* synaptic organization. It is not surprising that these mechanisms also contribute to

disease or injury states like neurodegeneration. We hope that the topics covered here will bring readers insights on the fundamental principles underlying neurodevelopment, and at the same time ignite the passion of our fellow scientists to address the intriguing questions that remain mysterious in the field.

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·Review·

Activity-dependent signaling mechanisms regulating adult hippocampal neural stem cells and their progeny

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Adult neural stem cells (NSCs) reside in a restricted microenvironment, where their development is controlled by subtle and presently underexplored cues. This raises a significant question: what instructions must be provided by this supporting niche to regulate NSC development and functions? Signaling from the niche is proposed to control many aspects of NSC behavior, including balancing the quiescence and proliferation of NSCs, determining the cell division mode (symmetric *versus* asymmetric), and preventing premature depletion of stem cells to maintain neurogenesis throughout life. Interactions between neurogenic niches and NSCs also govern the homeostatic regulation of adult neurogenesis under diverse physiological, environmental, and pathological conditions. An important implication from revisiting many previously-identified regulatory factors is that most of them (e.g., the antidepressant fluoxetine and exercise) affect gross neurogenesis by acting downstream of NSCs at the level of intermediate progenitors and neuroblasts, while leaving the NSC pool unaffected. Therefore, it is critically important to address how various niche components, signaling pathways, and environmental stimuli differentially regulate distinct stages of adult neurogenesis.

Keywords: neural stem cell; neuronal development; neuronal plasticity

Introduction

Neurogenesis occurs throughout life in discrete regions of the mammalian brain and substantial evidence supports critical roles of adult-born neurons for specific brain functions, such as learning, memory, and olfactory processing[1-3]. It is widely accepted that there are two primary neurogenic regions in the adult brain: the olfactory bulb where newborn neurons arise from the subventricular zone (SVZ) of the lateral ventricles, and the dentate granule cell layer of the hippocampus where newborn neurons are generated locally within the subgranular zone (SGZ). The origin of the new neurons is from a resident population of adult neural stem cells (NSCs)[4-7]. Although NSCs are also known to arise from other adult brain regions under pathological conditions and with injuries[8], it remains controversial whether active neurogenesis normally occurs outside of the SVZ and SGZ.

The adult mammalian brain is a plastic structure, capable of dynamic cellular and molecular remodeling in response to various environmental stimuli and pathological conditions. The adult hippocampus is a primary neuronal structure involved in memory formation and synaptic plasticity. Within the hippocampus, circuit dynamics in the dentate gyrus (DG) is facilitated by continuously generating new neurons throughout life. Adult hippocampal neurogenesis has attracted much interest because newborn neurons have been suggested to adapt the brain to various behavioral tasks, including spatial learning

and retention, pattern discrimination, and the clearance of memory traces[9, 10]. An emerging concept is that the amenability of newborn neurons confers advantageous properties toward higher usage in the hippocampus. For instance, newborn neurons at specific stages of maturation are preferentially recruited into circuitry due to their unique properties, including hyper-excitability, high excitation/inhibition balance, and enhanced synaptic plasticity[11-13]. In addition, adult hippocampal neurogenesis is involved in responses to antidepressants[14], stress[15], brain injuries, and mental disorders[16-18]. A basic understanding of precursor properties and their niche interactions will illuminate how precursor cells sense and respond to changes in the external environment to promote tissue homeostasis or repair.

It is commonly believed that adult neurogenesis arises from precursors with the properties of NSCs[5], but the developmental origin of adult hippocampal NSCs remains unclear. Recently, Li *et al*. showed that NSCs initially originate from the ventral hippocampus during late gestation and then relocate to the dorsal hippocampus, suggesting that the ventral hippocampus is the primary location that contributes to the NSCs in the adult hippocampus[19]. NSCs were originally defined by their potential to both self-renew and generate neurons and glia from a single cell *in vitro*[5, 20]. However, reprogramming studies have raised the question of whether cultured lineage-restricted neural progenitors acquire increased potential not evident *in vivo*[21-23]. Therefore, investigations of NSC properties *in vivo* are critical in interpreting neurogenesis under both physiological and pathological conditions. Moreover, the cellular targets of environmental effects have been shown to influence later stages of neurogenesis[16]. Signaling from the niche is proposed to control many aspects of NSC behavior, including their mitotic state, cell fate specification, and precursor maintenance. Therefore, understanding how various niche components, signaling pathways, and environmental stimuli differentially regulate NSC behavior will reveal how they contribute to homeostasis and repair. In this review, we summarize recent progress in understanding how adult NSCs and their progeny are regulated by intrinsic and extrinsic factors in an activity-dependent manner, and how they are affected by various environmental stimuli and pathological conditions.

Adult Neurogenesis Exhibits Distinct Developmental Stages

Significant progress has been made in identifying the major milestones and processes underlying adult neurogenesis[16]. In the adult mouse DG, lineage-tracing studies have shown that nestin$^+$MCM2$^-$ quiescent radial and non-radial NSCs give rise to highly proliferative Tbr2$^+$MCM2$^+$ intermediate progenitors, which in turn generate mitotic DCX$^+$MCM2$^+$ neuroblasts to become DCX$^+$MCM2$^-$ immature post-mitotic neurons and finally DCX$^-$NeuN$^+$ mature dentate granule neurons (GCs) (Fig. 1).

Neural Stem Cells

Radial glia-like cells have been classified as Type-1 cells, which are infrequently labeled by retroviruses and thymidine analogs, such as BrdU or EdU, indicative of a low proliferative capacity. Morphologically, their cell bodies reside in the SGZ region, and possess an apical process that extends into the inner molecular layer. These cells express glial fibrillary acidic protein (GFAP), intermediate filament protein (nestin), brain lipid-binding protein, and Sry-related HMG-box transcription factor (Sox2). Despite some overlap with the expression of astrocytic markers, Type-1 cells are morphologically and functionally different from mature astrocytes. Recent fate mapping studies using inducible Cre recombinase driven by various promoters or enhancers, including Gli, GFAP, nestin, and glutamate aspartate transporter, have shown radial glia-like cells to be the primary NSCs in the adult brain[24]. In another model, it has been shown that a single Sox2$^+$ cell can self-renew, or give rise to a neuron or an astrocyte *in vivo*, suggesting that non-radial/horizontal neural progenitor cells possess stem-cell properties[7]. While still under vigorous debate, these models may represent the coexistence of multiple NSC types in the adult brain[25] (Fig. 2).

Activation and maintenance of radial NSCs In the adult mammalian brain, adult NSCs are currently thought to be a slowly-dividing, relatively quiescent population with radial morphology. The function of quiescence may serve as a protective mechanism that counteracts stem-cell exhaustion similar to that of somatic stem cells[26, 27]. Thus, the activation and maintenance of NSCs are inseparable processes in which a change of one would correspondingly alter the other. The balance of NSC maintenance and

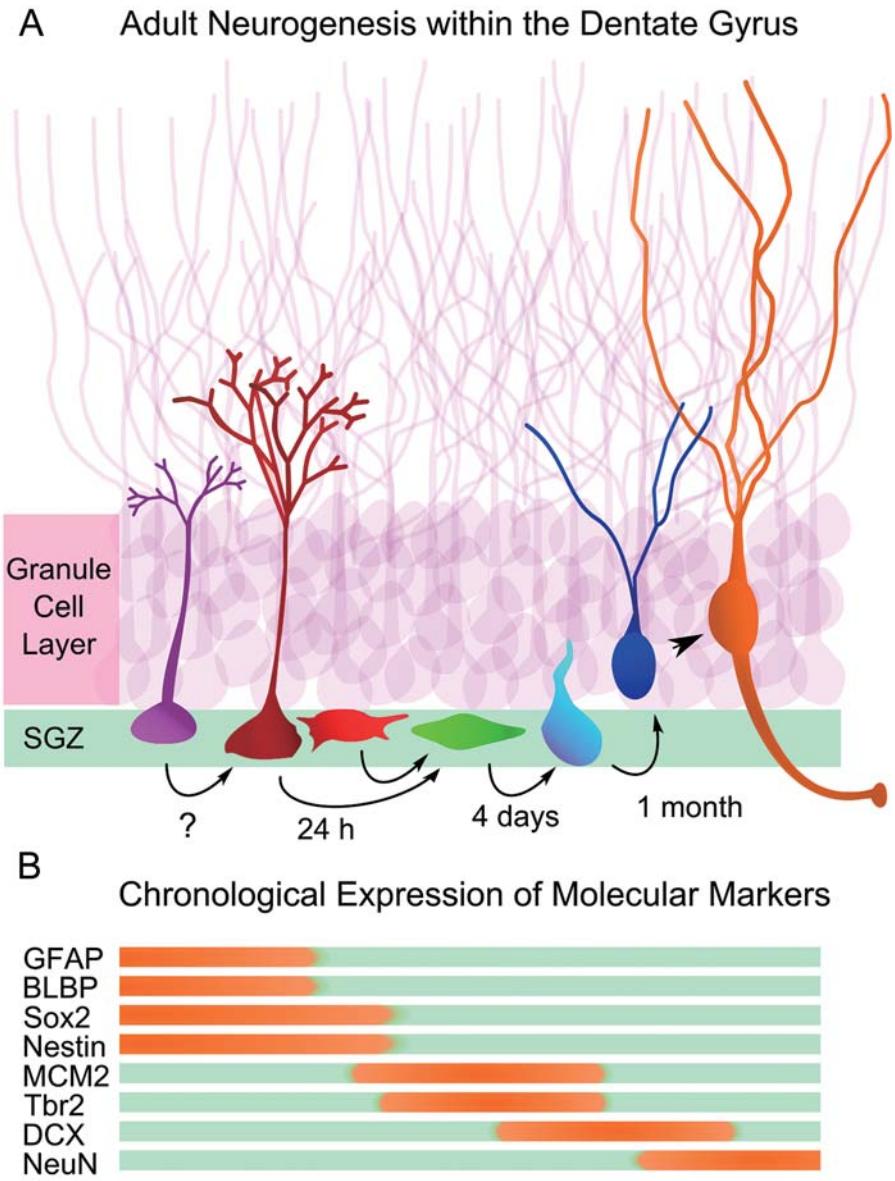

Fig. 1. Adult neurogenesis in the dentate gyrus of the hippocampus. A. Schematic summary of the development of newborn cells as characterized by the estimated timeline for each developmental stage. B. Expression of specific molecular markers at each stage.

neurogenesis is essential for ensuring the continuous generation of new hippocampal neurons throughout life without depleting the NSC pool. Incomplete maintenance and premature differentiation can cause depletion of the NSC pool and subsequent loss of neurogenesis; while excessive maintenance at the expense of neuronal differentiation compromises the neurogenesis rate necessary for proper hippocampal functions.

Fate choice of NSCs Multipotency and self-renewal are hallmarks of NSCs. In the adult brain, the neuronal lineage is thought to begin with the asymmetric cell division of a radial NSC to generate a highly proliferative intermediate progenitor, then the radial NSC returns to quiescence. Radial NSCs exhibit a low frequency of symmetric self-renewal under normal conditions, suggesting a capacity of the adult brain to amplify the NSC pool. Activated

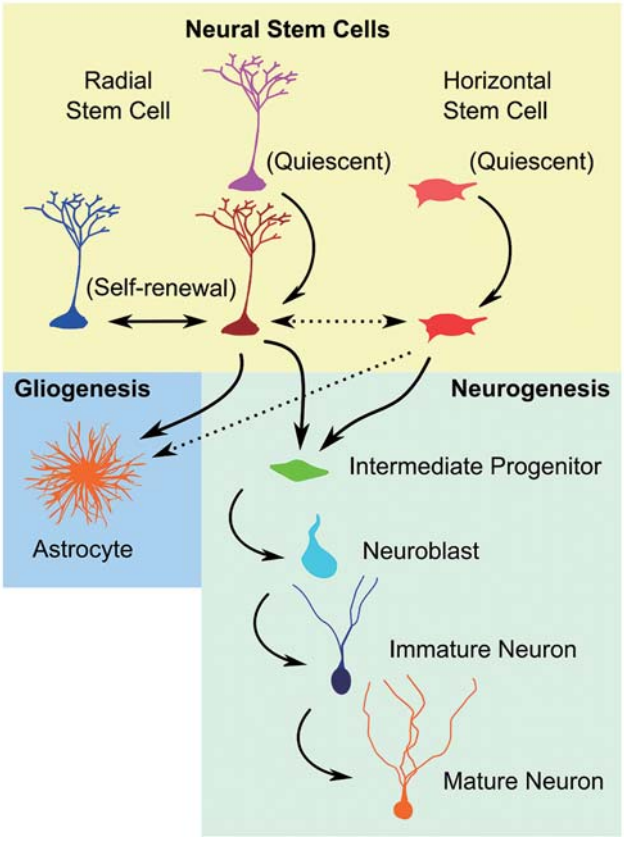

Fig. 2. Lineage of radial and horizontal NSCs and their progeny. Radial NSCs cycle between quiescent and mitotic states. Once activated, radial NSCs can divide symmetrically to generate additional radial NSCs, or asymmetrically to produce the neuronal and astroglial lineages. Though still to be further confirmed, horizontal NSCs are thought to be capable of generating neurons, astrocytes, and even radial NSCs.

NSCs have the potential to make various fate choices during multiple rounds of self-renewal, thus the total radial NSC pool reflects maintenance through quiescence or asymmetric self-renewal, reduction through terminal differentiation, and expansion through symmetric self-renewal.

The fate specification of radial NSCs is subject to dynamic regulation under diverse physiological, environmental, and pathological conditions. Furthermore, fate specification is a form of cellular plasticity which reflects brain adaptation to the environment. For example, social isolation stress promotes the expansion of radial NSCs, which in turn prepares the brain for increased neurogenic potential when more favorable conditions return[28]. The signals and molecular mechanisms dictating the fates of the NSC lineage remain to be determined. Of particular interest is to address how niche components couple the activity of neuronal circuitry to the regulation of NSCs under both physiological conditions and after specific experiences.

Interactions between NSC subtypes Recent studies have started to challenge the notion that radial NSCs are the only primitive stem cells in the adult brain, with the demonstration of the existence of a second morphologically distinct NSC population which in general is referred to as non-radial or horizontal NSCs[7, 25]. Within this pool, radial and horizontal NSCs can shuttle between mitotic activity and quiescence and respond selectively to neurogenic stimuli, pointing to the heterogeneous nature of the NSC population. The predominant evidence comes from the manipulation of Notch signaling in primitive NSCs which distinguishes two morphologically distinct populations: quiescent radial NSCs and active $Sox2^+$ horizontal NSCs. Interestingly, they respond differentially to physiological (exercise) and pathological (seizure) stimuli and aging. However, it remains unclear how these two subpopulations interact to orchestrate the precise regulation of adult neurogenesis. Accumulating evidence supports the view that radial NSCs are a reserve pool that can be recruited into the active pool to increase the neurogenic process in response to changes in conditions, while horizontal NSCs (possibly coming from the activated radial NSCs) can amplify themselves through symmetric expansion. Therefore, in these two subpopulations, “tissue-on-demand” constitutes their main mode of regulation. In future studies, it will be fundamentally important to define the relationships among distinct NSC populations (quiescent radial, active radial, quiescent horizontal, and active horizontal), how they are differentially regulated by various physiological and pathological stimuli, and the underlying molecular mechanisms of how they are influenced by neuronal activity to produce differentiated progeny.

Intermediate Neural Progenitors and Neuroblasts

In the adult SGZ, proliferating radial and non-radial NSCs give rise to intermediate progenitors (Type-2 cells), which

then become neuroblasts (Type-3 cells). Several types of highly proliferative intermediate progenitors have been identified according to their specific morphologies, electrophysiological properties, and expression of unique molecular markers[6, 29]. Type-2 cells are further divided into two subtypes: one subset maintains expression of the glial marker GFAP, but lacks radial processes (Type-2a); the other lacks GFAP and expresses the transcription factors Prox1 and NeuroD (Type-2b). Morphologically, horizontal cellular processes are still prominent in these cells[30, 31]. Type-2 cell proliferation is promoted by activity-dependent regulation through both physiological stimuli such as voluntary wheel running[32] or pharmacological stimulation such as treatment with the antidepressant fluoxetine[33]. Type-3 cells exit the cell cycle and express markers of the neuronal lineage, including DCX, PSA-NCAM, NeuroD, Prox1, and calretinin[34]. Morphologically, neuroblasts possess processes of various lengths, complexities, and orientations. Under pathological conditions, such as seizures, Type-3 cells display an aberrant state characterized by dramatically increased proliferation[35]. Many studies have revealed a substantial loss of newborn progeny during the first 4 days after they are born, when the majority of these precursors are still proliferating and express DCX[32, 36-38]. Due to the proliferative capacity of neural progenitors and neuroblasts, regulation at this stage would have a profound impact on the ultimate number of mature adult-born neurons.

Integration and Maturation of Immature Neurons

After precursor cells exit the cell cycle, most newborn neurons are eliminated within a short time. The mechanisms underlying the cell death of newborn neurons soon after birth are poorly understood. In the SGZ, the survival of newborn neurons at 1–3 weeks of age is influenced by the experiences of the animals, such as spatial learning and exposure to an enriched environment[39]. Glutamatergic signaling *via* NMDA receptors plays a cell-autonomous role in survival during the third week after birth, which coincides with the formation of dendritic spines and functional glutamatergic inputs[7, 40]. Those neurons that survive the early elimination phase are generally believed to be stably and persistently integrated into the DG neuronal networks.

The functional integration of newborn neurons *in vivo* requires the extension of dendrites and axons, and the formation of synapses with other neurons. Immature neurons send their axons to the CA3 region to form appropriate synapses within two weeks after cell-cycle exit. Dendrites of these cells reach the DG molecular layer within one week and continue to elaborate for at least 4 weeks. At 6–8 weeks of age, newborn neurons display overall morphological and functional characteristics similar to those of fully mature GCs[11, 41-43].

Regulation of Adult Neural Stem Cells and Their Progeny

The processes controlling adult neurogenesis depend on intrinsic and extrinsic variables that are responsible for NSC activation and maintenance, progenitor proliferation and differentiation, and immature neuron integration, survival, and maturation. A number of molecular players and signaling pathways have been identified, including niche factors/receptors, cytoplasmic factors, transcription factors, and epigenetic factors (Table 1). Most of the molecular players identified are involved in the later stages of adult SGZ neurogenesis. Recently, with the availability of promoter-specific transgenic mouse lines that selectively label distinct NSC and progenitor populations during adult neurogenesis, the molecular mechanisms responsible for the early events of adult neurogenesis are beginning to be elucidated.

Neurotransmitter-mediated Regulatory Mechanisms

Neurotransmitters are likely candidates to relay experiential information that influences adult neurogenesis. SGZ progenitor cells reside within a complex microenvironment and are potentially influenced by a plethora of synaptic inputs from local circuitry and distant brain areas through different neurotransmitters, including the main neurotransmitters gamma-aminobutyric acid (GABA) and glutamate, and other modulatory neurotransmitters such as acetylcholine, serotonin, and dopamine.

GABA Studies using engineered onco-retrovirus[44] and transgenic reporter mice[45, 46] have revealed that the synaptic integration of newborn neurons recapitulates embryonic neurogenesis by following a stereotypic sequence: (1) Initial GABA inputs to NSCs are non-synaptic and are mediated through GABA spillover from the mature synapses formed between presynaptic local interneuron terminals and mature GCs. (2) Then, neural progenitors begin to be innervated by local interneurons through input-

Table 1. Regulation of adult neurogenesis at distinct developmental stages

Signaling mechanism	Developmental stage							
	Neural stem cells		Neural progenitors		Neuroblasts to immature neurons		Immature to mature neurons	
	Activation	Maintenance	Proliferation	Differentiation	Dendritic development	Integration	Maturation	Cell Survival
Neurotransmitters	GABA↓, ACh↑		ACh↑, 5-HT↑	GABA↑, 5-HT↑	Glutamate↑	GABA↑, Glutamate↑		GABA↑, Glutamate↑
Morphogens	Notch1/ RBP-J↓, Wnt↓, BMP↓	Notch1/ RBP-J↑, Wnt↑, PTEN↑	Notch1/ RBP-J↓, BMP↓		Wnt↓		Wnt↓	
Transcription factors		Sox-2↑, shh↑					NeuroD↑, Prox1↑, Klf9↑	
Epigenetic regulators			Mbd1↓, Gadd45b↑	Mbd1↑	Gadd45b↑			
Environmental regulators	Exercise↑, social isolation↑, learning↑	Aging↓, social isolation↓	Exercise↑			Enrichment↑, learning↑	Learning↑	Enrichment↑, learning↑
Disease genes		FMRP		FMRP	DISC1, Mecp2	DISC1	Mecp2	
Disease effects	Seizure↑	Neurode-generation↓	Seizure↑		Seizure↑	Seizure↑, Neurode-generation↓	Seizure↑	Neurode-generation↓

specific GABAergic signaling[47, 48]. This initial synaptic transmission is slow and displays immature properties due to the relatively low concentration of GABA receptors on the newborn progeny[49]. (3) Between 2 and 3 weeks of cellular age, GABAergic inputs are converted from excitatory to inhibitory, and meanwhile, excitatory glutamatergic dendritic inputs start to form on newborn neurons. (4) Finally, inhibitory GABAergic synaptic inputs begin to appear on the cell body to form perisomatic synapses.

GABA is the major inhibitory neurotransmitter in the adult brain and acts *via* two main receptor types: ionotropic $GABA_A$ and G-protein-coupled metabotropic $GABA_B$ receptors. GABA can promote or suppress proliferation depending on the developmental stage, brain region, and the fate of distinct progenitor populations[50-52]. In the adult hippocampus, $GABA_A$ receptors have been reported to decrease the proliferation of quiescent NSCs[50, 53], promote the differentiation of neural progenitors[49], and promote the integration and survival of immature neurons[44, 54]. Recently, a study by Giachino *et al.* showed that NSCs of the SGZ also express metabotropic GABA receptors, and selective deletion of $GABA_{B1}$ receptors increases the proliferation of quiescent NSCs, supporting a role of $GABA_{B1}$ receptors in maintaining the quiescence of NSCs[55]. It remains unclear how these two types of receptors synergize with $GABA_A$ receptors to inhibit NSC activation/proliferation within the neurogenic lineage.

Though informative, previous *in vivo* studies have mostly used systemic manipulation and cell-autonomous manipulation by genetically knocking down a gene of interest through a genetic or retrovirus-mediated approach. Therefore, little is known about the source of neurotransmitters within the neurogenic niche and the underlying neuronal circuitry. One major advance in recent years has been the identification of functional inputs to newborn neurons and their synaptic partners during adult neurogenesis and the functional impact of existing neuronal circuits on the neurogenic process[2]. A recent study using

paired recording in acute slices showed that interneurons of the neurogliaform cell family provide a source of GABA for immature neurons labeled with POMC-EGFP at 11–12 days after birth[46] in the adult mouse DG[56]. Using a combination of optogenetics and lineage-tracing to target the quiescent radial glia-like NSCs, Song *et al.* showed that parvalbumin-expressing (PV^+) interneurons are a critical and unique niche component among different interneuron subtypes that couples neuronal circuit activity to regulate radial NSC activation through γ_2-containing $GABA_A$ receptors[53]. In contrast to the direct synaptic inputs onto immature neurons in POMC-EGFP mice[48], no apparent functional GABAergic synaptic responses were detected when radial NSCs were recorded in this and previous studies[57], suggesting that GABA spillover from activated PV^+ interneuron-mature GC synapses indirectly regulates nearby radial NSCs. Tonic GABA signaling spillover from presynaptic/postsynaptic neurons provides a means of acting on cells that might be located some distance from the signaling synapse. Therefore, it is an especially attractive candidate signal that reflects the overall local network activity for potential translation to local neural progenitors. Interestingly, a recent study showed that tonic and phasic GABA activation of neural progenitor cells and immature neurons is modulated by chemokine stromal cell-derived factor 1 co-released with GABA from local interneurons[58]. The mechanisms underlying such regulation remain to be determined.

In contrast to the inhibitory role in quiescent radial NSC activation, PV^+ interneuron activity positively regulates the survival of proliferating neuronal progeny[49]. Specifically, proliferating neuronal precursors in the adult mouse DG exhibit immature GABAergic synaptic inputs originating from local PV^+ interneurons. Moreover, PV^+ interneurons promote the survival of proliferative newborn progeny during the early phases of adult hippocampal neurogenesis upon optogenetic activation, whereas their suppression leads to decreased newborn progeny survival under both basal and enriched environment conditions. Taken together, these studies identify a novel niche mechanism involving PV^+ interneurons that couples local circuit activity to diametric regulation of quiescent NSC activation and survival of their proliferating neuronal progeny, two sequential phases of adult hippocampal neurogenesis. These findings provide the basic mechanisms underlying the dynamic control of adult neurogenesis during early developmental stages.

Glutamate The three pharmacologically-defined classes of ionotropic glutamate receptors in the adult brain were originally named after selective agonists — NMDA, AMPA, and kainate. The most studied subtype in adult neurogenesis is NMDA receptors. Accumulating evidence suggests that NMDA receptor-mediated glutamatergic signaling regulates distinct stages of adult neurogenesis. For example, injection of NMDA rapidly decreases cell proliferation in the adult rat DG, whereas injection of an NMDA receptor antagonist has the opposite effect[59, 60]. On the other hand, induction of long-term potentiation (LTP) at glutamatergic medial perforant path-granule cell synapses promotes the proliferation of adult neural progenitors and the survival of newborn neurons in an NMDA receptor-dependent fashion[61, 62]. These findings highlight the complexity of glutamate signaling in regulating adult neurogenesis, which is likely to involve both cell-autonomous effects in immature neurons and non-cell-autonomous effects through modulation by existing neuronal circuits. Genetic deletion of NR1, an obligatory subunit of the NMDA receptor, in proliferating adult neural progenitors reduces the survival of their neuronal progeny 2 to 3 weeks after birth[40]. Interestingly, injection of an NMDA receptor antagonist (CPP) diminishes differences in NMDA receptor signaling in all newborn neurons and promotes the survival of NR1-deficient neurons, suggesting a critical period for NMDA receptor-dependent competitive survival of newborn neurons in the adult brain[40]. This critical period coincides with a transition from excitatory to inhibitory GABA signaling. Whether GABA cooperates with glutamate signaling in regulating the survival of new neurons during this critical period remains to be determined. Analysis of the plasticity of glutamatergic synaptic inputs on newborn GCs during their maturation process has identified another critical period during which newborn neurons exhibit enhanced LTP. When 4–6 weeks old, newborn neurons exhibit both a reduced induction threshold and increased LTP amplitude in response to a physiological pattern of stimulation[7]. This critical period is associated with developmentally regulated NR2B-containing NMDA receptors in newborn neurons, since pharmacological inhibition of these receptors completely abolishes LTP in these neurons, but not in mature neurons[7].

In contrast to the regulatory role of glutamate in

later stages of neurogenesis, evidence that glutamate receptors regulate adult NSCs is still lacking. Kainate-induced seizures significantly stimulate the proliferation of NSCs[63], indicating the involvement of kainate receptors in the regulation of progenitor proliferation. Recently, a study using comparative recordings from patches excised from the soma and main process of NSCs has demonstrated the presence of AMPA receptors on the radial processes[64]. The functional roles of AMPA and kainate receptors in the regulation of NSCs remain to be determined.

Acetylcholine Accumulating evidence suggests that cholinergic signaling is involved in the regulation of adult hippocampal neurogenesis. For example, selective lesioning of the medial septum system negatively affects the proliferation of neural precursor cells[65, 66] and the administration of acetylcholinesterase inhibitors promotes NSC/neuronal progenitor cell proliferation and leads to a rapid Ca^{2+} rise in NSCs[67, 68]. Newborn neurons in nicotinic receptor α_7-knockout mice show delayed dendritic development and stunted maturation[69]. These studies indicate that neural precursors and their progeny are stimulated by cholinergic activation; however, direct evidence of how cholinergic activity regulates distinct stages of adult neurogenesis is still lacking. In addition, it remains unclear how various cholinergic receptor subtypes in neural precursor cells and their progeny work together to coordinate their responses to acetylcholine release. Future studies using targeted manipulations of components of this circuit are required to elucidate the nature of cholinergic signaling in neurogenesis.

Serotonin Studies of serotonergic signaling have been limited and conflicting[70-72], probably due to the diversity and complexity of serotonin (5-HT) receptor expression in the DG. The 5-HT receptor families are extremely diverse[73], and almost all fifteen receptor subtypes are expressed in the DG[74-79]. Depending upon which subsets of the 5-HT receptors are activated, DG neurons may be either depolarized or hyperpolarized by 5-HT and therefore increase or decrease their excitability. The opposing effects of activating different subsets of 5-HT receptors may explain the conflicting results in some studies. For example, selective 5-HT depletion has been reported to have no effects on the proliferation, survival, and differentiation of SGZ neuronal progenitors in the adult hippocampus[80]. Despite various manipulations leading to inconsistent results, it has been shown that an increase in the level of 5-HT enhances neural progenitor proliferation and differentiation[81], whereas depletion of 5-HT reduces these processes[82]. Future studies targeting the serotonergic-hippocampal circuitry in combination with genetic manipulations of their targets will help to tease out the complicated mechanisms associated with serotonergic circuitry and the relevant receptor subtypes.

Dopamine It has been proposed that dopamine (DA) plays a role in regulating the proliferation of neural precursor cells in the SGZ, although conflicting results have been reported[83, 84]. Denervation of dopaminergic neurons decreases the proliferation of NSCs in the SGZ[85]. Despite emerging studies that enhance our understanding of the role of DA during adult neurogenesis, studies targeting dopaminergic regulation of distinct stages of adult neurogenesis are still largely lacking. Therefore, it remains unclear whether the effect of DA on hippocampal neurogenesis is direct or indirect. Recently, a study using patch-clamp recording suggested that DA has distinct modulatory effects on dentate GCs at different developmental stages and through different receptor subtypes. DA modulates the strength of cortical inputs that newborn neurons receive from the medial perforant path through D1-like receptors, whereas D2-like receptors mediate the modulation of medial perforant path inputs to mature adult-born neurons[86]. It remains to be determined whether DA regulates early stages of adult neurogenesis.

Non-neurotransmitter-mediated Mechanisms

Morphogens A number of morphogens serve as niche signals to regulate the maintenance, activation, and fate choice of adult hippocampal neural precursors, including Notch, Wnts, and bone morphogenetic proteins (BMPs). Conditional disruption of BMP or Notch/RBP-J signaling in NSCs results in rapid initial activation of NSCs accompanied by a transient increase in the proliferation of intermediate neural progenitors and the production of new neurons. However, the long-term consequences of excessive activation of Notch signaling are depletion of the NSC compartment and impaired maintenance of NSCs, which ultimately lead to loss of the regenerative capacity of the radial NSC population and neuronal production[87, 88]. Direct evidence is still lacking in regard to whether the failure of stem cell maintenance is

due to increased astrocytic differentiation of radial NSCs or cell death of their downstream neuronal progeny. In addition to the intrinsic factors that regulate adult NSC development, extrinsic niche factors also play an important role in the regulation of distinct stages of adult neurogenesis. Using lineage-tracing and retrovirus-mediated approaches, the naturally-secreted Wnt inhibitor sFRP3 expressed by local mature GCs has been identified as an inhibitory niche factor, capable of suppressing multiple phases of adult neurogenesis[89]. Although the sources of most niche signals remain to be fully characterized, it is clear that they play important roles in fine-tuning the number of quiescent NSCs and the level of neurogenesis in the adult brain.

Transcription factors The sequential activation of different transcription factors ensures the proper development of adult neural precursors. Sox2 is a major mediator of Notch signaling in maintaining the precursor pool in the adult SGZ[87]. Shh appears to be a direct target of Sox2 in neural precursors, and deletion of Sox2 in adult mice results in a loss of hippocampal neurogenesis[90]. The orphan nuclear receptor TLX is also required for self-renewal and maintenance of neural precursors in the adult brain, likely through the canonical Wnt/β-catenin pathway[91]. Inhibitor of DNA binding (Id) genes encode dominant-negative antagonists of the basic helix-loop-helix transcription factors, and Id1 is highly expressed in radial NSCs in both the adult SVZ and SGZ[92]. In contrast, the transcription factors Prox1, NeuroD, and Kruppel-like factor 9 are sequentially required for the maturation and survival of new neurons in the adult hippocampus[93-95].

Epigenetic factors Various epigenetic mechanisms play important roles in fine-tuning and coordinating gene expression during adult neurogenesis, including DNA methylation, histone modifications, and non-coding RNAs[96]. For example, the epigenetic regulator methyl-CpG-binding domain protein 1 suppresses the expression of FGF-2 and several microRNAs controlling the balance between proliferation and differentiation during adult hippocampal neurogenesis[97]. Another epigenetic regulator, Gadd45b, is involved in maintaining the proliferation of neural precursors and the dendritic growth of newborn neurons by promoting BDNF and FGF1 expression in mature GCs in response to neuronal activation[98].

Cell-cycle regulators Cell-cycle inhibitors play major roles in maintaining the quiescence of adult neural precursors; deletion of these factors leads to transient activation and subsequent depletion of the precursor pool. A recent study has shown a requirement for the cyclin-dependent kinase inhibitor p57 in the maintenance of NSC quiescence; when p57 is deleted from NSCs *in vivo* a transient increase in neurogenesis through uncontrolled NSC activation is sharply followed by NSC pool exhaustion and reduced adult neurogenesis[99]. These findings fall among various other studies supporting a critical role of endogenous cyclin-dependent inhibitors and cyclin-dependent kinases in the cell-autonomous mechanics of adult neurogenesis[100]. Another recent study using an *in vivo* clonal approach clearly demonstrated that quiescent radial NSCs with PTEN deletion fail to be maintained over time due to increased astrocytic differentiation at the expense of neuronal differentiation[4]. How cell-cycle components dictate NSC fate choice is particularly important when considering the necessity of maintaining this population over a lifetime and neurogenic deficiencies that arise during aging.

Adult Neural Stem Cells in Experience-Mediated Plasticity and Disease

The generation of new neurons from adult NSCs is a dynamic and regulated process. Under physiological conditions, adult neurogenesis is regulated by controlling NSC activation, neuronal precursor proliferation/differentiation, and the survival of the newly-generated cells. Several physiological stimuli contribute to the dynamic regulation of adult neurogenesis, including physical exercise, various environmental and experiential conditions, learning, and aging. Physical activity enhances the generation of new neurons by inducing the proliferation of radial $Sox2^+$ progenitors and neuronal precursors[7]. The stress of social isolation promotes the expansion of radial NSCs, while exercise within enriched environments increases their neurogenic potential[28]. Chronic social isolation stress induces the activation and symmetric cell division of quiescent NSCs, and the long-term consequences of such an experience contribute to decreased adult neurogenesis. Aging is associated with a continuous decline in the number of new neurons, which could be due to increased quiescence of horizontal

NSCs[25] or the disappearance of radial NSCs *via* their conversion into mature hippocampal astrocytes[101]. Different neurogenic stimuli appear to affect cells at distinct stages of neurogenesis, and each of these stimuli can act at one or multiple levels of the neurogenic lineage. For example, voluntary running increases cell proliferation, while exposure to an enriched environment promotes new neuron survival. Learning modulates neurogenesis in a complex yet specific fashion, presumably by inducing the activation of NSCs and subsequently enhancing their survival and incorporation into neuronal circuits[102, 103]. Though a causal link between altered neurogenesis and animal behavior has not been established, it is likely that altered adult neurogenesis partially contributes to animals' overall behavioral outcomes.

Adult NSCs are also influenced by pathological conditions. Acute seizure activity robustly induces the production of aberrant dentate GCs at nearly every stage of adult neurogenesis. This includes increased activation of radial and horizontal NSCs[25], increased proliferation of neural progenitors and neuroblasts[35], ectopic migration, and aberrant dendritic and axonal development in immature neurons[104]. Chronic neurodegeneration impacts stem-cell maintenance, proliferation, survival, and functional integration in complex ways. For example, in mouse models of Alzheimer's disease, impaired GABA signaling leads to reduced hippocampal neurogenesis. This appears to occur, in part, through a mechanism involving a switch in NSC fate from a neurogenic to a gliagenic fate[105]. Abnormal dendritic growth and aberrant synaptic integration have also been reported[106]. In mice deficient in fragile X mental retardation protein (FMRP; a gene responsible for fragile X syndrome), both the proliferation and glial fate commitment of neural precursors are increased in the adult SGZ, through regulation of the Wnt/GSK3β/β-catenin/neurogenin1 signaling cascade[107]. Methyl-CpG-binding protein 2 (a gene mutated in Rett syndrome) regulates the maturation and spine formation of new neurons in the adult hippocampus[108]. Adult neurogenesis is also influenced by several additional pathological conditions, including inflammation induced by injury and irradiation, HIV infection, and drug addiction[3].

A number of neurological disease risk genes have been shown to regulate adult neurogenesis in a cell-autonomous fashion. Ablation of FMRP in adult nestin-expressing precursors disrupts hippocampus-dependent learning, and restoration of FMRP expression specifically in adult nestin-expressing precursors rescues these learning deficits in FMRP-deficient mice[18]. Disrupted-in-schizophrenia 1 (a gene implicated in major mental disorders) promotes the proliferation of neural progenitors through the GSK3β/β-catenin pathway[109], while limiting dendritic growth and synapse formation of new neurons through AKT/mTOR signaling in the adult hippocampus[110, 111]. These findings raise the intriguing possibility that aberrant postnatal neurogenesis may contribute to the juvenile and adult onset of many mental disorders[17].

It is becoming increasingly clear that adult neurogenesis is a multistep process modulated at different steps by various extrinsic and intrinsic neurogenic stimuli and influenced by pathological situations. Each neurogenic modulator may act at only one or at multiple levels of the neurogenic lineage. However, it is not clear whether changes in neurogenesis are NSC responses, adaptation in proliferation and survival of other cell types, or a combination of these effects. It is also unclear whether distinct NSC populations have different requirements for their maintenance and differentiation. Furthermore, it is also unclear if the most primitive NSCs in the adult brain, a quiescent population, can directly sense neuronal network activity and change their behavior. In future studies, it will be fundamentally important to define the relationships among distinct NSC populations (quiescent radial, active radial, quiescent horizontal, and active horizontal), how they are differentially regulated by various physiological and pathological stimuli, and the underlying molecular mechanisms of how they couple with neuronal activity to produce differentiated progeny. Identification of these mechanisms is critically important for harnessing this novel plasticity of adult neurogenesis to help repair the injured, diseased, and aged brain.

Concluding Remarks

Rapid progress in the field over the past decade has led to a better understanding of the distinct developmental steps of adult neurogenesis. Efforts have been made to elucidate different aspects of the regulation of adult neurogenesis

and a plethora of intrinsic and extrinsic factors have been associated with distinct steps of adult neurogenesis. Despite the identification of a variety of molecules involved in regulating distinct stages of adult neurogenesis, it remains unclear how extrinsic niche signaling is coupled to this intrinsic regulatory machinery. Moreover, the contributions of various anatomical and functional components within the SGZ remain to be determined. Future studies are needed to identify the molecular and cellular mechanisms underlying the activity-dependent circuitry regulation at distinct developmental stages of adult neurogenesis. Moreover, the heterogeneity of NSCs also raises the question of region-specific niche organization. It is important that further studies address how different niche components and signaling pathways interact to orchestrate the precise regulation of distinct stages of adult neurogenesis. Identification of new markers that dissect the neurogenic process into multiple stages and the availability of genetically-modified mice for cell-type-specific gain- and loss-of-function analysis will significantly accelerate these efforts. Understanding novel cellular and molecular mechanisms that regulate adult NSCs and the incorporation of newborn neurons into mature circuits will add greatly to our understanding of neuronal development and adult neurophysiology. This information is essential for designing strategies for the prevention and treatment of neurodevelopmental disorders, and also regeneration within the adult nervous system.

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·Review·

Regulatory mechanisms underlying the differential growth of dendrites and axons

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A typical neuron is comprised of an information input compartment, or the dendrites, and an output compartment, known as the axon. These two compartments are the structural basis for functional neural circuits. However, little is known about how dendritic and axonal growth are differentially regulated. Recent studies have uncovered two distinct types of regulatory mechanisms that differentiate dendritic and axonal growth: dedicated mechanisms and bimodal mechanisms. Dedicated mechanisms regulate either dendrite-specific or axon-specific growth; in contrast, bimodal mechanisms direct dendritic and axonal development in opposite manners. Here, we review the dedicated and bimodal regulators identified by recent *Drosophila* and mammalian studies. The knowledge of these underlying molecular mechanisms not only expands our understanding about how neural circuits are wired, but also provides insights that will aid in the rational design of therapies for neurological diseases.

Keywords: axonal growth; dendritic arborizations; developmental neurobiology

Introduction

Neurons are the building blocks of neural circuits. At the cellular level, each neuron typically forms an input compartment, the dendrites, which receive information, and an output compartment, the axon, which sends processed information to its target. These two different subcellular compartments are highly specialized so that each can perform its specific tasks. Dendrites and axons are distinguishable from each other in terms of electrical excitability, morphology, microtubule orientation, and distribution of specific molecules and organelles[1, 2] (Table 1). These structural and functional differences between dendrites and axons make neurons classical examples of polarized cells. The separation and differential growth of these two compartments are fundamental to the establishment and maintenance of neuronal polarity.

The sequence of events during neuronal morphogenesis, which seems to be evolutionarily conserved, has been described in detail in studies of mammalian neurons[1, 3, 4]. In general, the separation of the dendrites and axons requires two steps: specification of dendrites and the axon, followed by the differential growth of each compartment (Fig. 1). During the specification step, the dendrites and axon assume their respective compartmental identities to establish neuronal polarity[2, 4]. In the differential growth phase, the dendrites and axon develop the specific morphological characteristics that allow them to assume their specialized roles in the establishment of directional information transmission[1]. Following these two steps, many neuron types also undergo remodeling to assume their mature morphologies[5].

While significant effort has been aimed at understanding how dendrites and axons are specified[1, 6], less is understood about the molecular underpinnings of differential dendrite and axon growth. Although one might at first think that differential growth is solely controlled by the

Table 1. Commonly-used dendritic and axonal markers in different types of neurons

Neuronal types	Dendritic markers	Axonal markers
Mammalian hippocampal neurons	MAP-2	Tau
Mammalian cortical neurons	MAP-2	NF-H, Tau
Mammalian granule neurons	MAP-2	Tau
Drosophila sensory neurons	Nod::βGal, DenMark	Kin::βGal
Drosophila CNS neurons	Nod::βGal, DenMark	Syt::GFP

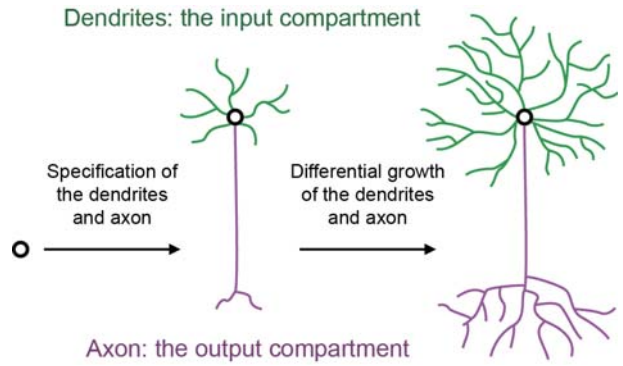

Fig. 1. A schematic illustration of the two steps of neuronal morphogenesis. Neuronal polarization is achieved in two steps. First, the nascent neuron (black circle) projects several processes, one of which commences rapid growth and becomes the axon[2, 3]. The remaining neurites then become dendrites as labeled by dendritic molecular markers[2, 3]. After acquiring their compartmental identities, the axon and dendrites extend additional branches to form the final branching patterns[2, 88]. The black circle indicates the soma; the green and purple processes indicate the dendrites and the axon, respectively.

compartmental differences set up during the specification step, this is unlikely to be the case for two reasons. First, the fact that different types of neurons exhibit distinct growth patterns of dendrites and axons argues for the existence of regulatory mechanisms that specifically control differential dendrite and axon growth. For example, during the differential growth phase, cerebellar Purkinje cells exhibit more dendritic growth than axonal growth, which leads to the formation of more elaborate dendritic than axonal arbors[7]. In contrast, cerebellar granule cells exhibit more axonal than dendritic growth, resulting a larger axonal than dendritic arbor[7]. Second, the existence of transcriptional programs that differentially regulate dendrite and axon development also supports the notion that the differential growth phase is controlled by *de novo* mechanisms and not simply by the cell-biological differences established during the specification phase[8-13].

Therefore, the regulatory mechanisms that operate in the differential growth phase play a major role in determining the final dendritic and axonal morphologies of mature neurons, and thus provide the basis for the morphological diversity observed in the nervous system.

Both mammalian and *Drosophila* neurons have been employed to identify the regulatory mechanisms that control differential dendritic and axonal growth. Mammalian neuronal cultures are robust systems for assessing dendritic and axonal arbor sizes, and are easily accessible to the application of pharmacological agents for manipulating the activity of molecules of interest. Despite this advantage, the cell culture environment differs from that *in vivo*. Thus, the roles of regulators identified in culture need to be further validated *in vivo*. Two major technical hurdles for the *in vivo* study of dendritic and axonal growth in the mammalian nervous system are the difficulty of achieving single-cell labeling and that of tracing the entire dendritic and axonal structures of a single neuron. As an alternative, the much smaller *Drosophila* nervous system offers an excellent system for studying the differential growth of dendrites and axons *in vivo*. Importantly, *Drosophila* is genetically tractable, allowing the use of advanced genetic mosaic techniques such as flip-out[14, 15] and mosaic analysis with a repressible cell marker (MARCM)[16]. Both of these techniques allow not only single-cell labeling, but also single-cell genetic manipulation.

In this review, we will focus on the roles of regulators identified in mammalian and *Drosophila* systems in

differentiating dendritic and axonal growth. Studies in these experimental systems have led to the discoveries of regulators dedicated to either dendrite or axon development ("dedicated mechanisms") and those that differentially direct dendritic and axonal development in opposite manners ("bimodal mechanisms") (Table 2). We will discuss these two major mechanisms separately.

Dedicated Mechanisms That Differentiate Dendritic and Axonal Growth

A number of molecular mechanisms operate in the differential growth phase[8, 9]. Although shared regulators, such as MAP1B (Futsch)[17] and histone deacetylase HDAC6[18, 19], are known to respectively promote or inhibit dendritic and axonal growth concurrently, other regulatory mechanisms are required to differentially regulate dendritic and axonal growth. For instance, differential regulation at the subcellular level can be achieved through "dedicated mechanisms", referring to regulators that specifically promote or inhibit the growth of one neuronal compartment without affecting the other[12] (Fig. 2). Based on their effect on dendritic and axonal growth, dedicated regulators can be further categorized into dendrite- or axon-dedicated regulators.

Dendrite-dedicated Regulators

In general, dendrite-dedicated regulators specifically control the growth of the dendritic compartment. These mechanisms can be either extrinsic, like growth factors, or intrinsic, like transcription factors. Extrinsic and intrinsic mechanisms may interact locally to promote the specific dendritic architectures of different neuron types. Besides *de novo* mechanisms, the cell-biological differences between axons and dendrites set up during the specification step may also influence the growth of one compartment and not the other. In this section, we will discuss our current knowledge of growth factors, transcription factors, and regulators of ER-Golgi transport in dendrite-dedicated regulation.

Table 2. Summary of dedicated regulators and bimodal regulators covered in this review

Molecules	Molecular function	Types of neurons studied	Role in dendritic growth	Role in axonal growth
Dedicated regulators				
BMP7/OP-1	TGF-β growth factor	Rat cultured sympathetic neurons/ cultured cerebral cortical neurons/ cultured hippocampal neurons	Positive regulator	None
NeuroD	bHLH transcription Factor	Cultured primary granule neurons	Positive regulator	None
Dar2	Homolog of Sec23	*Drosophila* da neurons	Positive regulator	None
Dar3	Homolog of GTPase, Sar1	*Drosophila* da neurons	Positive regulator	None
Dar6	Homolog of G-protein, Rab1	*Drosophila* da neurons	Positive regulator	None
Sar1	GTPase	Cultured hippocampal neurons	Positive regulator	None
Dar1	KLF transcription Factor	*Drosophila* da neurons	Positive regulator	None
SnoN-p300	Transcriptional complex	Cultured primary granule neurons	None	Positive regulator
Rac1	Small GTPase Rac	*Drosophila* PNS neurons and Purkinje cells	None	Positive/negative regulator
Bimodal regulators				
Sema3A	Secreted ligand	Cultured hippocampal neurons/ cortical neurons	Positive regulator	Negative regulator
CLASP2	Microtubule binding protein	Cultured cortical neurons	Positive regulator	Negative regulator
Rit	GTPase	Cultured hippocampal neurons	Negative regulator	Positive regulator
DLK	MAP Kinase Kinase Kinase	*Drosophila* C4da neurons	Negative regulator	Positive regulator

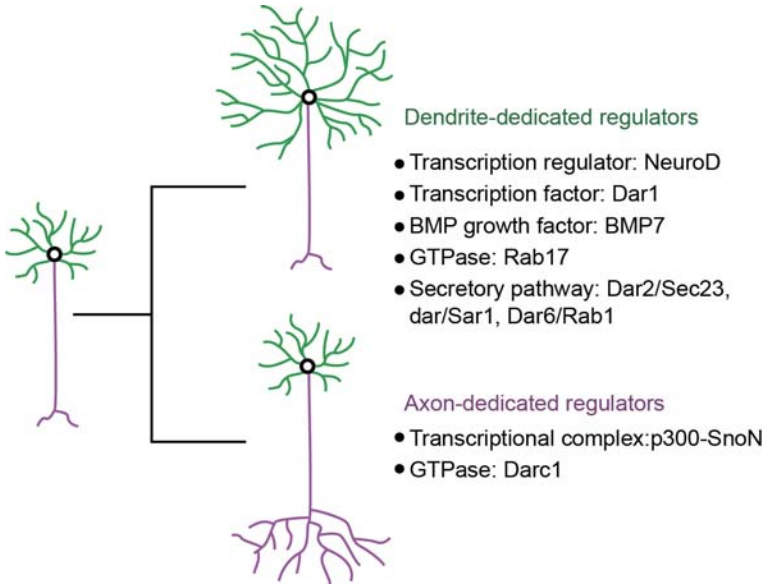

Fig. 2. Dedicated mechanisms of dendritic and axonal growth. Listed are known regulators that are dedicated to either dendrite-specific or axon-specific growth. The black circle indicates the soma; the green and purple processes indicate the dendrites and the axon respectively.

Growth factor BMP7 specifically promotes dendritic growth in mammalian cultured neurons The bone morphogenetic protein growth factor 7 (BMP7) (also termed osteogenic protein-1 or OP-1), a member of the transforming growth factor β (TGF-β) superfamily[20], is expressed in the nervous system. It induces the initial growth of dendrites in cultured rat sympathetic neurons, which typically develop a single axon without forming any noticeable dendritic structures in culture. Treatment of these neurons with recombinant human BMP7 leads to the formation of several dendrites without altering the number of axons[21]. BMP7 also selectively enhances dendritic arbor complexity after the initiation of dendrite formation. Exposure to BMP7 increases total dendritic length and the number of higher-order dendritic branches in CNS neurons *in vitro* without affecting axonal growth[22, 23].

How does BMP7 specifically promote dendritic growth? BMP signaling in general is transduced through ligand-receptor binding, which subsequently induces the phosphorylation of SMAD proteins and downstream transcriptional programs[24]. Consistent with this model, Garred and colleagues found that Actinomycin-D, a transcriptional inhibitor, blocks BMP7-induced dendritic growth in cultured sympathetic neurons. Microarray analysis of cultured sympathetic neurons treated with BMP7 for six hours showed changes in the transcript level of a number of transcriptional repressors belonging to the inhibitor of DNA binding (Id) family[25], an effect which may subsequently lead to the regulation of other transcriptional programs. BMP7 might also promote dendritic growth by enhancing the expression of the microtubule-associated protein MAP2[26]. Taken together, these studies suggest that the secreted molecule BMP7 may serve as an extrinsic mechanism that specifically promotes dendritic growth in mammalian neurons in culture.

Transcription factor NeuroD specifically promotes activity-dependent dendritic growth NeuroD is one of the basic helix-loop-helix (bHLH) transcription factors that control neuronal fate specification[27]. In addition to promoting neurogenesis[28], NeuroD expression persists in differentiated neurons[29] and controls dendrite morphogenesis in granule neurons[30, 31]. Gaudillière and colleagues found that knock-down of NeuroD inhibits dendritic growth but spares axonal morphogenesis in cultured primary granule neurons and granule neurons in cerebellar slices[30]. Furthermore, granule neuron dendritic branching is impaired in *NeuroD* conditional knock-out mice[31]. These results suggest that NeuroD specifically promotes dendritic growth.

NeuroD also plays a role in the neural activity-dependent patterning of dendritic arbors[32-34]. In cultured granule neurons, high neural activity induced by membrane-depolarizing concentrations of potassium chloride leads to more exuberant dendritic growth[30]. Knock-down of NeuroD blocks activity-induced dendritic overgrowth, suggesting that NeuroD may translate increased neural activity into a dendritic growth response[30]. Consistent with this notion, biochemical analysis revealed that NeuroD is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[30], a critical mediator of cellular responses to neural activity[35]. Gaudillière and colleagues further demonstrated that phosphorylation of NeuroD by CaMKII is indispensable for NeuroD to instruct activity-dependent dendritic growth[30]. Taken together, these studies suggest that NeuroD specifically mediates activity-dependent dendritic growth in granule neurons.

In addition to NeuroD, the calcium-responsive transactivator CREST[36] and the transcriptional complex AP-1[37] regulate activity-dependent dendritic growth in mammalian cortical and hippocampal neurons and *Drosophila* CNS neurons respectively. However, it remains unknown whether CREST and AP-1 function in axonal growth in these neuron types and thus whether they are dendrite-dedicated regulators.

Transcription factor Dar1 specifically promotes microtubule-based dendritic growth To perform a systematic search for genes that differentially regulate dendrite and axon development, Ye and colleagues used forward genetic screen that selected for mutants with dendrite- or axon-specific defects. To do this, they took advantage of the class IV dendritic arborization (C4da) sensory neurons in the *Drosophila* larva[38, 39]. In contrast to CNS neurons, C4da neurons directly sense multiple nociceptive stimuli[40-42] and their dendrites do not receive synaptic inputs. Nonetheless, the C4da neuron system has many advantages that make it well-suited for the study of dendrite and axon differential growth. First, the dendrites and axons of C4da neurons are easy to visualize with the help of a highly specific marker[43]. Second, unlike most invertebrate neurons, which are predominantly unipolar, da neurons resemble mammalian CNS neurons in terms of their multipolar morphology. Third, the dendrites and axons of these neurons exhibit similar cell-biological differences to those in mammalian CNS, including microtubule orientation[38, 44, 45] and organelle distribution[38, 46].

From their genetic screen of C4da neurons, Ye and colleagues isolated several mutants that displayed dendrite-specific growth defects, which they named *dendritic arbor reduction* (*dar*) mutants. The *dar1* gene encodes a *Drosophila* homolog of the Krüpple-like family of transcription factors (KLF), featuring three zinc-finger domains at the C-terminal region of the protein. Loss of *dar1* restricts dendritic growth in all classes of da neurons[47]. In sharp contrast, the growth of axons, including axon terminals, in these same neurons remains indistinguishable from wild-type controls[47]. Dar1 appears to preferentially promote microtubule-based, but not actin-based, dendritic growth. Overexpressing Dar1 specifically results in the appearance of microtubule-based higher-order dendritic branches. Moreover, loss of *dar1* function does not block the formation of F-actin-based dendritic filopodia caused by Rac1 overexpression. These results suggest that Dar1 preferentially regulates microtubules to promote microtubule-driven dendritic growth.

How does Dar1 influence the dendritic microtubule cytoskeleton? Ye and colleagues examined Spastin, a microtubule-severing protein, and found that the amount of *Spastin* mRNA was significantly elevated in *dar1* mutant neurons. These data indicate that Dar1 controls the transcription of *Spastin* to influence microtubules in the dendrites. Consistent with the change in *Spastin* transcript level, overexpression of Spastin impairs dendritic growth, leading to a phenotype reminiscent of that seen in neurons lacking *dar1*[47]. Further transcript profiling analysis may uncover additional Dar1 transcriptional targets involved in microtubule-based dendritic growth. In summary, these studies reveal Dar1 as a dendrite-dedicated mechanism that promotes dendritic growth *via* regulation of the microtubule cytoskeleton.

Regulators of ER-Golgi transport are preferentially required for dendritic growth Among the *dar* genes, three encode regulators of ER-to-Golgi transport: *dar2*, *dar3*, and *dar6*[38]. The mammalian homologs of these genes, Sec23, Sar1, and Rab1, respectively, are critical for ER-to-Golgi transport *via* COPII vesicles[48]. When mutations in *dar3* are introduced into single C4da neurons using the MARCM technique, not only do Golgi structures

become abnormal in the soma and dendrites, but total dendritic length is also markedly reduced[38]. When the Golgi apparatuses in the dendrites (termed dendritic Golgi outposts) are damaged using laser illumination, dendritic extension and retraction events become less dynamic. Moreover, redistributing the Golgi outposts in different parts of the dendritic arbor leads to a redistribution of dendritic branches. These findings highlight the idea that dendritic Golgi outposts may contribute locally to dendritic growth. Despite the changes in C4da neuron dendritic arbors, loss of *dar3* does not alter the axonal growth of these neurons[38]. Because the secretory pathway is a major source for the building blocks of the plasma membrane, these results suggest that growing dendrites have a greater demand for membrane supply during development than the axon.

Consistent with these findings in *Drosophila* C4da neurons, knock-down of the mammalian Dar3 homolog, Sar1, impairs dendrite-specific growth in cultured hippocampal neurons[38]. Taken together, these studies reveal a fundamental and evolutionarily-conserved difference in the reliance of dendritic *versus* axonal growth on the secretory pathway.

Implications of dendrite-dedicated mechanisms The diverse types of dendrite-dedicated mechanisms may form the basis for dendritic diversity in the nervous system. Since different neuron types require varied dendritic architectures to carry out their specific tasks, growth factors may induce the expression or activity of specific transcription factors to promote growing dendrites to assume the correct shapes. Because of the vast range of dendritic morphologies, it seems likely that many other dendrite-dedicated mechanisms that rely on growth factors and transcription factors remain to be discovered. In addition, the importance of secretory pathway regulators may be to transduce the signals provided by growth factors and transcription factors into physical changes in dendritic architecture.

Axon-dedicated Regulators

The axon-dedicated regulators identified so far include transcriptional and cytoskeletal regulators. These mechanisms are of particular interest for the development of axon regeneration therapies to treat spinal cord injuries and degenerative diseases. This section will discuss our current knowledge of axon-dedicated regulators, including transcription factors and cytoskeletal regulators.

The transcriptional complex SnoN-p300 specifically promotes axonal growth Ski-related novel protein N (SnoN) acts as a transcriptional repressor in TGF-β signaling[49]. In the nucleus of primary cerebellar granule neurons, SnoN is targeted for protein degradation by the Cdh1-APC ubiquitin ligase complex[50], which is indispensable for axonal growth in mammalian neurons[51]. Knock-down of SnoN inhibits granule neuron axonal growth. Conversely, elevated SnoN expression caused by either overexpression of a mutant form of SnoN resistant to degradation by the Cdh1-APC complex or by Cdh1-APC knock-down, results in elongated axons[50]. These results suggest that SnoN is both necessary and sufficient for axonal growth.

Further studies found that SnoN interacts with a histone acetyltransferase transcriptional activator, p300 or CREB-binding protein (CBP), to regulate axonal growth[52]. Knock-down of p300 impairs axonal growth without changing dendritic growth[52], suggesting that the SnoN-p300 complex is dedicated to axonal growth. Further, microarray analysis has led to the finding that expression of the actin-binding protein Ccd1[53] is reduced by knock-down of SnoN or p300[52]. Ccd1, like SnoN-p300, specifically promotes axonal growth in granule neurons[52]. Therefore, axon-dedicated regulation by the SnoN-p300 transcriptional complex is likely mediated by Ccd1.

Kirilly and colleagues found that knockdown of the *Drosophila* homolog of p300 leads to simplified dendrite arbors in pupal C4da neurons[54]. This suggests that the role of a specific regulator in differentiating dendritic and axonal growth could be cell-type specific or that p300 might mediate dendritic growth through a distinct mechanism.

The GTPase Rac1 specifically controls axonal growth The small GTPases of the Rac/Rho/Cdc42 subfamily are important regulators of the actin cytoskeleton in many cell types[55]. The *Drosophila* homolog of Rac, DRac1, plays an important role in the initiation and elongation of axonal growth in *Drosophila* PNS neurons[56]. Overexpressing either a constitutively active or a dominant-negative form of DRac1 inhibits axonal outgrowth and elongation without affecting the dendrites[56], suggesting that appropriate levels of actin polymerization are important for axonal growth.

The axon-dedicated role of Rac1 has been tested in mammalian Purkinje cells[57]. Consistent with the findings in *Drosophila*, overexpression of a constitutively-active

form of human Rac1 in cerebellar Purkinje cells leads to a reduction in axon terminals[57], while overall dendritic branching patterns remain normal. These results show that Rac1 is dedicated to axonal growth. It is noteworthy that constitutively-active Rac1 also reduces the size, while increasing the number, of dendritic spines on Purkinje cells[57]. Hence, although Rac1 is a dedicated regulator of axonal growth, it is also indispensable for organizing dendritic spine structures. This dichotomy may stem from an underlying imperative for proper actin regulation in both axonal growth and dendritic spine development.

Implications of axon-dedicated mechanisms Most current studies that aim to regenerate axons do not investigate the consequences of the interventions at the "other end" of the neuron—the dendrites. As a result, although many molecules are known to regulate axonal growth, very few are known to do so in an axon-specific fashion. Interventions that promote the regrowth of injured axons may not rescue defective dendrites or, even worse, may cause dendritic defects. Thus, it is imperative that we understand the intricacies of each growth program at both ends of the neuron to avoid unintended, adverse consequences of regenerative therapies.

Bimodal Mechanisms That Differentiate Dendritic and Axonal Growth

In addition to dedicated mechanisms, another strategy for differentially instructing dendritic and axonal growth is to direct their development in opposite manners at the same time. This mode of regulation is termed "bimodal regulation"[58] (Fig. 3). Unlike dedicated regulators, bimodal regulators might coordinate dendritic and axonal growth during development or in response to neuronal injury. This section discusses what is currently known about bimodal regulators.

Sema3A promotes dendritic growth but restricts axonal growth Semaphorin 3A (Sema3A) is a member of the Semaphorin family. Prior studies found Sema3A functions in an early step of neuronal polarization that specifies dendritic and axonal identities[59, 60]. In cultured hippocampal neurons, Sema3A inhibits cyclic adenosine monophosphate (cAMP) activity but enhances cyclic guanosine monophosphate (cGMP) activity[59]. cAMP, in turn, promotes axon initiation but suppresses the formation of dendrites; whereas cGMP has the opposite effect[61]. As a result, Sema3A preferentially promotes dendrite formation while suppressing axon formation. Similarly, Sema3A

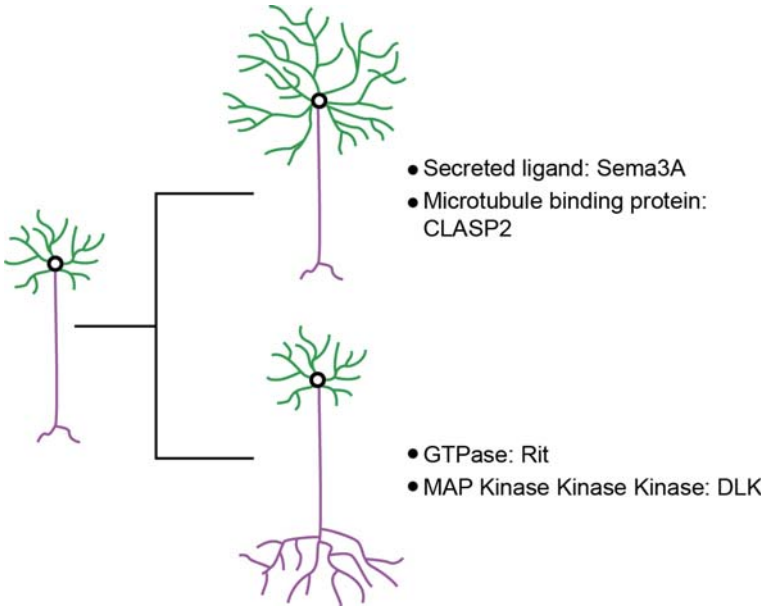

Fig. 3. Bimodal regulation of dendritic and axonal growth. Several bimodal regulators have been identified to oppositely alter dendritic and axonal growth. Sema3A and CLASP positively regulate dendritic growth but restrict axonal growth[59, 65], whereas Rit and DLK exert the opposite actions on these compartments[58, 68]. The black circle indicates the soma; the green and purple processes indicate the dendrites and the axon respectively.

acts as a chemoattractant for cortical apical dendrites but a chemorepellent for cortical axons[60]. The downstream effectors of Sema3A-cGMP/cAMP include protein kinase A (PKA), protein kinase G (PKG), and the serine/threonine kinase LKB1[59, 60, 62].

After dendrites and axon are specified, Sema3A-cAMP/cGMP continues to oppositely regulate the development of the dendritic and axonal compartments[59]. Exposure to Sema3A or cGMP results in more complex dendritic structures in cultured hippocampal neurons, and this is reversed by application of a PKG inhibitor[59]. These results demonstrate that Sema3A promotes the initiation and continued growth of dendrites while inhibiting these aspects of axonal growth.

CLASP2 promotes dendritic growth but restricts axonal growth Cytoplasmic linker protein (CLIP) and CLIP-associated protein (CLASP) bind to the plus end of microtubules and regulate microtubule dynamics in different cell types[63]. It is speculated that CLIP and CLASP proteins may be involved in the differential organization of the dendritic and axonal microtubule cytoskeleton[64]. In support of this, CLASP2 is reported to be a bimodal regulator. Knockdown of CLASP2 causes axonal over-branching but impairs dendritic extension in cultured cortical neurons[65].

It remains unknown how the bimodal function of CLASP2 is achieved. CLASP2 exhibits two microtubule-binding behaviors: it binds to the plus end of microtubules and also associates with microtubule lattices[63, 66]. The intriguing hypothesis that these two microtubule-binding activities may mediate the two opposite actions of CLASP2 on dendritic and axonal outgrowth remains to be tested.

Rit GTPase restrains dendritic growth but promotes axonal growth Rit is a member of the Ras GTPase family and is widely expressed in the mammalian nervous system[67]. Overexpression of a dominant-negative form of Rit inhibits axonal growth but leads to longer dendrites in cultured hippocampal neurons[68]. Conversely, overexpressing a constitutively active form of Rit markedly increases axonal length but reduces total dendritic length and number[68]. Lein *et al.* further found that extracellular signal-regulated kinase 1/2 (ERK1/2) mediates the bimodal regulation of Rit, as inhibition of mitogen-activated protein kinase/ERK 1 (MEK1) blocks the changes in both dendritic and axonal growth caused by constitutively active Rit. These data suggest that, in contrast to the axon-dedicated Rac1 GTPase, Rit GTPase functions as a bimodal regulator.

DLK pathway promotes axonal growth but restrains dendritic growth *in vivo* The evolutionarily-conserved dual leucine zipper kinase (DLK) pathway regulates axonal growth, regeneration, and degeneration[69-77], and organizes the presynaptic structures of axon terminals[78]. This pathway consists of two major components. The first is an E3 ubiquitin ligase named Pam/Highwire/RPM-1 (PHR). PHR targets DLK, a mitogen-activated protein kinase kinase kinase (MAPKKK), for protein degradation[69, 71]. Upregulated DLK expression, caused by either loss of PHR or overexpressing DLK, causes axon terminal overgrowth in various neuron types in *Caenorhabditis elegans*, *Drosophila*, and mammals[58, 69, 72, 79-82]. Moreover, loss of *DLK* blocks new axon outgrowth after nerve injury[70, 74-76, 83].

A recent study by Wang and colleagues demonstrates that overabundant DLK promotes axonal growth but negatively regulates dendritic branching in *Drosophila* C4da neurons[58]. The *Drosophila* homologs of the E3 ubiquitin ligase and DLK are named Highwire (Hiw)[81] and Wallenda (Wnd)[69], respectively. Either loss of *hiw* or overexpression of Wnd leads to exuberant axon terminal growth but markedly impairs dendritic growth in C4da neurons[58]. These dichotomous actions of the DLK/Wnd pathway are mediated by divergent downstream components. The transcription factor Fos and Down syndrome cell-adhesion molecule (Dscam) are required for axon growth in response to up-regulated DLK/Wnd[58, 84]. In contrast, dendritic regulation by DLK/Wnd is mediated by the transcription factor Knot[58]. It is noteworthy that in Knot-negative neurons, such as class I, II, and III da neurons, DLK/Wnd specifically promotes axonal growth and does not regulate dendritic growth[58].

The bimodal function of DLK/Wnd might serve to coordinate dendritic and axonal growth after nerve injury. Previous studies reported an increase in DLK/Wnd protein level after nerve crush injuries in both *Drosophila* motor neurons[75] and mouse optic nerves[74]. Based on the work of Wang and colleagues in C4da neurons, an elevated DLK/Wnd level likely restrains dendritic growth in injured neurons while promoting axonal regeneration. Indeed, it has been observed that axotomy not only triggers axon

regeneration but also causes more simplified dendrites in C4da[85] and mammalian neurons[86, 87]. These observations suggest that neurons may promote axonal regeneration at the expense of dendrites and that the bimodal regulator DLK/Wnd may coordinate these distinct dendritic and axonal responses to injury.

Implications of bimodal regulators The functional significance of bimodal regulation remains to be determined. We speculate that bimodal regulators might determine the ratio of dendritic arbor size to axonal arbor size[58]. For instance, high levels/activity of Rit or DLK might result in more elaborate axon branching but simpler dendritic structures; whereas high Sema3A and CLASP2 likely cause the opposite changes in dendritic and axonal patterns. It will be informative to determine whether bimodal regulators are differentially expressed in distinct neuron types and underlie the morphological diversity among them. Besides their functions during development, little is known about how these bimodal regulators control dendritic and axonal responses to injury or pathological conditions. Further investigation may shed light on how manipulating the activity of bimodal regulators might correct dendritic and axonal defects in disease conditions.

Summary

The differential growth of dendrites and axons is of fundamental importance to the establishment of connectivity and communication in neural circuits. It is also essential for generating the diverse neuronal morphologies that we observe in the nervous system. The molecular mechanisms that differentiate dendritic and axonal growth can be categorized into "dedicated" and "bimodal" mechanisms. Dedicated mechanisms specifically control the growth of only one compartment, while bimodal mechanisms promote the growth of one compartment while inhibiting the other. Moreover, it is likely that these distinct regulatory methods converge to pattern the distinct dendritic and axonal architectures of each neuron. Although just a few examples of each category have been discovered, our current knowledge hints at the possible complexity involved in patterning the nervous system. On one hand, many thousands of axon- and dendrite-dedicated regulators may be required to develop the diversity of neuronal architectures that we observe. On the other hand, this diversity may arise from various combinations and levels of just a few regulators. Furthermore, we do not yet fully appreciate the importance of having these various modes of regulation, especially bimodal regulation. Although we speculate that bimodal regulators function to coordinate axon and dendrite growth in both development and regeneration, further investigation is required to fully appreciate the role of these regulators. Further understanding of how these regulatory mechanisms operate during development and how to manipulate the activity of these regulators will also be instructive for designing strategies to restore defective neurons under pathological conditions. In some neurological disorders, only axons or dendrites are affected; in others, only a specific brain region or subset of neurons. Increasing our understanding of axon-specific, dendrite-specific, and bimodal regulators may allow us to specifically regrow, reshape, and regenerate many different types of neurons without adverse consequences for the remainder of the nervous system.

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·Review·

Microtubule dynamics in axon guidance

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Precise modulation of the cytoskeleton is involved in a variety of cellular processes including cell division, migration, polarity, and adhesion. In developing post-mitotic neurons, extracellular guidance cues not only trigger signaling cascades that act at a distance to indirectly regulate microtubule distribution, and assembly and disassembly in the growth cone, but also directly modulate microtubule stability and dynamics through coupling of guidance receptors with microtubules to control growth-cone turning. Microtubule-associated proteins including classical microtubule-associated proteins and microtubule plus-end tracking proteins are required for modulating microtubule dynamics to influence growth-cone steering. Multiple key signaling components, such as calcium, small GTPases, glycogen synthase kinase-3β, and c-Jun N-terminal kinase, link upstream signal cascades to microtubule stability and dynamics in the growth cone to control axon outgrowth and projection. Understanding the functions and regulation of microtubule dynamics in the growth cone provides new insights into the molecular mechanisms of axon guidance.

Keywords: axon guidance; growth cone; microtubule dynamics; signal transduction

Introduction

In the developing nervous system, proper axon outgrowth and pathfinding are essential for neurons to reach their final destination and establish precise neuronal circuits. Extracellular guidance signals including guidance cues, growth factors, and cell adhesion molecules, are responsible for directing the navigation of the growth cone (GC) of an extending axon through the modulation of cytoskeleton dynamics including filamentous (F) actin and microtubules (MTs), fundamental cytoskeleton components of GC motility[1-7]. Research in the past two decades has gained significant knowledge of the functional importance of actin dynamics in axon guidance, which has been the focus of several excellent reviews[3-6]. Here, we review recent studies examining direct modulation of MT dynamics in axon outgrowth and guidance.

MTs in the GC

The GC is the specialized, highly-motile tip of an extending axon, probing extracellular guidance signals and leading axon projection along specific pathways in the developing nervous system[1-3]. The GC has two general regions: the central (C) and peripheral (P) regions, and forms two kinds of protrusions: filopodia, finger-like projections, and lamellipodia, flat sheet-like protrusions[3-5, 8] (Fig. 1). These regions and protrusions of the GC are dynamic and persistently undergo shape changes *in vivo*, depending on both actin and MT dynamics in the GC. MTs are polarized hollow polymers of tubulins assembled by the lateral interaction of 11–15 protofilaments, in which α/β tubulin heterodimers hold together in a head-to-tail fashion. In general, MTs are bundled together in the axon shaft, whereas some are defasciculated crossing the C region of the GC as single MTs[9] (Fig. 1). In the C region of the GC, MTs may be relatively straight or form prominent loops, while some occasionally invade the P region as well as filopodia[9]. Individual MTs in the GC are pioneered by their plus ends, the fast-growing ends that favor polymerization compared to the minus ends[5]. MTs in the

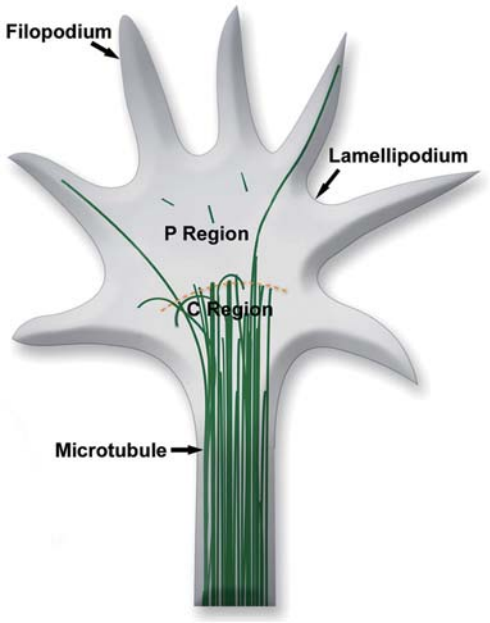

Fig. 1. Organization of microtubules in the growth cone (GC). The GC, an expanded tip of the axon, includes two regions: the C and P regions (delineated by an orange dotted line) with veil-like lamellipodia and finger-like filopodia. The P region of the GC contains unpolymerized tubulins and 'pioneer polymerized MTs', while the C region consists of stable, bundled MTs. MTs are shown in green.

GC spontaneously switch between phases of growth and shortening, a behavior termed dynamic instability, which may function as a direct sensor to control GC steering[10].

Role of α- and β-Tubulin in Axon Guidance

The importance of tubulin isotypes in axon guidance has emerged from recent discoveries of patients carrying mutations in genes encoding α- and β-tubulin (e.g. *TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBA8*)[11-14]. In addition to classic lissencephaly and hypoplasia of the hippocampus, cerebellum, and brainstem, brain malformations in patients harboring *TUBA1A* mutations include partial or complete absence of the corpus callosum and commissural fiber tracts, as well as hypoplasia of the internal capsule and corticospinal tract associated with dysmorphic basal ganglia[15, 16]. Mutations of *TUBB2B or TUBA1A* are associated with both lissencephaly and polymicrogyria[16, 17] which have in common axon-guidance defects including partial or complete agenesis of the corpus callosum and the internal capsule[12, 16]. Patients with homozygous deletions in *TUBA8* have extensive polymicrogyria, callosal anomalies, and optic nerve hypoplasia[18]. Missense mutations in *TUBB3*, encoding the neuron-specific β-tubulin isotype III, result in various neurological disorders, such as ocular motility disorder, congenital fibrosis of the extraocular muscle type 3, facial paralysis, intellectual and behavioral impairments, and axonal sensorimotor polyneuropathy[13, 14]. Fetopsy and imaging studies have demonstrated that *TUBB3* mutations cause a spectrum of axonal-projection defects such as agenesis or hypoplasia of the commissural axon tracts, the corticospinal tract, the anterior commissure, and oculomotor nerves[13, 14]. The *TUBB3*$^{R262C/R262C}$ knock-in mouse model reveals axon-guidance defects in commissural axons and cranial nerves[14], in which the anterior commissure is thinned and/or absent and the corpus callosum is composed of stalled commissural axons adjacent to the midline compared to the wild-type mouse[14]. In addition, knockdown of TUBB3 inhibits spinal cord commissural axon outgrowth and causes their misguidance, suggesting that TUBB3 is specifically involved in commissural axon projection[19]. All tubulin isotype mutations (e.g. *TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBA8*) commonly cause a generalized defect in axon guidance (Table 1), indicating that MTs play an essential role in controlling axon outgrowth and projection during brain development. The disease-associated tubulin isotype mutations impair tubulin heterodimer formation and alter MT instability[11, 13, 14], further suggesting that modulation of MT dynamics is required for proper axon guidance.

Modulation of MT Dynamics during GC Steering

Although the major function of MTs has been thought to be to consolidate and provide mechanical support to GC steering initiated by actin dynamics, an increasing number of studies suggest that they play an essential and instructive role in GC behavior[4, 30-33]. For example, dynamic MTs are oriented and stabilized preferentially in the direction of the GC turn, and distal dynamic MT ends in the P region of the GC are required for GC repulsion at substrate borders[32-34]. During adhesion-mediated GC steering of *Aplysia* bag-cell neurons, dynamic MTs in the P region explore adhesion sites prior to changes

Table 1. Summary of tubulin-related deficits in axon guidance and brain development

Tubulin isotype	TUBA1A	TUBB2B	TUBB3	TUBA8
Number of reported mutations	32[15, 20-27]	15[12, 16, 17, 27-29]	14[13, 14]	1[18]
Mutation site(s)	p.I5L, E55K, L70S, L92V, V137D, Y161H, I188L, Y210C, D218Y, V235L, I238V, P263T, R264C, A270T, L286F, V303G, N329S, A333V, G366R, M377V, A387V, R390C, R390H, L397P, R402L, R402C, R402H, S419L, R422C, R422H, M425K, G436R	p. G98R, L117P, G140A, S172P, L207P, I210T, L228P, N256S, F265L, T312M, R380S, R380C, D417N, E421K, c.1080-1084 deletion	p. R62Q, G82R, T178M, E205K, R262C, R262H, A302V, A302T, M323V, R380C, M388V, E410K, D417H, D417N	14-bp deletion in intron 1
Cortex	Lissencephaly, pachygyria, and/or PMG	Lissencephaly, pachygyria, and/or PMG	MCD (microgyria, gyral disorganization and PMG)	Lissencephaly and PMG
Basal ganglia	Dysmorphisms and hypoplasia	Dysmorphisms	Dysmorphisms	N/A
Corpus callosum	Dysgenesis, Probst bundles (bundles of stalled axons)	Dysgenesis and dysmorphisms	Dysgenesis, Probst bundles adjacent to the midline	Dysgenesis
Anterior commissure	Hypoplasia in 1 patient, N/A in most cases	Hypoplasia in 1 patient, N/A in most cases	Dysgenesis, tortuous and aberrant axon projections at the midline in a knock-in mouse model	N/A
Internal capsule	Hypoplasia	Hypoplasia	Dysgenesis	N/A
Corticospinal tracts	Hypoplasia	N/A	Dysgenesis	N/A
Cerebellum	Hypoplasia	Hypoplasia	Hypoplasia	N/A
Brainstem	Hypoplasia	Dysmorphisms and hypoplasia	Dysmorphisms	Dysmorphisms
Cranial nerves	Hypoplasia in II	Hypoplasia in II and III	Hypoplasia of I, III, IV, VI, VII and X, axon projection defects of IV and V in a knock-in mouse model	Hypoplasia in II
Guidance signaling	N/A	N/A	Netrin/DCC	N/A

MCD, malformations of cortical development; N/A, not available; PMG, polymicrogyria.

in GC behavior and retrograde actin flow[35]. Laminin/integrin signaling promotes directional MT assembly and stabilization in axon development[36]. Combination of L1, laminin, and EphB alters the MT organization and distribution in paused retinal GCs, with increased numbers of MTs that extend into the P region of the GC and filopodia[7]. Disruption of MT dynamics in the GC by MT-stabilizing or destabilizing drugs completely abolishes both the GC attraction and repulsion induced by diffusible cues, such as Netrin-1[30]. The local stabilization of MTs in one side of the GC, using the focal pipette application approach or direct focal photoactivated release of the MT-stabilizing drug taxol, induces GC attraction toward the side of application[37]. Disruption of MT stabilization on one side of

a GC, using the MT-disrupting drug nocodazole, is sufficient to induce GC repulsion (turning away from that side)[30]. Intriguingly, the application of low concentrations of taxol promotes MT polymerization at plus ends and enhances axon outgrowth *in vitro* and *in vivo via* MT stabilization[38]. These studies suggest that intrinsically polarized MT dynamics in the GC may initiate and instruct the axon projection in response to extracellular guidance cues. Bath incubation with Semaphorin 3A (Sema3A) decreases MT exploratory behaviors in the GC and collapses MTs into the MT loop, whereas Netrin-1 causes opposite changes in MTs, increasing their splaying in the GC and axon shaft[31]. Wnt3a rapidly reduces the rate of axonal extension and subsequently increases GC enlargement and pausing *in vitro* through changes in the organization of MTs[39]. Time-lapse imaging reveals that Wnt3a regulates MT directionality and increases MT looping in the remodeled GC[39]. Thus, guidance cue-mediated GC navigation occurs in a MT dynamics-dependent manner.

However, whether MT dynamics are directly or indirectly regulated by guidance cues is still unclear. A recent study from our lab suggests that Netrin-1 directly regulates MT dynamics through the coupling of its receptor deleted in colorectal cancer (DCC) to TUBB3 during axon attraction[19]. TUBB3 co-localizes with DCC in the P region of developing spinal cord commissural and cortical neuron GCs, including both lamellipodia and filopodia[19]. Biochemical assays indicate that TUBB3 interacts directly with DCC and that Netrin-1 induces this interaction in primary neurons[19]. The Netrin-1-induced interaction of TUBB3 with DCC is dependent on MT dynamics because the disruption of MT dynamics either with taxol or nocodazole abolishes this interaction[19]. Results from an MT co-sedimentation assay demonstrate that Netrin-1 induces MT polymerization in dissociated neurons with more polymerized TUBB3 in the pellet *versus* the supernatant fraction, suggesting that Netrin-1 directly modulates MT dynamics[19]. Remarkably, DCC co-sediments with polymerized MTs and Netrin-1 further increases the co-sedimentation of DCC with stabilized MTs[19]. In addition, TUBB3 knockdown blocks both Netrin-1-induced spinal commissural axon outgrowth and attraction *in vitro* and causes defects in commissural axon projection *in vivo*[19], suggesting that TUBB3 is specifically involved in Netrin-1-promoted attraction. These results lead to a simple functional model that Netrin-1 signaling directly regulates MT dynamics through the coupling of its receptor DCC to TUBB3 (Fig. 2). Netrin-1-dependent initial local stabilization of MTs within the DCC complex on one side of the GC could lead to a differential increase in MT growth and a higher number of MT plus-ends on this side, which might influence actin dynamics and enable MTs to differentially protrude into this side of the GC and further promote GC protrusion on that side. At the same time, lamellipodia and filopodia on the other site of the GC collapse and the GC eventually turns towards the Netrin-1 source. In this model, in response to Netrin-1, the 'capture' of dynamic MTs by DCC in the GC is a critical step, which could stabilize filopodia against retraction and promote axon outgrowth and turning (Fig. 2). Interestingly, Src family kinase-dependent TUBB3 phosphorylation appears to be required for the subsequent interaction of TUBB3 with DCC and modulation of MT dynamics, suggesting that DCC serves as a signaling platform for the recruitment of a multiprotein complex, including TUBB3, Src family kinases, and other key signaling molecules to modulate MT dynamics in Netrin-1-induced axon outgrowth and turning[19].

Intriguingly, TUBB3 missense mutations lead to specific axon projection defects in commissural axon midline crossing (the corpus callosum and anterior commissure), considering it is widely expressed in the developing brain. The role of TUBB3 in Netrin-1 signaling fits well, albeit not exclusively, in the dysgenesis and organization of these axonal tracts in patients with TUBB3 mutations. Future studies are required to determine how TUBB3 mutations affect Netrin-1-mediated MT dynamics and axon guidance in the developing nervous system. In addition to Netrin functions, other guidance cues and cell-adhesion molecules are implicated in commissural axon guidance, such as bone morphogenetic proteins[40, 41], sonic hedgehog[42], Slits[43], Wnts[44, 45], Draxin[46, 47], axonin-1, and NrCAM[48]. It would be interesting to determine whether TUBB3 is involved in signal transduction cascades downstream of these guidance molecules. Since mutations of TUBB2B, TUBA1A, and TUBA8 share similar commissural axon projection defects in midline crossing, it is plausible that these tubulin isotypes also play a differential role in the aforementioned guidance signaling.

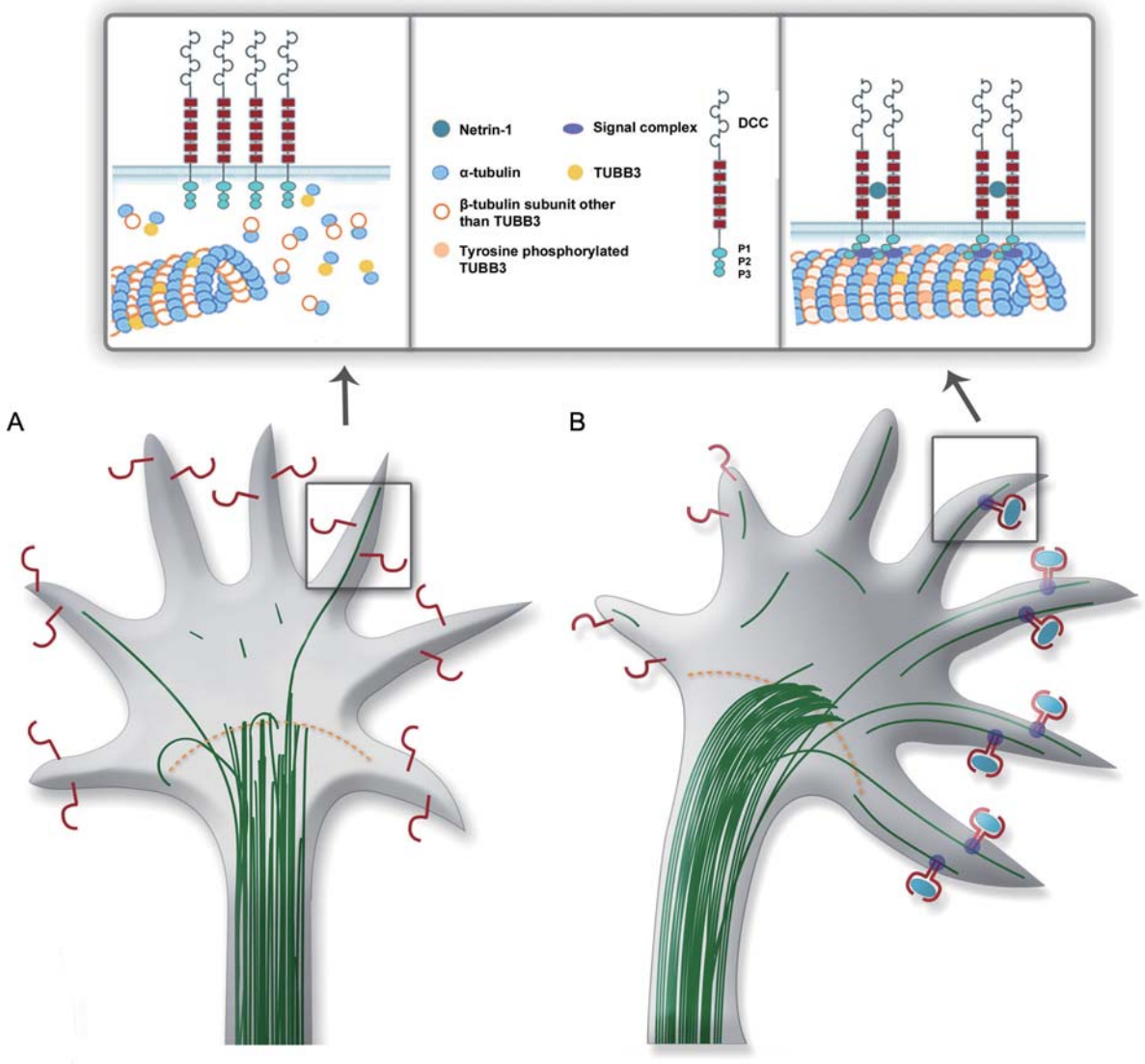

Fig. 2. Generalized model of direct involvement of MT dynamics in Netrin-1-promoted GC turning. Monomer DCC is evenly present on the GC (A) with unpolymerized tubulins in the P region (top left inset) in the absence of Netrin-1. Binding of Netrin-1 to DCC results in DCC homodimerization (B) and the recruitment of TUBB3, Src family kinases, and other key signaling molecules to form a 'molecular clutch' on the side of the GC close to the Netrin-1 gradient (top right inset). Netrin-1-induced MT polymerization/ stabilization occurs in the clutch site to polarize the GC and further maneuver GC steering (B). Actin dynamics in the GC is not included in this simplified model.

Microtubule-Associated Proteins (MAPs) in Axon Guidance

MT dynamics is regulated by various MAPs including the classical MAPs, which bind MTs along their entire length, and MT plus-end tracking proteins (+TIPs), which localize to the ends of growing MTs[3-5, 49]. MAP1B, an MT-stabilizing MAP, is highly expressed in the developing nervous system[50] and plays an important role in axon outgrowth and pathfinding[3, 4, 49]. Homozygous *MAP1B* mutants display a striking axon projection defect in the brain, the selective absence of the corpus callosum[51]. A follow-up study using microscale chromophore-assisted laser inactivation revealed that MAP1B is directly involved

in GC turning, suggesting that this is an axon guidance defect[52]. MAP1B knockdown by RNA interference in cultured cortical neurons alters the speed of MT growth in axons, resulting in axon outgrowth inhibition[53]. MAP1B binds mainly to dynamic MTs and promotes MT nucleation, polymerization, and stabilization *in vitro* and *in vivo*[54, 55]. In developing neurons, MAP1B is prominently located at the distal part of an extending axon and the GC, where the proportion of dynamic MTs is very high[56]. Primary neurons from hypomorphous *MAP1B* mutant mice present a reduced proportion of dynamic MTs in the distal part of the axon[57]. Phosphorylated isoforms of MAP1B are present at the highest concentrations in the distal axon and the GC of chick retinal ganglion cells and phospho-MAP1B inactivation on one side of the GC changes GC motility, morphology, and growth direction[52]. These results indicate that MAP1B involvement in GC steering relies on its function as a regulator of MT stability and dynamics.

Interestingly, the lack of MAP1B *in vivo* leads to dramatic abnormalities in the pontine nuclei and major axonal tracts such as the corpus callosum, the hippocampal commissure, the anterior commissure, and the reciprocal corticothalamic pathway. Most of these deficits are similar to the phenotypes of *Netrin-1*- and *DCC*-deficient mice[58-61], suggesting that MAP1B plays a role in Netrin-1-mediated axonal guidance. In addition, Netrin-1 stimulation of hippocampal and dorsal spinal cord explants from *MAP1B*-null embryos fails to induce axon outgrowth and attraction[61]. Netrin-1-directed axon outgrowth of developing neurons requires MAP1B phosphorylation through the activation of serine/threonine kinases cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3)[61]. Thus, it is likely that MAP1B plays an essential role in promoting MT dynamics preferentially on one side of the GC during Netrin-1-mediated chemoattractive turning. We propose that, in the developing GC, asymmetrical assembly of a signaling complex including DCC, TUBB3, Src family kinases, and MAP1B in response to a Netrin-1 gradient leads to a polarized increase in MT growth and stability on one side of the GC, which in turn promotes GC protrusion on that side and eventually controls GC turning towards the Netrin-1 source. While the defects in some axonal tracts, such as the anterior commissure and the entorhinal-hippocampal pathway, are almost identical to those in *Netrin-1*- and *DCC*-mouse mutants, other connections such as the reciprocal corticothalamic projections are much more severely affected in *MAP1B* mutants than in *Netrin-1*- and *DCC*-mutants[59], suggesting that MAP1B is involved in several signaling cascades governing axon projections in the developing nervous system. Consistent with this, canonical Wnt signaling modulates MT dynamics through a Dishevelled-dependent inhibition of GSK3β with the consequent regulation of MAP1B phosphorylation[62, 63].

Other classic MAPs, such as collapsin response mediator proteins (CRMPs), MAP2, and Tau, are also involved in axon guidance and neurite outgrowth. For example, CRMPs, cytosolic phosphoproteins highly expressed in developing neurons, are involved in the regulation of MT dynamics and axon outgrowth[64]. CRMP1–5 associate with tubulin[65, 66] and CRMP2 promotes axon growth through direct binding to tubulin and modulating MT dynamics[65, 67]. Hyperphosphorylation of CRMP2 disrupts MT assembly in neurites and is implicated in Alzheimer's disease[68]. CRMP2 regulates the transport of soluble tubulin to the distal parts of growing axons through binding to the kinesin-1 light chain[69]. In addition, CRMP2 is required for Sema3A-mediated repulsive signaling *via* the induction of GC collapse[68, 70]. Interestingly, CRMP5 forms a ternary complex with MAP2 and tubulins (α- and β-tubulin) which antagonizes CRMP2-induced axon outgrowth through a tubulin-based mechanism[66]. Thus, the interaction of CRMP5 with tubulin and MAP2 inhibits the tubulin polymerization and neurite outgrowth induced by CRMP2[66]. While MAP2 is involved in regulating neurite outgrowth through the association with MTs and other cytoskeleton elements[71], the role of MAP2 in axon guidance is unclear.

+TIPs bind to the rapidly-growing (+) ends of dynamic MTs, which are concentrated in the P region of the GC, and form comet-like assemblies along the ends of polymerizing MTs[3-5, 9, 72]. Many of these +TIPs are involved in a wide range of guidance signaling. For example, the cytoplasmic linker protein-associated protein CIs/Orbit/MAST/CLASP promotes MT rescue (the change from MT depolymerization to polymerization) and stabilization in the GC and is one of the first +TIP proteins implicated in Slit/Robo-mediated axon guidance *via* the non-receptor tyrosine kinase Abl[73, 74]. In the GC, CLASP associates with

both MT plus-ends and MT lattices with opposite functions: the plus-end-binding activity promotes axon outgrowth *via* MT stabilization, whereas the lattice-binding activity inhibits GC navigation *via* suppression of GSK3 activity[75]. The adenomatous polyposis coli (APC) protein, another +TIP, is a well-characterized signaling molecule that mediates the canonical Wnt/β-catenin signaling pathway. APC is highly expressed in the developing brain and concentrated in the GCs of dissociated neurons, where it binds to a subset of MTs to direct GC steering[76]. Local disruption of the interaction of APC and extending MTs abolishes GC turning behavior, including both attraction and repulsion[76]. Wnt3a increases APC loss from MT-plus ends and induces MT looping in the GC, resulting in a guidance defect[39]. Although APC2, the second APC family member, does not contain the APC domain associated with MT or +TIP binding, it is preferentially expressed in the nervous system and has been shown to stabilize MTs and play a role in the ephrin-A2-mediated guidance of retinotectal neurons[77]. APC associates with plasma membranes[78], so it is plausible that APC interacts with guidance receptors in GCs to regulate MT dynamics and stability in axon guidance.

Most +TIP proteins associate with end-binding (EB) proteins and require these 'core' +TIPs for plus-end tracking on growing MTs[49]. There are three EB proteins, EB1, EB2, and EB3. EB1 was first identified as an APC-binding protein and is required for the recruitment of APC to MT plus-ends[79]. In neuroblastoma cells, EB1 is localized to MT plus ends in neurites and GCs and plays an essential role in determining neurite length by regulating MT growth rate, growth distance, and duration[80]. Similarly, EB3 is preferentially expressed in brain, particularly in neuronal GCs, and involved in neuritogenesis *via* the coordination of dynamic F-actin-MT interactions[81]. Interestingly, MAP1B sequesters EB1 and EB3 in the cytosol of developing neurons through direct interactions, which do not require MT integrity[82]. The binding of MAP1B to EB3 is regulated by phosphorylation mediated by proline-directed kinases such as GSK-3 and CDK5, but not non-proline-directed kinase casein kinase 2 (CK2)[82]. Overexpression of MAP1B in N1E-115 cells inhibits EB protein binding to MT plus-ends, whereas MAP1B knockdown increases EB binding to MT growing-ends and to the MT lattice[82]. The interaction of EB3 with MTs is also enhanced in the GCs of primary MAP1B-knockdown neurons[82]. The excessive EB3 binding to MTs and induction of MT looping in the GC of *MAP1B*-deficient neurons likely lead to changes in MT dynamics, in particular overstabilization, which impairs GC navigation and affects axon outgrowth[82]. Therefore, too much or too little MAP1B disrupts EB protein-dependent MT growth and stability in the GC and further blocks axon projection. These results suggest that MAP1B functions as a direct regulator of EBs to modulate MT dynamics during neurite and axon extension. Although these studies have shown that +TIPs are essential for the regulation of axon outgrowth from developing neurons, results from a yeast two-hybrid screen and a GST pull-down assay reveal that all three EB protein members interact with plexin-A2, B1, and B3[83] and these interactions play an important role in regulating neurite growth of Neuro 2A cells[83], suggesting that they play a role in ephrin/Eph-mediated axon projection. Further studies are required to determine whether other guidance receptors also associate with EBs and whether the functional importance of +TIPs in GC turning is mediated by multiple guidance cues.

MT-regulating kinesins belong to the unconventional kinesin family which modulates MT assembly and/or disassembly rather than functioning as a molecular cargo transporter. These regulatory kinesins act as either MT elongases, pause factors, or depolymerases to regulate MT organization and dynamics[84]. Non-motile kinesin-13 family members, such as KIF2A, KIF2B and KIF2C/MCAK (mitotic centromere-associated kinesin), can also identify and stabilize curved protofilaments at MT ends to promote MT depolymerization[85]. KIF2A is highly expressed in developing neurons and *KIF2A*-knockout mice exhibit neuronal migration defects, abnormally-elongated collateral branches of axons, and severe sensory axon target hyperinnervation[86, 87], suggesting that KIF2s play an important role in axonal branching and pruning during brain development. Kinesin-4 KIF21A, a cortical MT growth inhibitor, strongly accumulates in the axonal GC[88]. Heterozygous missense mutations in *KIF21A* cause CFEOM1, a dominant neurodevelopmental disorder associated with axon-guidance defects[88, 89]. Expression of wild-type or mutant KIF21A in primary neurons increases the accumulation of KIF21A in the GC, and reduces the proportion of the GC with a fan-like morphology and

GC motility, as well as suppressing the responsiveness to Sema3F, a repulsive guidance cue[88]. Although the formation of shorter and branched axons induced by increased KIF21A levels is believed to cause alterations in axonal target innervation, the exact signaling mechanism underlying KIF21A-mediated Sema3F repulsion remains elusive.

Signaling Pathways That Regulate MT Dynamics in Axon Outgrowth and Guidance

Intracellular signal transduction pathways initiated by different guidance cues likely engage in cooperative crosstalk during axon guidance, which eventually converges on the modulation of MT stability and actin dynamics. Several key regulators appear to regulate MT dynamics directly in GCs[39, 73, 90]. For example, GSK3 is involved in multiple guidance pathways including the Wnt, Netrin-1[61], Sema3A[91], Slit2[92], and neurotrophin pathways, and it is known to regulate MT dynamics and assembly by phosphorylating multiple MAPs including APC, MAP1B, CRMPs, CLASPs, Tau, MAP2, and stathmins[4, 93, 94]. In general, inhibition of GSK3β-dependent phosphorylation of these MAPs directly modulates MT behavior which affects axon outgrowth and guidance. In the axonal GC, GSK3β-mediated MAP1B phosphorylation is required for maintaining MTs in a dynamic state and axon outgrowth and pathfinding[95]. Netrin-1 regulates mode I MAP1B phosphorylation and MAP1B activity through GSK3 and CDK5 both *in vivo* and *in vitro*[61]. MAP1B is required for Netrin-1-mediated chemoattraction *in vitro* and *in vivo*. Slit2 induces GSK3β phosphorylation and inhibits neurite outgrowth in adult dorsal root ganglion neurons[92]. In addition, the sequential phosphorylation of CRMP2 by CDK5 and GSK3β is necessary for Sema3A-induced GC collapse through MT reorganization[68, 70]. Inactivation of GSK3β by Wnts results in a significant decrease in the phosphorylation of MAP1B[63, 96], which leads to an increase in MT stability affecting axon outgrowth. The effects of Wnts on MT dynamics and GC behavior could be achieved through inhibition of phosphorylation of other MAPs induced by GSK3β[97]. Inactivation of GSK3β reduces CRMP2 phosphorylation, increasing its ability to bind tubulin and promoting MT assembly, whereas APC phosphorylation by GSK3β reduces the binding of APC to MT plus-ends[90, 98, 99]. Interestingly, NGF-induced axon growth is dependent on local inactivation of GSK3β at the distal axon, which results in the accumulation of dephosphorylated APC at MT plus ends and the promotion of MT assembly[90]. However, suppression of GSK3 activity to a greater extent inhibits axon growth in embryonic cortical neurons, suggesting that a precise balance of GSK3 activation and inactivation is required for efficient axon outgrowth in the developing nervous system[75, 100]. This dual function of GSK3 on axon growth is mediated by its physiological substrate CLASP2[75].

Mitogen-activated protein kinases (MAPKs) control the phosphorylation status and activity of several MAPs and are implicated in the regulation of axon outgrowth, guidance, and regeneration. c-Jun N-terminal kinases (JNKs) are strongly expressed in the developing nervous system and play an important role in axon outgrowth and guidance *in vitro* and *in vivo*[101, 102]. *JNK1*-deficient mice reveal defects in anterior commissure formation and axonal MT integrity[101]. JNK1 has recently been shown to play an essential role in Netrin-1-mediated axon outgrowth and attraction[102]. Activated JNK is strongly expressed in spinal cord commissural axons before and as they cross the floor plate[102]. Bath incubation with Netrin-1 dramatically increases the level of endogenous phospho-JNK in commissural axon GCs[102]. Netrin-1 increases JNK1 activity in the presence of DCC or Down syndrome cell adhesion molecule (DSCAM), two Netrin receptors, and the expression of both receptors further enhances Netrin-1-induced JNK1 activity[102]. Netrin-1-induced JNK1 activity is blocked by inactivation of the JNK pathway both *in vitro* and *in vivo*[102]. DCC collaborates with DSCAM to regulate JNK activity in Netrin signaling[102]. Netrin-1-induced axon outgrowth and attraction is inhibited either by JNK1 knockdown or a JNK inhibitor[102]. Expression of JNK1 shRNA *in ovo* causes defects in spinal cord commissural axon projection and pathfinding[102]. These studies indicate that JNK1 is specifically involved in the coordination of DCC and DSCAM in Netrin-1-mediated attractive signaling. Furthermore, JNK-deficient mice exhibit hypophosphorylation of MAP1B[101], suggesting that the JNK1 pathway is specifically involved in axon guidance *via* regulation of MAP-mediated MT dynamics in the GC.

Many JNK substrates are MAPs, such as doublecortin (DCX), superior cervical ganglion 10 (SCG10), and Tau, which control MT dynamics and stability in the GC. DCX, a MAP expressed in the developing nervous system, plays an important role in neuronal migration and axon guidance[103, 104]. DCX is enriched in axonal GCs[105] and stabilizes MTs in developing neurons[104, 106]. Although *DCX*-knockout mice display defects in axon tracts[104], there is no direct evidence to link DCX to specific guidance cues. Whether the effects of Netrin-1 on axon outgrowth and pathfinding might be mediated through the JNK-DCX-MT dynamic pathway needs further evaluation. SCG10, a MAP in axons, is also a JNK substrate and plays an important role in axonal outgrowth by modulating MT stability[107, 108]. In developing neurons, phospho-JNK1 and SCG10 are enriched in GCs[102, 109] and control the balance of MT assembly and disassembly *via* the sequestration of tubulin dimers or the severing of polymerized MTs[110, 111]. SCG10-mediated regulation of the GC MT cytoskeleton is also involved in EphB-mediated axon guidance[7]. Whether the JNK-SCG10 pathway is involved in Netrin signaling is not clear. Tau functions as a MAP and is differentially localized in the distal end of the axon[112]. Downregulation of Tau levels in neurons using antisense oligonucleotides inhibits axonal outgrowth[113]. Interestingly, *Tau*-deficient mice exhibit normal brain development[114, 115] due to possible compensatory increases in MAP1A[116]. Mice devoid of both *Tau* and *MAP1B* suffer premature death and manifest significant neuronal and axonal defects[117]. Tau stabilizes MTs through the regulation of tubulin-tubulin interactions along the protofilament[118] and promotes MT stability within the axon[118]. However, it remains unclear how Tau regulates MT dynamics in axon guidance. Tau actively interacts with various signaling partners, including Src-family kinases, phosphoinositides, and PLCγ[119]. Phosphorylation of Tau within its MT binding site at Ser262 by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) is required for Wnt5a-mediated axon outgrowth and repulsion through modulation of dynamic MTs in the GC[120].

Localized cytosolic Ca^{2+} signals in the GC are known to mediate axon turning[121-125]. CaMKII and calcineurin (CaN) phosphatase, two frequency-dependent Ca^{2+} effectors, function as a switch to control the direction of GC steering: preferential activation of CaMKII by a relatively large local Ca^{2+} elevation promotes attraction, whereas activation of CaN by modest local Ca^{2+} levels induces repulsion[121]. CaMKII/CaN has been shown to mediate multiple guidance signaling, such as Netrin-1, Sema2a, and Wnts[126, 127], indicating that differential activation of CaMKII and CaN phosphatase is specifically involved in GC steering. It is well known that tubulin proteins and MAPs, such as MAP2 and Tau, can be phosphorylated and dephosphorylated by CaMKII and CaN-PP1, respectively, which affects MT assembly, stability, and dynamics in the GC[128-130]. Therefore, differential activation of the Ca^{2+}-dependent CaMKII/CaN pathway controls GC navigation depending on asymmetric local modification of MT stability and dynamics in the GC. Indeed, Wnt5a gradients can induce CaMKII-dependent asymmetric redistribution of dynamic MTs in the GC, which is required for Wnt5a-mediated axon repulsion[120].

The Ras superfamily of small GTPases, consisting of Rho, Ras, Rap, Arf, Sar, and Ran, also plays a crucial role in controlling axon turning *via* modulation of the F-actin and MT dynamics in the GC[4, 5, 131-133]. Local stimulation with guidance cues or extracellular adhesion molecules triggers asymmetric signaling of the Rho family GTPases RhoA, Rac1 ,and Cdc42, which in turn locally modulate actin and MT assembly, disassembly, and organization in the GC to orient axon outgrowth and projection[131, 134]. Several MAPs, Short Stop/ACF7, MAP1B, CLASPs, CRMPs, and APC, are regulated by multiple small GTPases[4, 5, 131, 135]. Therefore, the asymmetrical localization and redistribution of guidance receptors in the GC in response to guidance cues or adhesion molecules may lead to the assembly of an asymmetrical signal complex including small GTPases, MAPs, and MTs to polarize GC navigation[134].

Final Thoughts

A 'search and capture' model of MT regulation has been proposed for more than two decades[136]. In this model, exploring MTs in the cytoplasm are captured at specific cellular sites and transiently stabilized to initiate several biological processes, such as directing vesicle traffic and chromosome separation[136, 137]. GC turning is preceded by asymmetric enrichment of F-actin and MTs. The discovery of the direct interaction of DCC and TUBB3 as essential linking factors between MTs and Netrin signaling validates

this model at the molecular level in GC turning. Dynamic MTs 'captured' by attractive receptors on one side of the GC lead to a differential increase in MT growth and stabilization on this side, which could stabilize filopodia against retraction and promote axon outgrowth and attraction (Fig. 2). It is tempting to speculate that dissociation of dynamic MTs and repulsive receptors may result in the collapse of GC lamellipodia and filopodia inducing axon repulsion. Further studies are required to evaluate this model.

Clearly, many important questions about the regulation of MT dynamics in axon guidance remain to be answered. For example, where are MTs in the GC generated: the cytoplasm, axon shaft, or the C or P region of the GC during axon turning? How many MAPs or guidance receptors are specifically involved in directly regulating MT stability and dynamics in axon outgrowth and pathfinding? How do multiple signal cascades initiated by different guidance cues coordinate to regulate MT dynamics in GC steering? Since the coordination of F-actin and MT dynamics plays an essential role in axon guidance, it would be interesting to untangle the role of actin dynamics in these models as well: how MTs and F-actin work together to influence axon turning; how they interconvert; and how they regulate, or are regulated by MAPs and/or actin-binding proteins in axon guidance. The combination of super-resolution microscopy with genetics, biochemical assays, sophisticated axon turning assays, and fluorescence cytochemistry will allow us to better understand how guidance cues spatiotemporally modulate intracellular MT dynamics in the GC to control axon outgrowth and pathfinding.

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·Review·

Glial cells in neuronal development: recent advances and insights from *Drosophila melanogaster*

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Glia outnumber neurons and are the most abundant cell type in the nervous system. Whereas neurons are the major carriers, transducers, and processors of information, glial cells, once considered mainly to play a passive supporting role, are now recognized for their active contributions to almost every aspect of nervous system development. Recently, insights from the invertebrate organism *Drosophila melanogaster* have advanced our knowledge of glial cell biology. In particular, findings on neuron-glia interactions *via* intrinsic and extrinsic mechanisms have shed light on the importance of glia during different stages of neuronal development. Here, we summarize recent advances in understanding the functions of *Drosophila* glia, which resemble their mammalian counterparts in morphology and function, neural stem-cell conversion, synapse formation, and developmental axon pruning. These discoveries reinforce the idea that glia are substantial players in the developing nervous system and further advance the understanding of mechanisms leading to neurodegeneration.

Keywords: glia; neuronal development; Gcm; neurodegeneration; neural stem cell; synapse formation; axon pruning

Introduction

Nervous systems sense environmental inputs and cellular cues and their development, which mainly relies on the correct differentiation of two cell types, neurons and glia, is a vital process for animals to execute functions such as cognition, learning, and memory. At the cellular level, neurons develop from undifferentiated progenitor cells (neural stem cells) to differentiated cells with compartmentalized structures like axons and dendrites that mediate pathfinding, information processing, and synaptic connections. Almost every aspect of this developmental process and subsequent neuronal activity are under precise regulation by factors such as signaling components and the surrounding milieu. Interestingly, a major part of these regulatory mechanisms is mediated by glia, the partners of neurons. It is known that glia play essential roles by providing extrinsic signals to neurons and acting as part of the niche required for neuronal development and function.

Conventionally, glia have been considered to play a passive supporting role due to a lack of electrical excitability for transducing information like neurons. Nonetheless, compelling evidence has demonstrated that glia participate actively in mediating a number of neuronal events such as axon guidance, peripheral axon ensheathment, and formation of the blood-brain barrier to protect the central nervous system (CNS)[1-5]. On the other hand, a tripartite model that includes glia has recently been proposed to revise the classical view of synaptic structure[6-8]. In addition to the presynaptic and postsynaptic compartments, adjacent glia, particularly mammalian astrocytes, are now envisioned as one of the major components integrating synaptic function by releasing gliotransmitters, promoting synapse formation, and regulating synaptic plasticity[9]. Intriguingly, studies from the invertebrate model organism *Drosophila melanogaster* have offered abundant insights

into how *Drosophila* glia, resembling their mammalian counterparts, function to interact with neurons and regulate development. These recent advances have now implicated glia in other previously-unrecognized functions.

Several recent articles have provided excellent overviews of the origin and development of glia[10-14]. In this review, we explicitly summarize glial functions that have emerged as key mechanisms in the regulation of neuronal development in *Drosophila*. We describe the distinct classes of *Drosophila* glia, followed by a discussion of how they modulate neural stem-cell behavior, an extrinsic regulatory step during the early stage of neural fate decision. Next, we discuss how glia secrete different factors to affect the development and function of the neuromuscular junction (NMJ). Finally, we compare the glia-derived two-step secretion/engulfment mechanism in NMJ remodeling with axon pruning of mushroom body (MB) γ-neurons. Altogether, these recent discoveries point to a significant role for glia during neuronal development, and provide novel insights into mechanisms leading to a destabilized state of the nervous system, as in neurodegeneration.

Drosophila Glia

The genetically-tractable organism *Drosophila* has been an excellent animal model in advancing our understanding of glial biology. Distinct classes of glia are based on their morphology and function similar to their mammalian counterparts[10, 13, 15-17] (Table 1 and Fig. 1). Surface glia, the outermost layer of protection surrounding the larval and adult CNS, comprises two subtypes, the perineurial and subperineurial glia (SPG). These glial cells exclusively function as a blood-brain-barrier to prevent unwanted molecules over a certain size from entering the CNS[2,5,16]. The protection is mainly mediated by SPG, which form

Table 1. *Drosophila* glial cells are categorized into four groups according to their function and distribution[9,10,13,16]

Drosophila glia	Subtype	Distribution	Primary function Corresponding mammalian counterparts
CNS			
Surface glia	Perineurial glia (PG)	CNS surface (outer layer)	Blood-brain barrier (BBB)
	Subperineurial glia (SPG)	CNS surface (underneath PG)	Blood-brain barrier (BBB) Glia-glia pleated septate junctions (pSJs)
Neuropil glia	Ensheathing glia	Synaptic neuropil (outside)	Axon ensheathment Mammalian counterpart: Oligodendrocytes
	Astrocyte-like glia (Reticular glia)	Synaptic neuropil (infiltrated)	Axon ensheathment and pruning Engulfment activity Mammalian counterpart: Astrocytes
Cortical glia		In the cortex around neuronal cell bodies	Gas and nutrient exchange Mammalian counterpart: Astrocytes
PNS			
Peripheral glia	PG	Peripheral nerves (outer layer)	Axon ensheathment Mammalian counterpart: Schwann cells
	SPG	Peripheral nerves (underneath peripheral PG)	
	Wrapping glia	Inner layer contacting axon	

CNS: central nervous system; PNS, peripheral nervous system.

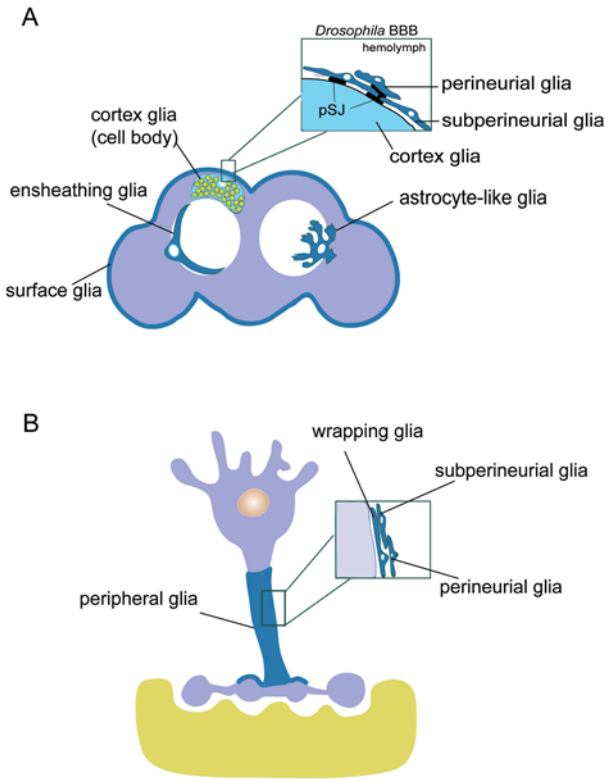

Fig. 1. *Drosophila* glia. A: In this schematic cross-section of *Drosophila* brain, four types of CNS glia are shown in blue: cortical glia (neuronal cell bodies in green), surface glia, and neuropil glia, which include ensheathing glia and astrocyte-like glia. The insert shows the subtypes of surface glia. Perineurial and subperineurial glia are shown in close association with the cortical glia. These glial cells function as a blood-brain-barrier to protect the CNS. Pleated septate junctions (pSJs) within subperineurial glia are in black. B: Schematic of *Drosophila* peripheral glia in the neuromuscular junction with a presynaptic axon (purple) and postsynaptic muscle (yellow). Three subtypes of peripheral glia are shown in blue: perineurial, subperineurial, and wrapping glia. These glia wrap around the axons of motor neurons up to the proximal synaptic bouton and regulate synapse formation and function.

pleated septate junctions among themselves. The components of pleated septate junctions are known to be homologs of proteins forming the paranodal junctions between axons and glia at the node of Ranvier in mammals[3,18]. In regards to size, SPGs have large and flatted cell bodies and are few in number, whereas perineurial glia have smaller cell bodies and higher numbers (Fig. 1A).

Cortical glia, also termed cell-body-associated glia, are structurally similar to mammalian astrocytes. Cortical glia wrap around the neuronal cell bodies at the outer surface of the brain, mediate gas exchange between cell bodies and the trachea, and provide trophic support. The third glial subtype is neuropil glia; these are closely associated with the neuropil regions containing bundles of axons and ensheath the synaptic neuropil like mammalian oligodendrocytes. Two types of neuropil glia are present in *Drosophila*: ensheathing glia, which surround the synaptic neuropil, and astrocyte-like glia that infiltrate into the inner region of the neuropil volume. Finally, CNS-derived peripheral glia are also subcategorized into three types[3-5,16]. The innermost type in contact with axons is termed wrapping glia; this is also considered to be a subtype of neuropil glia due to its association with nerves. Immediately above the wrapping glia is the peripheral perineurial glia and SPG. As in the CNS, these SPGs also form pleated septate junctions and provide insulation for axons (Fig. 1B).

It is noteworthy that microglia, the resident immune cells, engulf cell debris to protect the integrity of the nervous system. Unlike mammals, there is no corresponding microglial subtype in *Drosophila*. In terms of engulfing activity, at least two subtypes of *Drosophila* glia have been shown to execute this function[19, 20].

Glia Modulate Neural Stem-Cell Behavior

Drosophila neural stem cells, also termed neuroblasts (NBs), are plastic with an undifferentaited nature and serve as an excellent model to study stem-cell biology[21-23]. During the first wave of neurogenesis, embryonic NBs undergo asymmetric division to generate a smaller ganglion mother cell, which divides once more to produce differentiated neurons and/or glial cells, and another NB with self-renewal potential[21, 24]. These NBs generate most of the larval CNS neurons and enter a quiescence period for ~24 h at the end of embryogenesis[24-27]. How these NBs are reactivated during the larval stage remains largely unclear. However, once reactivated, they continue to divide and generate the neurons needed for the adult CNS. During larval neurogenesis, a different NB type, type II, produces a transient amplifying intermediate neural progenitor cell which undergoes extra rounds of division to

produce greater numbers of neurons than type I NBs[28-30]. These NBs, patterned by the distinct temporal and spatial expression of transcription factors, orchestrate the order and diversity of neural progeny in both the larval and adult CNS[22,23,28].

Glia participate in distinctive ways throughout this developmental process[31-37]. A fat body-glia-NB signaling relay has been demonstrated to regulate NB reactivation after quiescence[38, 39]. Within this relay, the insulin/insulin-like growth factor signaling pathway with the downstream effector PI3K/Akt, the central regulator of growth and metabolism, is activated in NBs by insulin-like peptides (dILPs) secreted by glia. These dILPs, in particular dILP2 and dILP6, bind to the single insulin/insulin-like growth factor receptor and are secreted upon the delivery of a nutrient signal from the fat body. This tripartite relay then allows the NBs to exit from quiescence and reactivate.

Typically, dILPs are secreted by insulin-producing cells in the larval brain to execute their function during cell growth and proliferation[40]. The discovery that glia are capable of secreting some of these peptides suggests an alternate route for converting the fat body signals into paracrine dILP function, hence diversifying their target list. This particular group of glia, surface glia, is adjacent to the NBs and associates with the surface to wrap around the CNS (note that Sousa-Nunes *et. al.* suggested that cortical glia are responsible for the secretion[39]). Surface glia are ideally positioned to transmit signals from the fat body to modulate NB reactivation. It is worth noting that glia also express additional factors such as the glycoprotein Anachronism (Ana)[41], dPerlecan[42-44], the RNA-binding protein FMRP implicated in Fragile X syndrome[45], and another type of secretory peptide, the activin-like peptides[46, 47], all of which have been reported to contribute to NB reactivation in different ways (Fig. 2A).

Later during development, after NB reactivation,

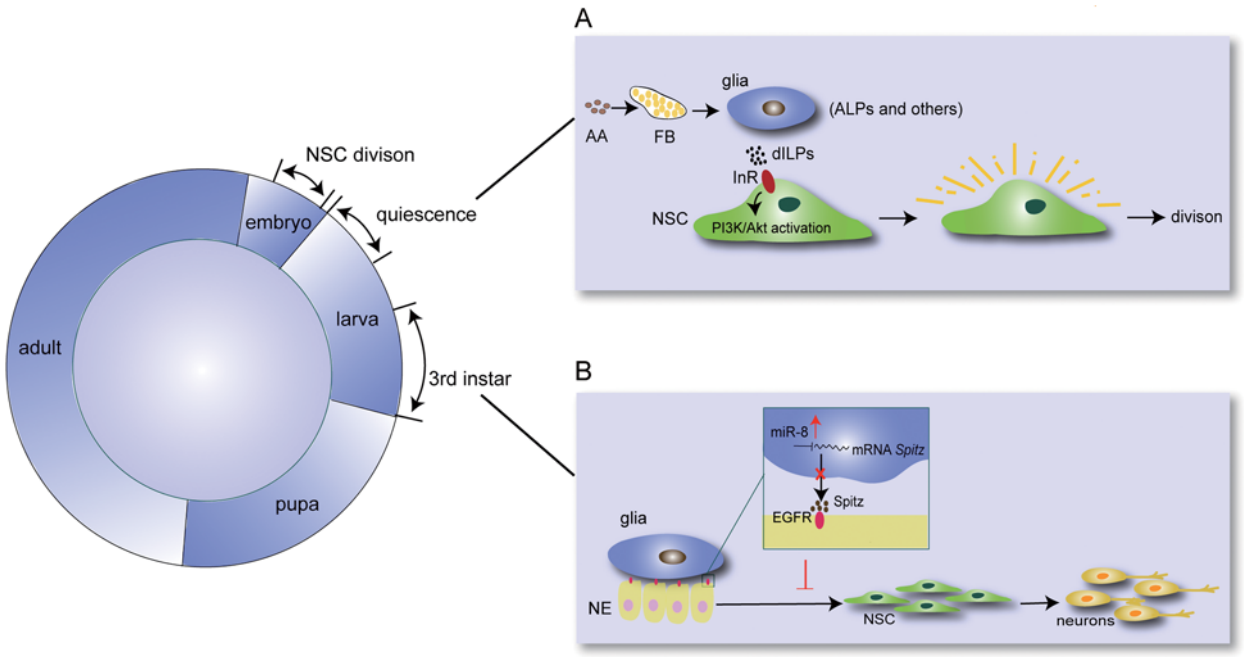

Fig. 2. ***Drosophila*** **glia modulate neural stem-cell behavior. The life cycle of** ***Drosophila*** **from embryo to adult is illustrated in the left panel. A: The three-step fat body-glia-NB relay. An amino-acid-triggered fat body signal is delivered to the surface glia, which are ideally positioned to release dILPs to activate the insulin receptor (InR) expressed in the NBs. This action in turn reactivates NBs from quiescence. Glia also secrete other factors such as activin-like peptides (ALPs) to modulate NB reactivation. B: In** ***Drosophila*** **optic lobe, neural stem cells (NSCs) are transformed from neuroepithelial (NE) cells and this transition is regulated by the optic-lobe-associated glia expressing the microRNA miR-8. miR-8 inhibits the translation of the epidermal growth factor receptor (EGFR) ligand** ***Spitz*****, abolishes its secretion by glia and interaction with EGF receptors on NE cells. This glial regulation suppresses the NE-to-NSC transition.**

glia regulate the transition from neuroepithelial (NE) to neural stem cells in the developing larval optic lobe. This transition, an event orchestrated in a manner similar to the epithelial-to-mesenchymal transition in mammals[48], is an ideal system to study an effect of the glial niche on stem-cell behavior. One of the recent studies using this system has revealed a specific glial subtype below the SPGs, the optic-lobe-associated glia, that express the microRNA miR-8, a homolog of mammalian miR-200. Glial-specific expression of miR-8 locally inhibits translation of the epidermal growth factor receptor ligand Spitz, affecting the ligand-receptor interaction on the NE cell membrane, and leading to the dysregulation of NE expansion and NB transition. In contrast, miR-8 positively regulates glial size, suggesting a dual effect on both glia and the neighboring NE cells[49] (Fig. 2B).

In summary, different populations of *Drosophila* glia function in diverse ways to regulate stem-cell behavior. Similar to mammals, a glial niche environment organized by glia and other cell types is required for NB conversion, reactivation after quiescence, and ultimately during the proliferative developmental phase (Fig. 2).

Glia-derived Factors during Synapse Formation and Function

The *Drosophila* NMJ is a widely-used model for studying synapse formation and activity. These synapses are glutamatergic, stereotypically positioned, and resemble mammalian central synapses in terms of the neurotransmitter used[50, 51]. Compelling evidence has shown that glia, closely associated with these synapses, modulate synaptic activity and synapse formation[52-56]. Among the three types of peripheral glia, perineurial glia and SPGs, but not wrapping glia, send processes into the NMJ[56]. These processes display a variety of morphological structures along the motoneuron axons to the point of nerve-muscle contact, and sometimes extend into the proximal synaptic bouton, yet never completely cover the NMJ[5, 53, 56, 57].

Recent advances have uncovered a critical role for peripheral glia during NMJ formation and function. Wingless (Wg)/Wnt, identified by chromatin immunoprecipitation analysis as a downstream target of the glial transcription factor Reversed polarity (Repo), is secreted by glia to mediate postsynaptic glutamate receptor clustering[55]. Unlike the Wg/Wnt released from motoneurons, which regulates both NMJ growth and postsynaptic glutamate receptor clustering in a manner dependent on dFrizzled2 (dFz2) receptors[58-61], glia-derived Wg/Wnt does not affect NMJ size, but regulates postsynaptic function as revealed by electrophysiological studies[55]. Furthermore, peripheral glia secrete another factor, the TGF-β ligand Maverick (Mav), that binds postsynaptically to a not-yet-identified receptor (likely the TGF-β type II receptor Punt) and turns on Gbb transcription *via* the cascade of Mad phosphorylation and Co-Smad Medea (Med) interaction. Gbb is the central effector of the retrograde signaling from muscle to presynaptic motoneuron and it does do by interacting with the bone morphogenetic protein (BMP) receptors Wishful thinking (Wit), Saxophone (Sax), and/or Thickvein (Tkv). Interaction with this receptor brings about Mad phosphorylation, hence regulating the expression of the Rac-activating gene *trio* and synaptic growth[52, 62, 63] (Fig. 3).

Bimodal Regulation of Synaptic Remodeling by Glia

In addition to synapse formation and function, remodeling events that occur during synaptogenesis to shape the synaptic contact are also regulated by glia. For instance, the tumor necrosis factor-alpha (TNF-α) factor Eiger expressed by peripheral SPGs mediates a glia-derived pro-degenerative signaling event that controls axonal and synaptic degeneration. Severe presynaptic degeneration of the NMJ, indicated by fragmentation of presynaptic membranes, occurs when the functions of cytoskeletal molecules like Spectrin or Ankyrin are disrupted[64-66]. Loss of Eiger significantly suppresses the presynaptic degeneration induced by the absence of Ankyrin, suggesting a role for Eiger in mediating the degeneration of these presynaptic materials[54]. Upon secretion from glia, Eiger interacts with the TNF receptor Wengen in neurons, triggering the downstream caspase Dronc-Dcp1 pathway that induces axonal and synaptic degeneration. In addition, mitochondria-based signaling mediated by DARK and Debc1 is proposed to work with the caspase pathway to augment the response to the glia-derived pro-degenerative signal[54].

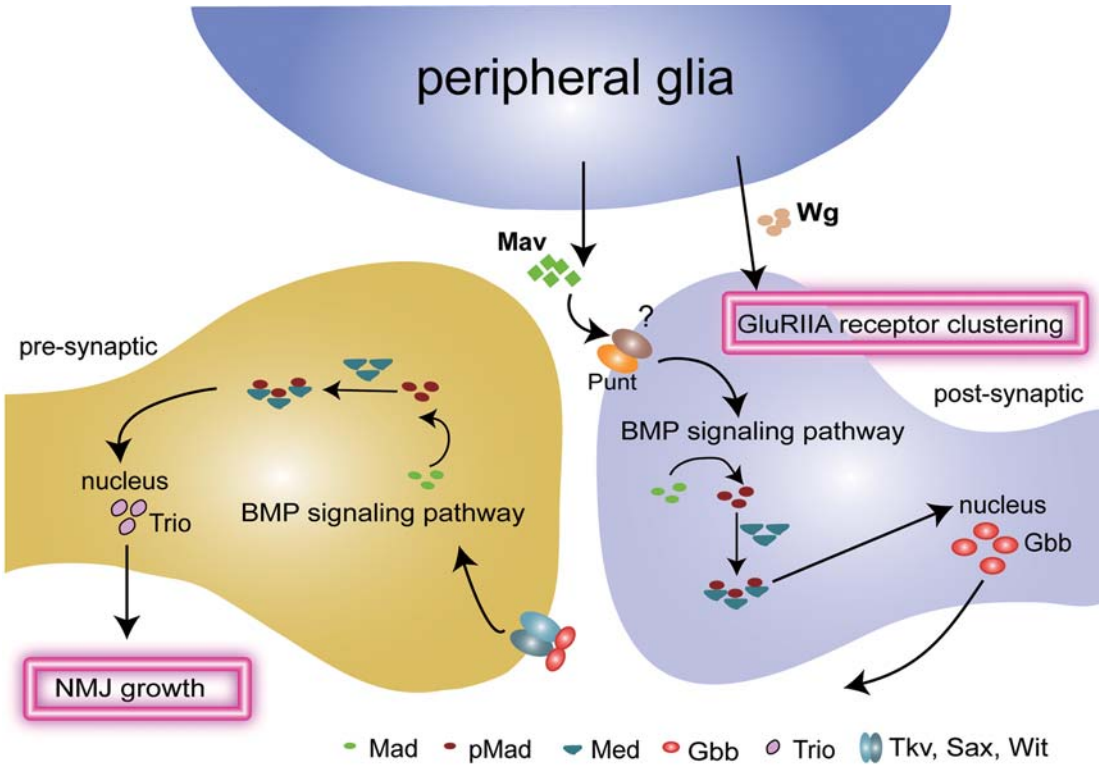

Fig. 3. Glia-derived factors regulate NMJ formation and function. In the *Drosophila* NMJ, adjacent peripheral glia secrete Wg to regulate postsynaptic function *via* glutamate receptor clustering. Glia also secrete another TGF-β ligand Mav, which acts postsynaptically to turn on BMP signaling *via* Mad phosphorylation and Mad-Med interaction. Upregulated BMP signaling tunes the transcription of Gbb, which is released from muscle to the presynaptic compartment to activate BMP signaling in motoneurons. This retrograde Gbb signaling controls Trio expression, which then regulates NMJ growth and size.

Reasonably, upon degeneration, these fragmented presynaptic membranes need to be removed to create an environment for the synapse to "remodel" under normal cellular dynamics. A recent study has shown that these extra presynaptic materials, including fragmented membranes (also termed presynaptic debris) and undifferentiated boutons (also termed ghost boutons) represented by the lack of active zones and postsynaptic proteins[53, 67], have been detected in motoneurons either naturally or upon light-stimulation of neurons expressing channelrhodopsin-2. In addition to the Eiger-dependent instructive signals provided by glia, these extra materials of presynaptic origin are removed *via* an engulfment process also mediated by adjacent peripheral glia. In particular, downregulating the expression of the engulfment receptor *draper* in glia results in an accumulation of presynaptic debris, but does not affect the presence of ghost boutons. It is worth noting that *draper* expression in muscle is also required for the engulfment process, but only for the disappearance of ghost boutons, indicating distinctive mechanisms by which glia and muscle control different presynaptic materials[53] (Fig. 4A).

Developmental Axon Pruning: Two-step Mechanism Mediated by Glia

A similar two-step glia-mediated mechanism has been ascribed to the axon pruning of MB γ-neurons. During metamorphosis, extensive remodeling of axons and dendrites occurs in order to accommodate the need for an adult neuronal circuitry. Notably, γ-neurons of the MB, the center for learning and memory in *Drosophila*, serves as an excellent model for understanding the mechanism underlying this dynamic process. Beginning in the late

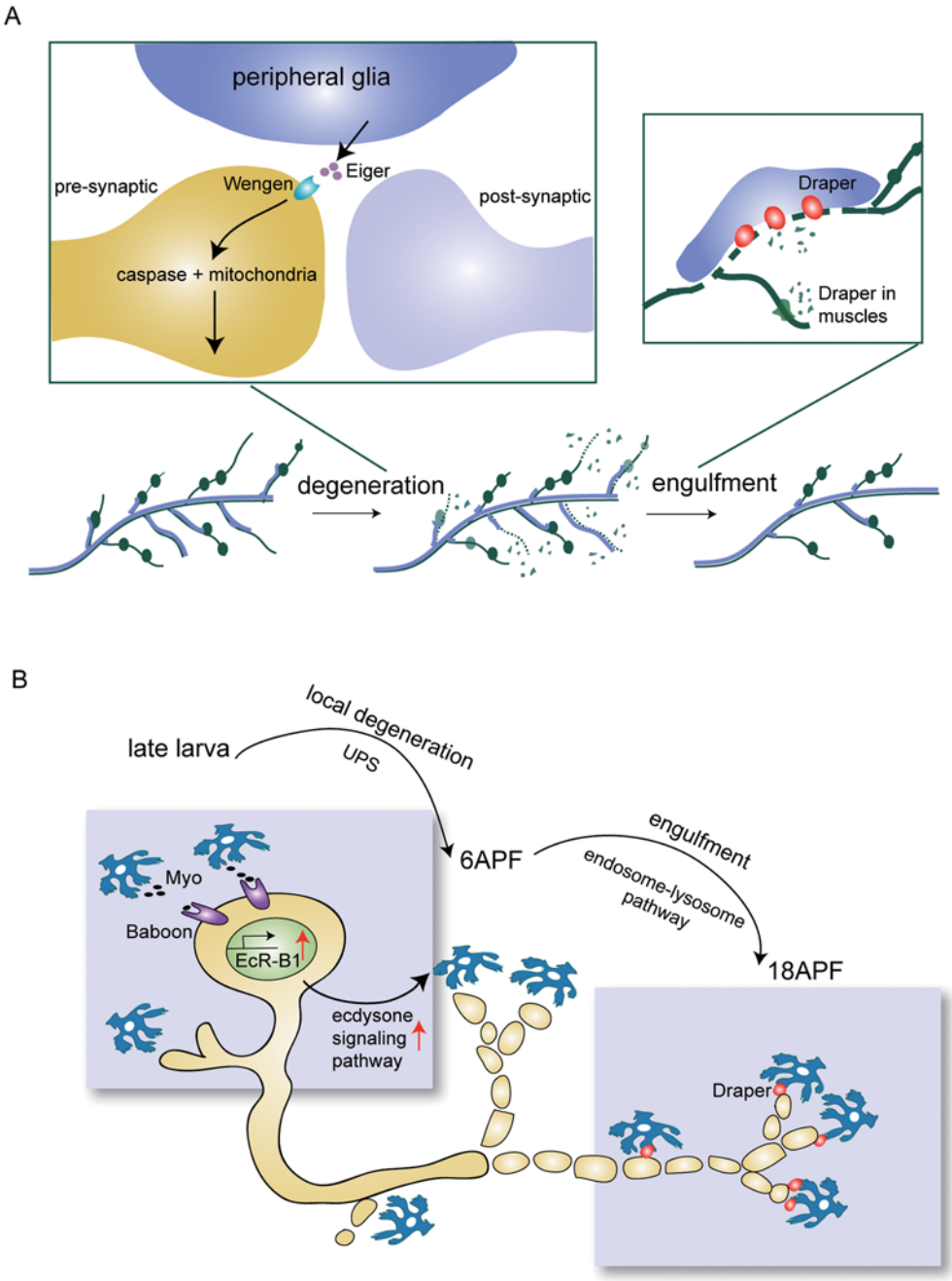

Fig. 4. Synaptic remodeling and axon pruning: two-step mechanism mediated by glia. A: In addition to releasing Wg and Mav, peripheral glia secrete the TNF ligand Eiger to interact presynaptically with the TNF receptor Wengen. This interaction activates the downstream caspase pathway (Dronc and Dcp1), which mediates axonal and synaptic degeneration in the NMJ. Two types of presynaptic degeneration materials, presynaptic debris and undifferentiated ghost boutons, are engulfed by glia and muscle respectively. Draper (red circles on the right), the engulfment receptor expressed in both glia and muscle, is responsible for engulfment activity in the NMJ. B: During axon pruning of MB γ-neurons, at an initial step, astrocytic glia (blue) secrete the TGF-β ligand Myo (black dots) to interact with the receptor Baboon (purple) on the neurons (upper left). This interaction activates TGF-β signaling in MB neurons, then upregulates ecdysone signaling by increasing the ecdysone receptor B1 (EcR-B1) levels. Upregulation of ecdysone signaling actively recruits astrocyte-like glia to infiltrate γ-neurons and initiate axon pruning. In the late pupa, glial cells engulf the degenerating axon materials *via* the activity of Draper (lower right). MB neurons are yellow and Draper is red.

larval stage, MB γ-neurons project with dendrites and axons that bifurcate into a dorsal and medial branch. At ~6 h after puparium formation, the axons and dendrites undergo a pruning process triggered by the metamorphic hormone ecdysone[68], so that local axon degeneration is induced and both the dorsal and medial branches are pruned, leaving only the axon peduncle. Later during the pupal stage, the medial branch re-extends and establishes the adult-specific axonal connection. It has been previously shown that, in addition to ecdysone signaling, the ubiquitin-proteasome system also plays a role in initiating the axon pruning of MB γ-neurons[68, 69].

Intriguingly, glia are involved in regulating the axon-pruning process by a consecutive two-step mechanism. Initially, upregulation of MB ecdysone receptor B1 (EcR-B1) expression is required to trigger pruning, and this upregulation is effected by the activation of TGF-β signaling in MB neurons. To achieve this, surrounding cortical and astrocyte-like glia secrete the TGF-β ligand myoglianin (Myo) that interacts with the type-I receptor Baboon in MB neurons to activate intrinsic TGF-β signaling[70]. Interestingly, the immunoglobulin superfamily molecule Plum has been shown to regulate TGF-β signaling at the receptor level and may participate in the glia-MB neuron interaction during developmental axon-pruning[71].

Upon the upregulation of ecdysone signaling, astrocyte-like glia infiltrate the MB and axon pruning is initiated. Further down the road, astrocyte-like glia also take on a scavenger-like role in cleaning up the degenerating axon fragments[72-75]. This glial degradation pathway, mediated by endosomes and lysosomes, is strictly required for axon pruning since inhibition of glial function in this case results in a delay in pruning and accumulation of degenerating materials. On the other hand, in the absence of ecdysone signaling from MB neurons, astrocyte-like glia do not infiltrate γ-neurons and engulfment activity is silenced. These results suggest that astrocyte-like glia take on an active role during pruning and that a bi-directional interaction between MB neurons and glia is required for the correct pruning process to occur[72].

Interestingly, similar to the NMJ, the glial engulfment receptor Draper is also required for the engulfment of axonal debris during MB γ-neuron pruning[73]. Although a notable amount of data has demonstrated that *draper* expression in glia is required for the engulfment of apoptotic neurons in embryos[76, 77], glial engulfment during γ-axon pruning differs from the engulfment mechanism for apoptotic cells. Expression of the caspase inhibitor *p35* in MB neurons does not lead to pruning defects[69], suggesting that apoptosis is not the major mechanism. On the other hand, γ-axon pruning is similar to the Wallerian degeneration of axon injury, which does not involve apoptosis, and the ubiquitin-proteasome system is one of the major mechanisms[78]. A Wallerian degeneration process has recently been well exemplified in *Drosophila* olfactory receptor neurons[79] and it has been shown that *draper* expression is similarly upregulated when axons undergo injury in this model[79-82]. Yet, unlike γ-axon pruning where astrocyte-like glia are the major subtype responsible[75, 83], ensheathing glia have been shown to engulf debris during axon injury of olfactory receptor neurons[84] (Fig. 4B).

Concluding Remarks

Understanding the mechanisms of how a nervous system develops from single progenitor cells to a functional unit integrating responses has always been the central area of interest in modern neuroscience. In-depth experimental analysis and pioneering work on model organisms like *Drosophila* have allowed researchers to draw conclusions about the important contributions of glia to the series of events leading to the maturation of neuronal circuitry. As both a long-term supporter and an active participant, glia modulate neural stem-cell behavior, secrete factors to regulate synapse formation, and are involved in redefining the nature of synaptic connections *via* degeneration and regrowth. Intriguingly, bi-directional communication between neurons and glia powerfully orchestrates developmental progression and serves as the basis for the mechanisms underlying neurodegenerative diseases. It is increasingly clear that glia, like neurons, are major mediators in regulating various aspects of neuronal development and function; their importance can no longer be neglected. Future work is required to further elucidate the glia-derived regulatory mechanisms, both intrinsic and extrinsic, in other developmental contexts and a fruitful outcome advancing our knowledge is envisioned.

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·Review·

Nematodes feel a craving - Using *Caenorhabditis elegans* as a model to study alcohol addiction

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Alcohol is the most frequently-used addictive drug. However, the mechanism by which its consumption leads to addiction remains largely elusive. Given the conservation of behavioral reactions to alcohol, *Caenorhabitis elegans* (*C. elegans*) has been effectively used as a model system to investigate the relevant molecular targets and pathways mediating these responses. In this article, we review the roles of BK channels (also called SLO-1), the lipid microenvironment, receptors, the synaptic machinery, and neurotransmitters in both the acute and chronic effects of alcohol. We provide an overview of the genes and mechanisms involved in alcoholism-related behaviors in *C. elegans.*

Keywords: *C. elegans*; substance abuse; ethanol; BK channel

Introduction

Substance abuse refers to the harmful or hazardous use of psychoactive substances including alcohol and illicit drugs. Due to its sophisticated genetics, *Caenorhabditis elegans* (*C. elegans*) has provided novel insights into the mechanisms of substance abuse. Unbiased forward genetic screening for drug-resistant or hypersensitive mutants permits the identification of new addiction-related molecules, and RNA interference (RNAi)[1, 2] allows the targeted inactivation of any gene. More importantly, the *C. elegans* nervous system is similar to that in mammals, including ion channels, signal pathways, synaptic machinery, and neurotransmitters, so using this model may lead to a better understanding of the molecular and neurobiological factors that underlie substance abuse.

Alcohol consumption is common in many societies. It is associated with an increased risk of acute and chronic health conditions related to its intoxicating, toxic and dependence-inducing properties (World Health Organization). Despite the prevalence of alcohol consumption and its propensity for abuse, the molecular targets and physiological mechanisms underlying intoxication and abuse remain elusive.

In humans, acute exposure to alcohol causes hyperactivity at low doses and physical impairment of coordination and balance, sedation, and even death at high doses. Interestingly, the same internal concentrations of alcohol cause incoordination and sedation in *C. elegans*. Moreover, both the metabolic enzymes for alcohol degradation and the molecular/physiological pathways mediating the actions of alcohol are similar in *C. elegans* and mammals, which suggest conserved function of alcohol targets in the nervous system. Thus, *C. elegans* has been widely used as a genetic model organism to identify the effectors of intoxication. Genetic studies in worms have provided a more complete understanding of alcohol preference, tolerance, and withdrawal.

C. elegans exhibits acute behavioral responses to ethanol comparable to those in higher species[3]. Acute exposure causes a dose-dependent depression in locomotion and egg-laying behaviors of *C. elegans* at the same internal concentration of ethanol (20 to 30 mmol/L) that induces intoxication in humans and other mammals, while acute tolerance is induced by continuous exposure[4]. Here, we summarize recent insights into the behavioral actions of ethanol in *C. elegans* and the diverse molecular effectors involved.

Ion Channels

BK (or SLO-1) channels play an important role in acute behavioral responses to ethanol. These channels, which are activated by both intracellular Ca^{2+} and membrane depolarization, play a prominent role in coupling intracellular Ca^{2+} with cellular excitability[5]. In the nervous system, BK channels function as an important regulator of neural transmission and network activity[6-8]. Ethanol reduces neuronal excitability by activating BK channels. This altered BK channel activation has the potential to limit neuropeptide and growth hormone release, nociception, and cerebrovascular tone.

A role for BK channels in the regulation of ethanol actions was first identified in a screen for ethanol-resistant *C. elegans* mutants by Davies *et al*.[3]. *slo-1* loss-of-function mutants are strongly resistant to the sedative effects of the drug, while s*lo-1* gain-of-function mutants display depression of locomotion and egg-laying behaviors to a degree similar to ethanol-treated wild-type animals[3]. The absence of BK channel activity in *slo-1* mutants provides a mechanism for resistance to the behavioral effects of ethanol. Furthermore, *in vivo* electrophysiological recording from dopaminergic CEP (cephalic) neurons in *C. elegans* shows that ethanol activation of SLO-1 does not require cytosolic factors and is not due to increased internal Ca^{2+} levels[3]. Ethanol activates BK channels, hyperpolarizing the neurons and inhibiting neuronal excitability, which is a major cause of the acute responses in worms.

Lipid Microenvironment

C-terminal-binding protein 2 (CTBP2), the mammalian homolog of CTBP-1, acts as a transcriptional repressor and shows a significant association with alcohol-dependence in a genome-wide association study of an Australian population[9]. In *C. elegans*, two transcriptional co-repressors (*ctbp-1* and *pag-3*) have been identified by a genetic screen for mutations that result in the defective development of acute functional tolerance[10]. Bettinger *et al*. found that transcriptional repression of the levels of the triacylglycerol lipase LIPS-7 modifies the phenotype of gain-of-function mutations in the BK channel[10]. This study suggests that the lipid microenvironment tunes the neuronal effects of ethanol, including the initial sensitivity as well as the development of acute tolerance.

Taken together, these findings suggest that the mechanisms of ethanol action on BK channels may be conserved between *C. elegans* and higher organisms. Overall, a variety of factors can fine-tune the action of ethanol on BK channels and result in changes in channel activity[11].

Presynaptic Proteins

It is believed that the synapse is the most ethanol-sensitive element in the central nervous system[12]. At the level of the synapse, ethanol indirectly inhibits neurotransmitter release through modulation of neuronal activity in *C. elegans* and vertebrates[3, 13, 14].

RAB-3/RAB3A

The *rab3* gene encodes a small G-protein that interacts directly with synaptic vesicles to regulate their release[15-17]. Genetic disruption of the function of presynaptic RAB-3/A protein alters ethanol-related behaviors. Loss-of-function mutations in RAB-3 and the RAB-3 exchange factor AEX-3 confer resistance to the locomotor effects of ethanol in *C. elegans*[18]. Similarly, mice lacking one or both copies of *Rab3A* are resistant to the ataxic and sedative effects of ethanol, whereas *Rab3A* haploinsufficiency increases voluntary ethanol consumption[18]. These data suggest a conserved role of RAB3/A-dependent neurotransmitter release in behavioral responses to ethanol. However, it remains unclear whether the resistance to ethanol in both species is the result of altered acute sensitivity or the abnormal development of tolerance.

Munc18-1

The synaptic protein Munc18-1 interacts with the SNARE protein syntaxin-1 and functions in exocytosis. A genetic study of two mouse strains with different ethanol preference indicated a correlation with a polymorphism (D216N) in Munc18-1[19]. Interestingly, *munc18-1* transgenic mutant worms (D214N) are strongly resistant to both the stimulatory and sedative effects of acute ethanol. Analysis of an alternative Munc18-1 mutation (I133V) supports the link between reduced SNARE complex binding and ethanol resistance[20]. The interaction between the SNARE complex and Munc18-1 may also be a target for the transduction of effects. This study pinpoints a role of ethanol at the level of vesicle fusion, whereby its acute effects are ameliorated by point mutations in UNC-18. Hence, vesicle recruitment and docking might be potential sites for the neuronal action of ethanol.

Neurotransmitters

Lee *et al*. created an ethanol-preference assay and found that *C. elegans* develops a preference for or attraction to ethanol as a result of prolonged pre-exposure to the drug[24]. *cat* (*catalase*)*-2* mutant worms (deficient in the synthetic enzyme for dopamine) fail to develop a preference for ethanol, suggesting that dopamine is required. *tph* (*tryptophan hydroxylase*)*-1* mutant worms that have defects in the synthetic enzyme for serotonin are also defective in ethanol preference, indicating that serotonin also plays a role. These results suggest that dopamine and serotonin are required for this form of behavioral plasticity.

Receptors

NPR-1

In vertebrates, neuropeptide Y (NPY) receptor genes play a role in alcohol addiction[21, 22]. The *npr-1* gene encodes an NPY-like receptor protein in *C. elegans*[23]. Acute tolerance develops more rapidly in *npr-1* loss-of-function mutants, and the mutant *npr-1* negatively regulates acute ethanol tolerance[4], which indicates that NPR-1 is implicated in the development of acute tolerance.

SEB-3

Jee *et al*. found that *seb-3* in *C. elegans*, which encodes a CRF (corticotropin-releasing factor) receptor-like G-protein-coupled receptor, contributes to acute tolerance to ethanol and to the development of tremor during ethanol withdrawal. Similarly, a specific CRF receptor antagonist reduces acute functional tolerance to ethanol in mice[25].

Conclusion and Perspectives

C. elegans has been widely used as a model system to study the mechanisms of addiction-related behaviors, such as acute responses, tolerance, sensitization, withdrawal, and dependence. Current studies in *C. elegans* indicate that the behavioral responses induced by ethanol are regulated by various factors and take place at presynaptic as well as postsynaptic sites. Ethanol activates presynaptic BK channels in *C. elegans*, causing a large efflux of K^+, hyperpolarizing the neuron, depressing neuronal excitability, and inhibiting neurotransmitter release (Fig. 1). Additional factors, including posttranslational modification and alternative splicing of SLO subunits[26], BK channel assembly with accessory subunits[27, 28], and the lipid milieu, may modulate BK channel activity[10]. Downstream of BK and Ca^{2+} channel function, ethanol may act on additional presynaptic effectors, such as Rab3 and Munc18, which function in vesicle fusion and recruitment/docking respectively, to modulate neurotransmitter release [18, 20, 29, 30] (Fig. 1).

The fact that the ethanol responses of genes and the mechanisms involved in *C. elegans* contribute to a large extent to our molecular understanding of ethanol-induced behavior in mammals underlines the existence of conserved targets and pathways in vertebrates and invertebrates. It is worth noting that the direct and indirect interaction of ethanol with these targets may result in effects through subsequent gene expression and synaptic activity. Moreover, chronic ethanol treatment may induce long-lasting alterations in neuronal networks and behavioral plasticity that could underlie compulsive alcohol consumption and drug-seeking behavior.

Recent Technological Advances

The community of investigators using *C. elegans* possesses a wealth of tools to investigate the neuronal and molecular mechanisms underlying the effects of various drugs. Combinations of forward and reverse genetic approaches[42] provide a means of understanding the correlation of genes and drug-induced behavior. The existence of numerous well-defined promoters can be used to regulate gene expression spatially and temporally. The role and dissection of neuronal circuits in drug-related behaviors can be achieved through laser ablation of specific sensory neurons and interneurons. Patch-clamp recording from neurons and neuromuscular junctions *in vivo*[43] is a powerful tool for exploring neuronal activity. The ability to perform Ca^{2+} imaging and optogenetic manipulations on free-moving worms[44, 45] permits the monitoring of neuronal activity in response to acute or chronic drug exposure. Strikingly, whole-brain imaging in *C. elegans*[46] is becoming feasible and will hopefully emerge for future studies of drug abuse.

Other Addictive Drugs

In addiction to cocaine and amphetamine, the dopamine

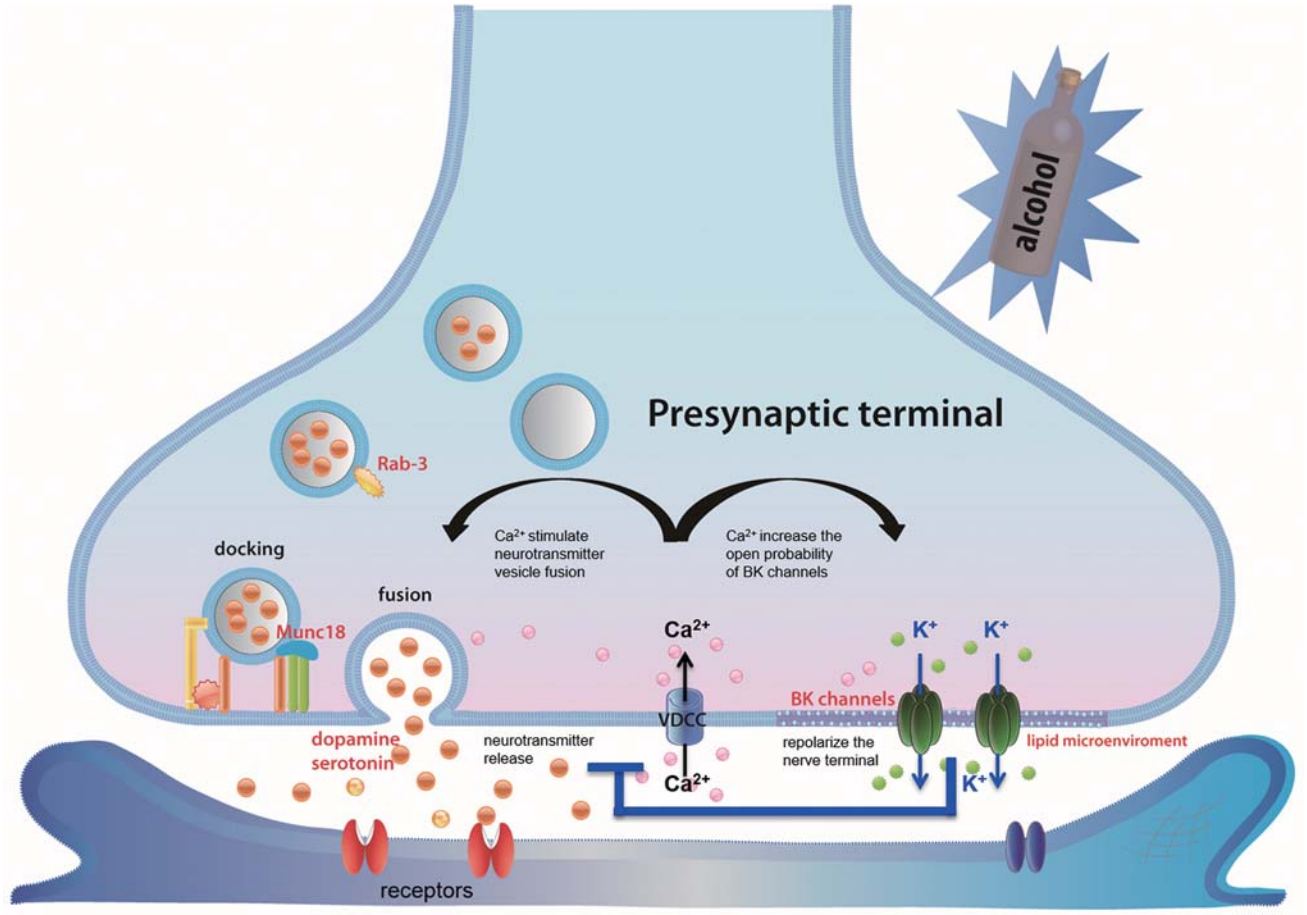

Fig. 1. Ethanol action on BK channels and the modulation of exocytosis in *C. elegans*. Ethanol activates presynaptic BK channels in *C. elegans*, causing a large efflux of K^+, hyperpolarizing the neuron, depressing neuronal excitability and inhibiting neurotransmitter release. Ethanol may act on additional presynaptic effectors (such as Rab3 and Munc18 that function in vesicle fusion and vesicle recruitment/docking respectively) to modulate neurotransmitter release.

reuptake transporter is believed to be a critical molecular target. Behavioral assays have been developed to assess responses and/or adaptation to cocaine and amphetamine in *C. elegans*[31, 32]. Acute cocaine treatment changes locomotor activity, and the neurotransmitter serotonin is required for the cocaine response in *C. elegans*[31].

Nicotine, the primary addictive substance in tobacco, acts on the brain through neuronal nicotinic acetylcholine receptors (nAChRs). There are 42 different predicted nAChR subunits in *C. elegans*[33]. Worms exhibit behavioral responses to nicotine including an acute response[34, 35], tolerance[36], withdrawal, and sensitization[37]. These nicotine responses require nAChRs, suggesting that they are functionally conserved. Importantly, mutant worms lacking TRPC (transient receptor potential, canonical) channels are defective in their response to nicotine, which can be rescued by a human TRPC channel. These results have uncovered a novel role for TRPC channels in regulating nicotine-dependent behavior[37].

Although no opioid and cannabinoid receptors have been identified so far[38-41], *C. elegans* serves as a useful tool to characterize the functions of known genes as well as to identify new genes that are involved in the regulation of addiction to other drugs. The fact that some substances are often used together makes studies of addiction more challenging. For instance, dependence on alcohol is correlated with dependence on tobacco. Revealing the underlying mechanism of the interactions between these

substances and their addictions will lead to a better understanding of co-occurring addiction and its treatment. *C. elegans* also holds the potential for further use in the screening of therapeutic targets and compounds for treating alcoholism.

The continued use of *C. elegans*, a simple, yet powerful *in vivo* model system, may help us to uncover the mysterious mechanisms by which drug exposure induces changes in synaptic and behavioral plasticity.

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·Review·

MeCP2: multifaceted roles in gene regulation and neural development

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Methyl-CpG-binding protein 2 (MeCP2) is a classic methylated-DNA-binding protein, dysfunctions of which lead to various neurodevelopmental disorders such as Rett syndrome and autism spectrum disorder. Initially recognized as a transcriptional repressor, MeCP2 has been studied extensively and its functions have been expanded dramatically in the past two decades. Recently, it was found to be involved in gene regulation at the post-transcriptional level. MeCP2 represses nuclear microRNA processing by interacting directly with the Drosha/DGCR8 complex. In addition to its multifaceted functions, MeCP2 is remarkably modulated by post-translational modifications such as phosphorylation, SUMOylation, and acetylation, providing more regulatory dimensions to its functions. The role of MeCP2 in the central nervous system has been studied extensively, from neurons to glia. Future investigations combining molecular, cellular, and physiological methods are necessary for defining the roles of MeCP2 in the brain and developing efficient treatments for MeCP2-related brain disorders.

Keywords: MeCP2; Rett syndrome; central nervous system; gene expression regulation; post-translational modification; post-transcriptional regulation; glia

Introduction

Methyl-CpG-binding protein 2 (MeCP2) was initially identified in 1992 as a classic methyl-CpG-binding protein[1]. Since DNA methylation was considered to be an important epigenetic mechanism for the regulation of gene transcription[2, 3] (Fig. 1A), MeCP2 was then regarded as a transcriptional repressor and the methyl-DNA-binding domain and transcriptional repression domain were revealed by subsequent biochemical studies[4, 5] (Fig. 1B). Furthermore, many proteins such as the Sin3A/HDAC and NCoR/SMRT co-repressor complexes interact with MeCP2[6] (Fig. 1B), confirming its key role in transcription repression.

The role of MeCP2 in the brain was originally discovered in 1999, when Dr. Huda Y. Zoghbi and her team found that MeCP2 mutations are the genetic root of a rare neurodevelopmental disorder named Rett syndrome (RTT, MIM312750)[7]. As *MECP2* is an X-linked gene, it is not surprising that Rett syndrome mainly occurs in females, as loss-of-function mutations of *MECP2* would largely be lethal in males. Classic RTT patients have normal development to 6–18 months of age, and then start to undergo growth arrest and appear to have defects in motor functions such as hand skills. Furthermore, neurological abnormalities including autistic features, seizures, and mental retardation are prevalent in the progression[8]. In accord with the clinical features of RTT patients, expression pattern analysis showed that MeCP2 expression is dominant in brain tissues, emphasizing its crucial role in neural development[9].

The *MECP2* gene is composed of four exons and alternative splicing results in two protein isoforms, MeCP2E1 and MeCP2E2, with different N-termini[10, 11]. *In situ* hybridization in mouse brain showed that *Mecp2e2* expression is restricted to the dorsal thalamus while

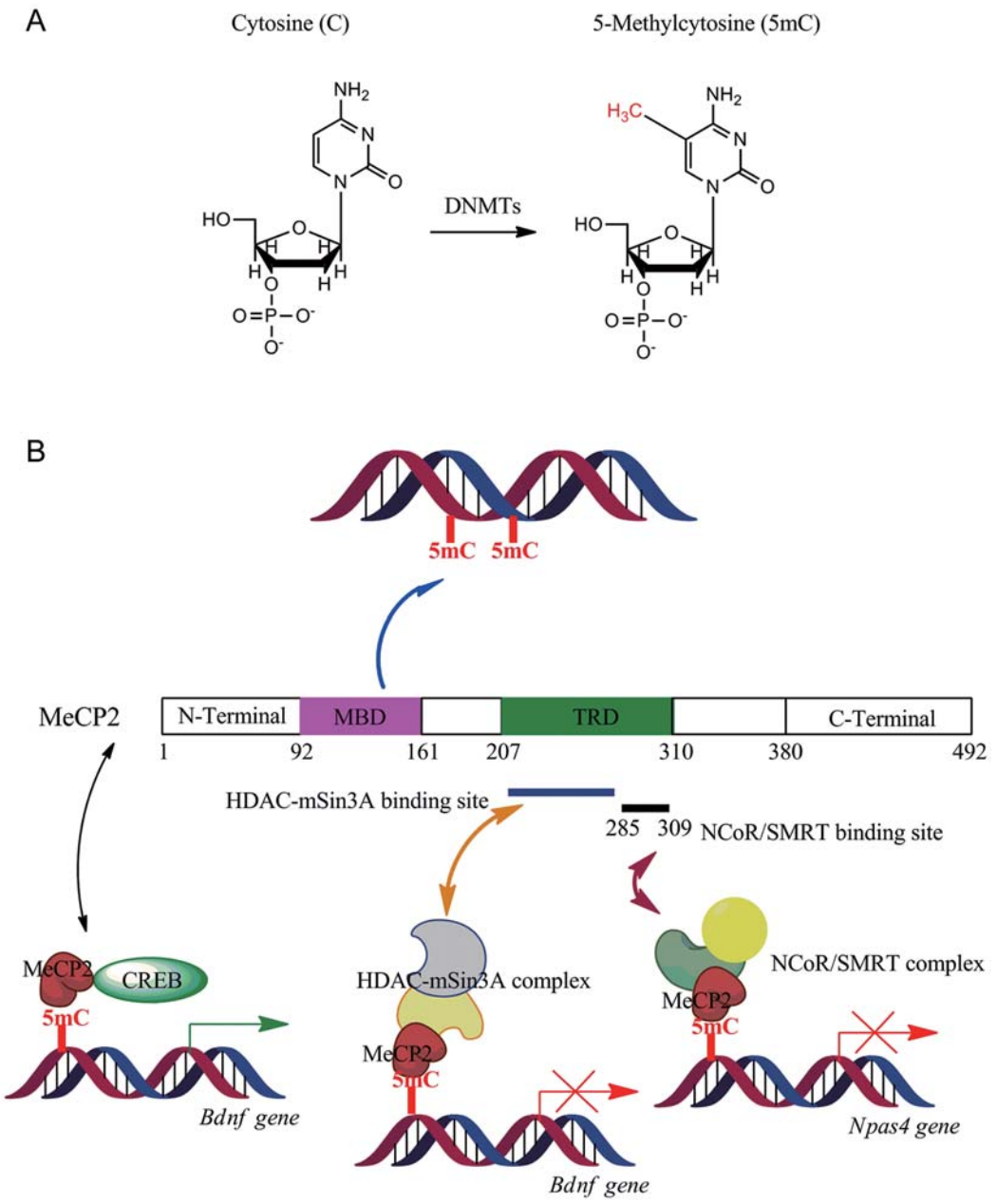

Fig. 1. MeCP2 regulates transcription in a bidirectional manner. A: 5-methylcytosine is the major form of DNA methylation occurring at cytosine. This methylation process is mediated by DNA methyltransferases (DNMTs). B: MeCP2 has two functional domains, a methyl-DNA-binding domain (MBD) and a transcriptional repression domain (TRD). MeCP2 binding to a methylated DNA site is mainly mediated by the MBD. Physical interaction between MeCP2 and co-repressor complexes (HDAC-mSin3A and NCoR-SMRT) is mainly dependent on the TRD. In addition to co-repressor complexes, MeCP2 binding to the transcriptional activator CREB1 has been identified.

Mecp2e1 is the major brain isoform outside the thalamus[12]. Furthermore, expression analysis in human brain tissues revealed that the *MECP2E1* mRNA level is significantly higher than *MECP2E2* in the whole brain or cerebellum[11]. With the development of MeCP2 isoform-specific antibodies, researchers have investigated the expression of both isoforms at the protein level during brain development and in various brain regions, and found

a later onset of MeCP2E2 expression than MeCP2E1[13]. We further showed that the MeCP2E2 expression patterns in different brain regions are quite different while the MeCP2E1 expression pattern is uniform[13]. Although both isoforms have been detected in many cell types including neurons, astrocytes, and oligodendrocytes, MeCP2E1 is significantly higher in neurons than in astrocytes[14]. In addition, MeCP2E1 and MeCP2E2 have quite different functions in neuronal survival[15], embryonic development[16] and responses to drugs[17].

RTT disorder is mainly caused by MeCP2 deficiency, but duplications of *MECP2*-containing loci are also detrimental to neural development and proper brain functions. Patients carrying *MECP2* duplications usually manifest autistic features, mild RTT phenotypes, and mental retardation[18]. All these results underscore the importance of the homeostatic modulation of MeCP2 expression, indicating that the dosage of MeCP2 protein is critical for the development of the central nervous system.

To investigate the underlying pathophysiology of RTT, mouse models in which MeCP2 is either deleted or overexpressed have been established[19-21]. Many clinical features manifested in RTT patients have been reproduced in mouse models and thanks to these models, studies in this field have accelerated and significant valuable insights into the pathogenesis of RTT have been achieved. In addition, RTT has been successfully modeled in cynomolgus monkeys by targeting the *MECP2* gene with TALENs technology[22, 23].

Initially, MeCP2 was thought to be present mainly in excitatory neurons[24]. However, after decades of study, the functions of MeCP2 protein have expanded from transcriptional repression to post-transcriptional regulation, and it is recognized as a key regulator in various cell types including excitatory neurons, inhibitory neurons, and glia. Furthermore, MeCP2 protein undergoes multiple posttranslational modifications such as phosphorylation, SUMOylation and acetylation, which impact its functions. Here, we summarize the multifaceted functions of MeCP2 in the central nervous system, emphasizing its diverse and indispensable roles in brain development and functions. We believe that more functions of MeCP2 remain to be uncovered and these will provide a more comprehensive view of its roles in the brain.

Dual Functions of MeCP2 in the Regulation of Gene Expression

Since methylation of CpG islands is recognized as a hallmark of gene silencing and MeCP2 specifically binds to methylated CpG, much effort has been devoted to identifying gene targets whose expression is repressed by MeCP2. In 2003, two studies showed that MeCP2 binds to the promoter of the *BDNF* (brain-derived neurotrophic factor) gene to repress its expression, while neuronal depolarization decreases the methylation level of *BDNF* regulatory regions and promotes MeCP2 phosphorylation, leading to MeCP2 release from the *BDNF* promoter and the activation of *BDNF* transcription[25, 26]. Recently, it was reported that MeCP2 represses GluR2 expression by binding to the promoter region to modulate synaptic scaling during which neuronal activation increases the expression of MeCP2, leading to further inhibition of GluR2 transcription and a decrease of neuronal excitability[27]. However, MeCP2 is not just a transcriptional repressor, as transcriptional profiling analysis in the hypothalamus and cerebellum of *Mecp2* knockout/transgenic mice revealed a bi-directional change of gene expression[28, 29]. Consistently, protein interaction analysis has shown that MeCP2 interacts not only with co-repressor complexes[6, 30, 31] but also with the transcriptional activator CREB[28] (Fig. 1B). So it was proposed that MeCP2 also serves as a transcriptional activator to stimulate gene expression (Fig. 2). The transcriptional-activation function of MeCP2 has been further confirmed in astrocytes[32]. However, the underlying mechanisms remain to be determined.

More intriguingly, the modulation of specific gene expression by MeCP2 can change dynamically between repression and activation. As noted above, BDNF expression can be repressed by MeCP2 in cultured cortical neurons. However, it has been demonstrated that MeCP2 overexpression promotes BDNF expression in cultured cortical neurons[33]. Furthermore, BDNF expression is upregulated in *mecp2* transgenic mice and downregulated in *mecp2* knockout mice, which suggests that MeCP2 promotes BDNF expression *in vitro* and *in vivo*[28, 33]. Several hypotheses have been proposed to explain the discrepancy between these studies. For example, it has been proposed that different MeCP2 phosphorylation status could recruit

distinct factors to repress or activate BDNF expression[34]. Of course, further experiments are needed to verify these hypotheses.

Gene Expression Regulated by MeCP2: from mRNAs to Non-coding RNAs

Early studies on the impact of MeCP2 on gene expression mainly focused on the transcriptional level. However, transcripts of protein-coding genes only account for one-fifth of all transcripts in the genome[35]. Indeed, non-coding RNAs including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are abundant in the nervous system and they are critical for neuronal functions and brain development[36, 37]. It is well-known that miRNAs modulate gene expression post-transcriptionally to influence various aspects of neuronal functions from cell fate determination to synaptic plasticity. For example, miR-124a and miR-9 are essential for neural lineage differentiation and are involved in determining neural progenitor differentiation into neurons or glia[38], while miR-134 is located in synapses and is involved in spine development[39]. lncRNAs physically interact with transcriptional factors and chromatin remodelers to modulate gene expression and regulate neuronal functions[40, 41]. For instance, the specific lncRNA RMST interacts with hnRNPA2/B1 and SOX2 to regulate gene transcription and finally influence neuronal differentiation[42]. As the transcriptional process is similar in protein-coding and non-coding genes, and MeCP2 is a transcriptional regulator, it is likely that the transcription of non-coding RNAs is regulated by MeCP2. As expected, deletion of *Mecp2* results in disrupted expression of miRNAs and lncRNAs in the mouse model of RTT[43-45]. These results underscore the essential role of MeCP2 in transcriptional regulation and suggest that MeCP2 might modulate genome transcription globally, independent of RNA type.

Modulation of Gene Expression at the Post-transcriptional Level by MeCP2: miRNA Processing, RNA Splicing, and Protein Synthesis?

The involvement of MeCP2 in regulating miRNA expression was first reported in adult neural stem cells, in which miR-137 was up-regulated after MeCP2 knockdown and proliferation/differentiation balance was impaired[46]. Then, further studies analyzed the expression profiles of miRNAs in the whole brain or cerebellum of *Mecp2*-null mice and found that the levels of many miRNAs were altered[43, 44]. Although studies indicate that the expression of several miRNAs is regulated by MeCP2 at the transcriptional level, recently it has been reported that MeCP2 can also regulate miRNA expression post-transcriptionally[47]. Solexa-based deep sequencing revealed that most mature miRNAs are up-regulated in the hippocampus of MeCP2-knockout mice. Real-time PCR experiments showed that MeCP2 depletion has little effect on primary miRNAs but significantly enhances the expression of precursor and mature miRNAs, indicating a post-transcriptional impact of MeCP2 on miRNA expression. Further analysis revealed that MeCP2 directly interacts with DGCR8, an essential component of the miRNA-processing machinery in the DGCR8/Drosha complex, to inhibit the expression of miRNAs at the post-transcriptional level[47] (Fig. 2).

MeCP2 is also essential for the regulation of RNA splicing. Protein interaction analysis by co-immuno-precipitation revealed that Y box-binding protein 1 (YB-1), a conserved RNA-binding protein involved in the regulation of RNA splicing, interacts directly with MeCP2. Functional studies using splicing mini-gene assays showed that MeCP2 affects the RNA-splicing process and abnormal alternative splicing events have been reported in a mouse model of RTT[48]. Consistently, MeCP2 depletion in non-neuronal cell lines also impairs the process of alternative splicing[49] (Fig. 2). Further studies using mass spectrometry have identified several RNA-binding proteins and splicing factors as MeCP2 partners, providing compelling evidence that MeCP2 is a critical factor in the RNA-splicing process[50].

It has been reported that protein synthesis is impaired and the translation rate is reduced significantly in *Mecp2* mutant brains[51]. As MeCP2 is located in the nucleus and is not a ribosome-resident protein, the involvement of MeCP2 in the regulation of protein translation may be an indirect effect. Since the AKT/mTOR pathway is critical for the regulation of protein translation and its impairment is implicated in various neurodevelopmental diseases, it is possible that MeCP2 modulates protein translation *via* the AKT/mTOR pathway. Consistent with this hypothesis,

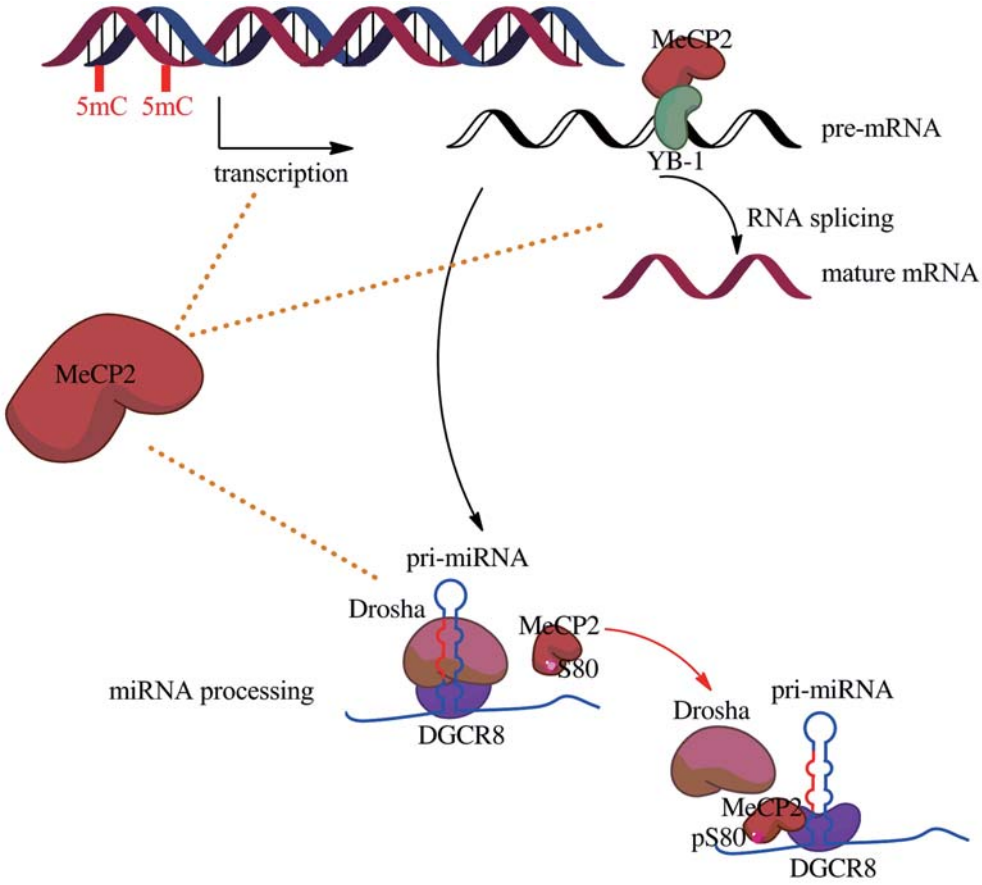

Fig. 2. Multifaceted functions of MeCP2 in the regulation of gene expression. MeCP2 regulates gene transcription bi-directionally. In addition, MeCP2 is involved in the regulation of RNA splicing *via* interaction with YB-1, which is essential for the maturation of pre-mRNA. Furthermore, microRNA (miRNA) processing is also regulated by MeCP2. MeCP2 phosphorylation at the Serine 80 (S80) site interferes with the intra-molecular interaction and promotes its interaction with DGCR8 to inhibit miRNA processing.

phosphorylation of rpS6, an important target of the AKT/mTOR pathway, is reduced in *Mecp2* mutant mice[51].

Impact of Post-translational Modifications on MeCP2 Functions

Protein functions can be modulated by post-translational modifications such as phosphorylation, acetylation, and SUMOylation. Such modifications alter protein structure and change their physical interactions with other components, leading to changes in protein localization and signal transduction in cells[52]. Various modifications of MeCP2 have been identified (Fig. 3). Repression of BDNF expression by MeCP2 is relieved by membrane depolarization in neurons and such de-repression depends on Ca^{2+}-mediated MeCP2 phosphorylation[25]. And using mass spectrometry, multiple phosphorylation sites in MeCP2 under different conditions have been identified[53]. Of these, Serine-421 (S421) has attracted much attention as its phosphorylation is triggered by physiological stimuli such as neuronal depolarization and behavioral stimuli[54, 55]. The importance of activity-dependent MeCP2 phosphorylation at S421 has been investigated extensively. Indeed, studies in brain slices have shown that S421 phosphorylation is essential for dendritic branching and spine morphogenesis[54]. Furthermore, MeCP2 S421A mutant mice show impaired responses to novelty[56]. Learning and memory tests have further shown that long-term potentiation in the hippocampus and hippocampus-dependent memory are enhanced, while excitatory

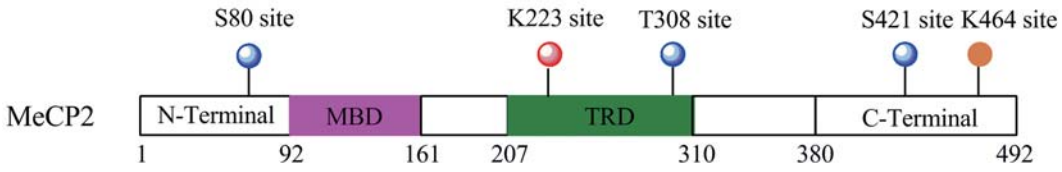

Fig. 3. Post-translational modification (PTM) of MeCP2. Diverse PTMs have been detected in MeCP2 protein. Of these sites, Serine(S) 80, Threonine (T) 308 and S 421 can be phosphorylated (blue spheres); Lysine (K) 223 can be SUMOylated (red sphere); and K 464 can be acetylated (orange sphere).

synaptogenesis is promoted in these mutant mice, implying a critical role of S421 phosphorylation in neuronal and brain circuit development[55]. Exploration of the molecular mechanisms has shown that phosphorylation at the S421 site modulates the binding of MeCP2 to the neuronal genome and in the case of BDNF, S421 phosphorylation blocks MeCP2 binding to the BDNF promoter, leading to the activation of BDNF expression under conditions of neuronal depolarization[25]. Consistently, another study showed that MeCP2 protein with the S421A mutation has a higher binding affinity to multiple target-gene promoters to either enhance or repress the expression of the target gene[55].

The Serine 80 site of the MeCP2 protein can also be phosphorylated. However, MeCP2 S80 is heavily phosphorylated under normal conditions while neuronal activity leads to its dephosphorylation[53]. Functional analysis showed that phosphorylation at S80 is essential for MeCP2 binding to the promoters of specific targets, while dephosphorylation induces the dissociation of MeCP2 from gene promoters. In addition, S80 phosphorylation inhibits the intra-molecular interaction of MeCP2, resulting in enhancement of the physical interaction between MeCP2 and DGCR8, and such interaction interferes with the miRNA processing mediated by the Drosha/DGCR8 complex[47].

Recently, researchers have found that MeCP2 represses gene transcription *via* interactions with NCoR/SMRT co-repressor complexes, and MeCP2 phosphorylation is essential for such interactions[30, 31]. Phosphotryptic mapping has identified a novel site, T308, phosphorylation of which is induced by neuronal activity, and functional analysis has revealed that phosphorylation of T308 interrupts the physical interaction between MeCP2 and the NCoR complex, thus inhibiting the transcriptional suppression effect of MeCP2[30].

Besides phosphorylation, other post-translational modifications such as acetylation and SUMOylation also occur in the MeCP2 protein. And functional analysis has shown that MeCP2 acetylation at K464 promotes its binding to the BDNF promoter and enhances the repressive effect of MeCP2 on BDNF expression. Acetylation and deacetylation of MeCP2 are mediated by p300 and SIRT1, respectively[57]. Furthermore, another modification called SUMOylation, in which MeCP2 is modified by small ubiquitin-like modifiers (SUMOs), is essential for the physical interaction between MeCP2 and the HDAC1/2 complex. SUMOylation at MeCP2 K223 is important for the transcriptional-repression effect of MeCP2 and for synaptic development[58].

In sum, phosphorylation, acetylation, and SUMOylation are just three types of post-translational modification and other kinds of modification exist. Exploring their relationships with MeCP2 is important and will deepen the understanding of MeCP2 functions in neuronal development. Furthermore, exploring and evaluating the role of aberrant MeCP2 modifications in RTT pathogenesis will pave the way for the development of treatment for RTT.

MeCP2 in Neurons: Importance for Excitatory and Inhibitory Neurons and Glia

Initially, studies on the effect of MeCP2 on neuronal functions mainly focused on excitatory neurons[24]. Electrophysiological studies showed that long-term potentiation recorded in cortical/hippocampal slices is reduced in *Mecp2*-null mice[59] and enhanced in *Mecp2* transgenic mice[21]. The magnitude of synaptic output in MeCP2-null and transgenic neurons showed that the amplitude/frequency of excitatory postsynaptic currents is inhibited/enhanced under MeCP2 knockout/overexpression conditions[24]. Consistent with these results, further

immunohistochemical analysis revealed that glutamatergic synapse formation is indeed regulated by MeCP2.

In addition to glutamatergic neurons, MeCP2 is also critical for dopaminergic and serotoninergic neurons, as MeCP2 deletion in either type leads to motor impairment and increased aggression, respectively[60]. Furthermore, though researchers initially thought that MeCP2 was only expressed in excitatory neurons, subsequent studies revealed that mice with MeCP2 depletion in GABAergic neurons manifest many of the phenotypes of RTT[61]. All these results confirm that MeCP2 is critical for normal functions of various neuronal types rather than being limited to excitatory neurons.

Neurons are not the only cell type in the central nervous system. In fact, the major type in brain is glia: astrocytes, oligodendrocytes, and microglia. MeCP2 expression was initially thought to be limited to neurons. However, subsequent studies showed that MeCP2 is expressed in both neurons and glia. Importantly, deletion of MeCP2 in astrocytes impairs neural development and functions in a non-cell-autonomous manner[62, 63]. And in support of these observations, MeCP2 re-expression solely in astrocytes in MeCP2-null mice alleviates many of the abnormal features of RTT mouse models, implying the involvement of astrocytes in the pathogenesis of RTT. Thus, targeting astrocytes is promising for the treatment of RTT patients. In addition to astrocytes, dysfunctions of two other glial types, oligodendrocytes and microglia, also contribute to RTT neuropathology[64]. Surprisingly, transplantation of wild-type bone marrow, the source of microglia, markedly arrests the progression of RTT symptoms and further investigations have shown that impaired phagocytic activity in microglia is critical for the development and progression of RTT symptoms[65]. This study also supports the idea that bone marrow transplantation is feasible for the treatment of RTT patients.

Concluding Remarks

Taken together, the findings show that MeCP2 is widely expressed in the central nervous system and not limited to specific cell types, indicating that the pathogenesis of RTT is not just a disruption of neuronal functions but is rather caused by multiple impairments of the central nervous system. The mechanisms behind RTT neuropathology are complicated and what we know is still limited. In addition, the nervous system is composed of brain and spinal cord and the brain is composed of different regions. The functions of MeCP2 in these different structures remain to be elucidated. As the expression of MeCP2 can also be assessed in tissues other than the nervous system, investigating their functions in these tissues will provide more valuable insights into the pathology of RTT.

Initially recognized as a transcriptional repressor, the functions of MeCP2 have been extensively expanded. Multi-layer control of gene expression by MeCP2 is now widely accepted. We believe that a comprehensive understanding of MeCP2 would not only benefit RTT patients but accelerate studies of neural development and other neurodevelopmental disorders, paving the way for the development of effective treatments for patients with neurodevelopmental disorders.

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·Review·

RNA binding proteins: a common denominator of neuronal function and dysfunction

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In eukaryotic cells, gene activity is not directly reflected by protein levels because mRNA processing, transport, stability, and translation are co- and post-transcriptionally regulated. These processes, collectively known as the ribonome, are tightly controlled and carried out by a plethora of *trans*-acting RNA-binding proteins (RBPs) that bind to specific *cis* elements throughout the RNA sequence. Within the nervous system, the role of RBPs in brain function turns out to be essential due to the architectural complexity of neurons exemplified by a relatively small somal size and an extensive network of projections and connections. Thus far, RBPs have been shown to be indispensable for several aspects of neurogenesis, neurite outgrowth, synapse formation, and plasticity. Consequently, perturbation of their function is central in the etiology of an ever-growing spectrum of neurological diseases, including fragile X syndrome and the neurodegenerative disorders frontotemporal lobar degeneration and amyotrophic lateral sclerosis.

Keywords: alternative polyadenylation; alternative splicing; amyotrophic lateral sclerosis; anti-Hu syndrome; CPEB; ELAV; fragile X syndrome; FMRP; FUS; HU; HuB; HuC; HuD; HuR; neuron; neurodegeneration; Nova-1; Nova-2; paraneoplastic opsoclonus-myoclonus ataxia; PTBP-2; PTBP-1; TDP-43; FTLD

Introduction: RBPs as Multi-tasking Modulators of Protein Output

The timing and dosage of gene expression are fundamental determinants of cellular phenotype and organismal complexity. Consequently, the regulation of gene expression is highly coordinated at multiple levels by ubiquitous and cell-specific *trans*-acting factors. Whereas for many years the specific focus has been on basal transcriptional regulation, post-transcriptional mechanisms regulating RNA metabolism have increasingly emerged as major determinants of gene output. The main reason is the high sequence plasticity, structural diversity, and agility of mRNA molecules that makes them ideal hubs for partners to bind and modulate protein output. RNA binding proteins (RBPs) are *trans*-acting factors that reversibly bind to these RNAs either alone or in conjunction with non-coding RNAs, particularly microRNAs (miRs)[1]. The transit interaction of RBPs and miRs with the RNAs results in the formation of ribonucleoprotein complexes that ultimately determine the fate of RNAs.

Post-transcriptional regulation confers several advantages to cells, some of which are particularly essential for neurons (summarized in Fig. 1). First and foremost, alternative pre-mRNA splicing allows the functional proteome to qualitatively expand; new proteins are generated from the same pre-mRNA with different binding partners and functions[2]. Then, there is alternative polyadenylation (APA) that allows either the formation of different proteins from the same pre-mRNA (if APA occurs in an internal exon), or, more often, the generation of transcripts with different 3'UTR sizes that can be quantitatively regulated by additional RBPs and/or miR complexes[3]. Modulation of RNA stability is

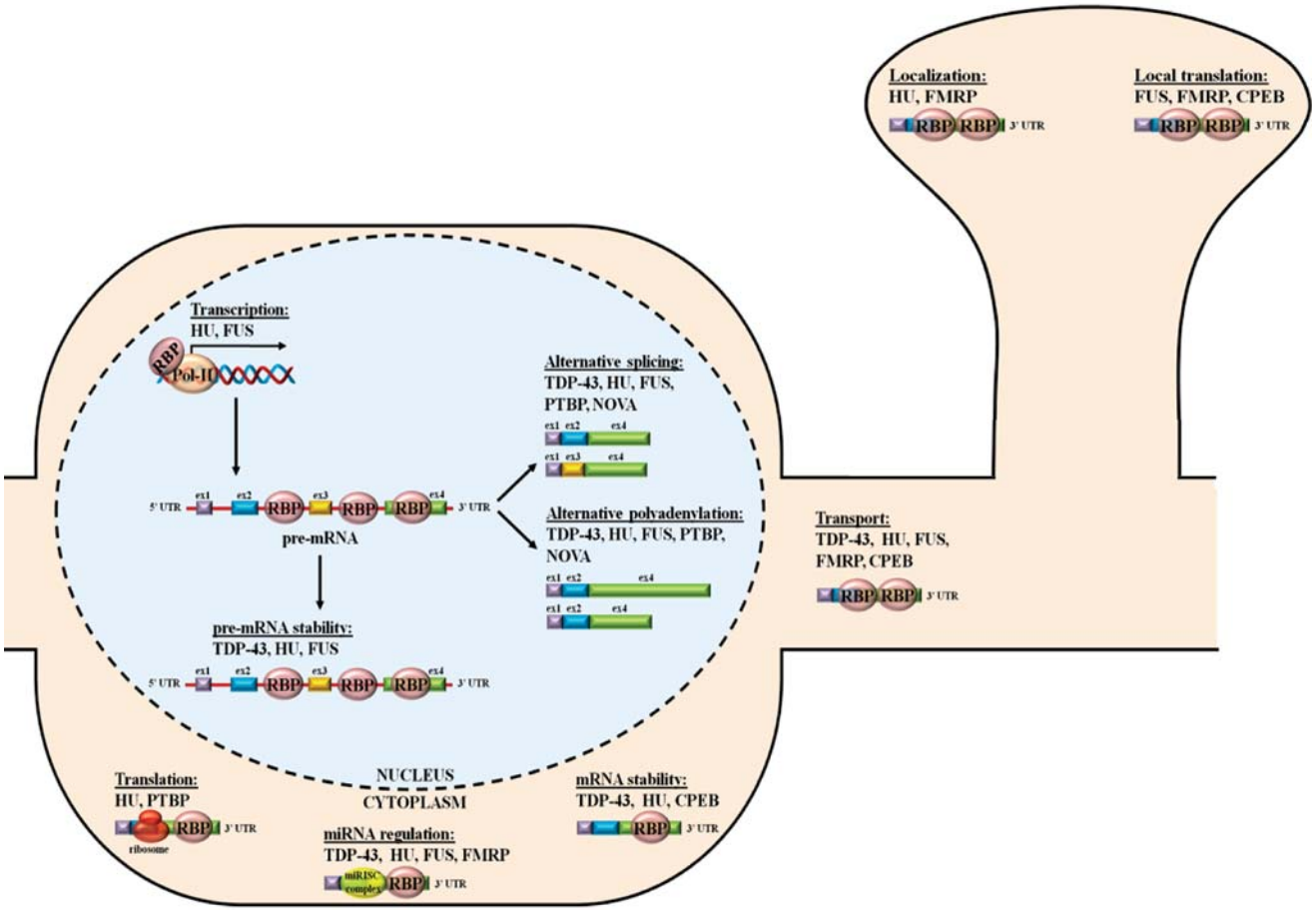

Fig. 1. Diverse mechanisms of RNA binding protein function.

another key target of post-transcriptional mechanisms. In this case, different RBPs stabilize or destabilize mRNA transcripts by binding to intronic and/or exonic sequences to quantitatively regulate protein output[4, 5]. Further, RBPs may also be involved in translation regulation by enhancing or reducing the translation efficiency of mRNAs[6, 7]. Finally, RBPs may be involved in mRNA transport along axons and dendrites, subcellular localization, or activity-dependent local translation[8]. Importantly, different RBPs can interact with the same RNA at different binding sites or compete for the same binding site, increasing the complexity of RNA regulation[9]. Not surprisingly, deregulation of RBPs leads to impaired protein homeostasis and cellular function. This may trigger the development of disease, especially in tissues where cells are long-lived, highly differentiated, and poorly replenished throughout the organism's life[10].

This review focuses on the multifunctional roles of RBPs in neurons, with special emphasis on those RBPs that are strongly associated with neuronal function and dysfunction. It concludes with the emerging view that RBPs may serve as nucleation centers for neurodegenerative processes, based on their requisite role in RNA metabolism and their strong intrinsic propensity for protein aggregations aggravated by stress.

RBPs and Pre-mRNA Splicing: Driving Phenotypic Diversity

In higher eukaryotes, alternative mRNA splicing is a key mechanism that allows expansion of the functional proteome from a genome of limited size depending on cell type, developmental stage, and stimuli. Alternative transcripts are generated by a series of splicing events that include exon skipping, intron retention, alternative first/

last exons, and mutually-exclusive exons. This happens in the nucleus and relies on the interaction between the spliceosome components, the splicing regulator proteins, and the pre-mRNA. The spliceosome is an RNA-protein complex consisting of the small nuclear RNAs U1-6 and several RBPs that catalyze splicing[11]. These core components are common to all cells, and their function is to bind at intron-exon boundaries and catalyze intron removal and exon joining. In addition, specific *cis* elements on these pre-mRNAs and cell-type specific splicing regulators that recognize them drive cell-specific alternative splicing[12]. Splicing events are highly prevalent since 92%–94% of human genes undergo alternative splicing[13], 86% of which express minor isoforms that amount to 15% or more of the total gene expression[14]. The majority of splicing events (88%) take place in the coding region and alter the protein products[15]. mRNA transcript diversity is most prevalent in the brain, in part as a result of high gene expression[13,16], with brain tissues expressing the greatest number of tissue-specific exons[17, 18]. Besides generating diversity, alternative pre-mRNA splicing indirectly influences the stability, transport, localization, and translation of mRNA transcripts. Even minor changes, not immediately appreciated, like the use of a longer 5'UTR, may lead to either reduced protein translation[19, 20] or altered subcellular distribution and enhanced translation activity under non-cap-dependent conditions as shown for postsynaptic proteins bearing IRES *cis* elements[21, 22]. In the nervous system, alternative splicing has been implicated in the control of neuron specification, differentiation, and the modification of synaptic strength. Five of the most relevant cell-specific splicing regulators in the brain are the PTBP-2, HU, NOVA, TDP-43 and FUS proteins, all of which are discussed below. Of note, besides their main role in pre-mRNA splicing, these RBPs have additional RNA regulatory functions that are described in other sections of this review (Fig. 1).

PTBP-2

Polypyrimidine tract binding protein 2 (PTBP-2) is expressed in early post-mitotic neurons, as well as muscle and testis, and has 73% homology to the ubiquitously-expressed PTBP-1[23-25]. Like PTBP-1, it contains four RNA recognition motifs (RRMs), nuclear import/export signals, and recognizes UCU-rich targets to regulate alternative splicing[26-29]. A recent high-throughput study has shown that as much as 96% of PTBP-2 binding sites are found in introns, consistent with a role in pre-mRNA processing[27]. Conforming to the splicing events of other splicing regulators (e.g. HU, Nova, and TDP-43), upon binding to downstream introns of pre-mRNA, PTBP drives exon inclusion, while upon binding to upstream introns, it drives exon exclusion. In most cases, PTBP acts as a repressor of alternative splicing[26-29]. To discern its physiological role, a series of elegant experiments has shown that undifferentiated neural cells express high levels of PTBP-1 protein that alternatively splices the *ptbp-2* pre-mRNA to generate a nonsense-mediated decay isoform that fails to translate into a mature protein. During neuronal differentiation, however, the increase in miR-124 expression reduces PTBP-1 levels and allows *ptbp-2* pre-mRNA to be efficiently spliced and translated[26, 30]. Subsequent, detailed analysis by Licatalosi *et al*. (2012) showed that the precise role of PTBP-2 is to maintain neural progenitor pools and prevent premature neurogenesis in the developing brain. They based this assessment on (1) the finding that *ptbp-2*-null mice display ectopic nests of neuronal progenitors, and (2) cross-linking immunoprecipitation high-throughput sequencing (HITS-CLIP) assays showing that PTBP-2 inhibits the incorporation of adult-specific alternative exons in mRNAs that encode proteins associated with the control of cell fate, proliferation, and the actin cytoskeleton[27].

HU

The mammalian homologs of the *Drosophila* embryonic lethal abnormal vision (ELAV) protein, also known as HU proteins (HuR, HuB, HuC and HuD), are by far the best characterized RNA-binding proteins with roles that span all stages of mRNA metabolism including pre-mRNA splicing, mRNA transport, stability, and translation[31-35]. HU proteins are 70% homologous at the protein level and contain three RRMs[36]. HuR is ubiquitously expressed, while HuB, HuC, and HuD are neuron-specific members of the family although HuB is also expressed in the gonads[37]. Each displays a characteristic expression pattern during development. Using *in situ* hybridization in the mouse brain, Okano *et al*., have shown that HuB is expressed in early post-mitotic neurons in the outer layer of the ventricular zone, continuing in the intermediate zone, and diminishing in the cortical plate. HuD is predominantly expressed in

the intermediate zone and less in the ventricular zone and cortical plate, while HuC is expressed in the cortical plate and is absent from the other two zones[36]. In the adult brain, all neurons express some set of *hu* mRNAs with different neuronal tissues expressing from one to all *hu* mRNAs. In the neocortex, for instance, all neurons express HuC but few express HuD or HuB mRNA. Overall, HuB and HuD show similar distributions and display similar or opposing functions during development. Both stimulate neurite outgrowth and neuronal differentiation *in vitro,* but HuB potentiates neural stem cell proliferation while HuD has a negative impact on this process[38-41]. Accordingly, *hud*-null mice contain increased numbers of self-renewing cells in the subventricular zone, indicating that HuD is required for the exit of neural stem cells from the cell cycle[38]. These mice also revealed a transient impairment in the neurite extension of cranial nerves during early embryonic development. Moreover, they displayed an abnormal clasping defect and poor performance on the rotarod test, suggesting a sensory/motor defect[38]. A study of *huc* null mice revealed significant defects on the rotarod test. However, when they were tested for tail-twitching they showed no defect, likely due to functional redundancy as dorsal root ganglia robustly express all HU proteins[36, 42]. Despite the fact that both *huc*- and *hud*-null mice (*hub*-null mice have not been generated yet) do not show any gross anatomical defects, *huc/d* double-nulls die shortly after birth, further supporting the idea of functional redundancy[42]. The most recent evidence suggests that HU proteins play important roles in neuronal plasticity. They are significantly upregulated in hippocampal neurons after contextual or spatial learning tasks and after glutamate receptor activation[43-47]. In addition, *hud* transgenic mice exhibit aberrant acquisition and retention of memories[48], while *huc*-null mice display spontaneous epileptic seizure activity as a result of reduced glutamate expression[42]. In humans, HU proteins have been associated with the anti-HU syndrome that resembles the phenotype of *hu*-null mice and is characterized by sensory neuropathies, autonomic, brain stem, and cerebellar dysfunctions, short-term memory loss, and epileptic seizures. This syndrome is the outcome of an immune response to neuronal HU proteins that are ectopically expressed in certain tumors such as small-cell carcinomas and neuroblastomas. These auto-immune responses involve the production of antibodies that cross the blood-brain barrier and injure neurons in a yet poorly characterized manner[49]. At the molecular level, HU proteins bind to AU- and GU-rich elements to stabilize mRNA and/or promote translation. Three recent high-throughput studies have shown that as much as 30% of HU binding sites are found in introns, answering the long-unresolved question of why the nuclear abundance of HU is high[4, 42, 50]. Further, these studies showed that intronic binding to regulate splicing is often coupled in *cis* with 3'UTR binding to enhance pre-mRNA stability[4]. In addition to these findings, HU proteins have been shown to bind nascent pre-mRNAs co-transcriptionally to modulate the speed of transcription and, thus, the inclusion of certain exons in a process that involves protein-protein interaction with RNA pol II and HDAC II[51]. Moreover, HU proteins influence alternative splicing indirectly, by enhancing the mRNA stability and translation as well as modulating the splicing activity of another neuronal splicing regulator, NOVA-1[52].

NOVA

As with the neuronal HU proteins, neuro-oncological ventral antigen (NOVA)-1 and -2 proteins were originally discovered as target antigens in the auto-immune neurological disorder paraneoplastic opsoclonus-myoclonus ataxia. In this disorder, patients with lung or fallopian tumors develop excessive motor movements as a result of impaired motor inhibition in the nervous system[53-56]. NOVA-1 and NOVA-2 are expressed in differentiated neurons with largely reciprocal expression in the central nervous system. NOVA-1 is expressed primarily in the hindbrain and spinal cord while NOVA-2 occurs in the neocortex[53, 57, 58]. Both NOVA proteins bind to clusters of a minimum of three YCAY (Y is either a C or U) motifs on target mRNAs and can tolerate variable spacing between these[58, 59]. *Nova-1*-null mice are born indistinguishable from their littermates but die after 2–3 weeks with profound motor failure that correlates with apoptotic death of motor neurons in the spinal cord and brainstem[60]. Similarly, *nova-2*-null mice die a couple of weeks after birth and are characterized by aberrant migration of cortical and Purkinje neurons, whereas the neural progenitor cell fate remains intact[61]. *Nova* double-null mice are born alive, but they do not move, even after noxious sensory stimuli (tail pinch),

and die immediately after birth. These mice are born stiff but otherwise have normal gross morphology with a beating heart. Histological analysis indicated that these animals never inhaled, because diaphragmatic muscle atrophy occurs and the lung alveoli fail to expand, pointing to a lack of functional motor innervation[62]. At the molecular level, Nova proteins possess three K-homology (KH) domains for RNA-binding and dimerization and shuttle between the nucleus and the cytoplasm with ~60% of Nova proteins residing in the nucleus[57, 59]. HITS-CLIP analysis revealed that NOVA crosslinks to both intronic and 3′ UTR clusters in many transcripts, suggesting, similar to HU, an ordered set of *cis*-actions on target mRNAs[63]. More recently, a study of >200 transcripts displaying significant steady-state changes between wild-type and *nova*-null mice revealed that NOVA binding is primarily to intronic rather than stability-associated 3'UTR elements. Further analysis indicated that binding of NOVA to intronic sequences of these pre-mRNAs regulates the inclusion of cryptic exons that trigger nonsense-mediated decay leading to the reduced synthesis of functional proteins[64]. Interestingly, most of these NOVA targets encode for synaptic proteins, including several implicated in familial epilepsy. Accordingly, NOVA was found to shift from the neuronal nucleus to the cytoplasm in response to seizure treatment with pilocarpine. Moreover, *nova* haplo-insufficient mice display spontaneous epilepsy[64].

TDP-43

Transactive response DNA-binding protein of 43 kDa (TDP-43) is a predominantly nuclear protein, ubiquitously expressed and highly conserved. Its mis-localization in the cytoplasm is a hallmark of sporadic and familial amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and some synucleinopathies. In affected neurons and glia, TDP-43 is bound in the cytoplasm in the form of biochemically-insoluble inclusion bodies[65]. At the molecular level, TDP-43 contains two RRM motifs and shows clear preference in binding to at least five UG repeats[66, 67]. It contains a Gly-rich domain that mediates protein-protein interactions and all but one of the 48 identified ALS mutations occur in this domain[68]. This domain also contains a Q/N-rich region, described as prion-like domain that mediates co-aggregation with poly-glutamine misfolded proteins[69]. TDP-43 interacts with proteins in the spliceosome machinery and it is largely thought to be an important component of pre-mRNA splicing[70]. Recent high-throughput studies revealed that most TDP-43 binding occurs in introns (~70%) and to a lesser extent in 3'UTR and non-coding RNA (~10%)[71]. Binding of TDP-43 to long/deep (>2 kb from the nearest intro-exon junction) intronic sequences correlates positively with protein expression, suggesting that it may suppress cryptic splice site expression and/or regulate mRNA stability[5]. TDP-43 influences alternative splicing in a position-dependent manner, similar to the other RBPs. Hence, TDP-43 binding further upstream of an alternatively-spliced exon promotes its exclusion, while binding to proximal intronic sequences downstream of the alternatively-spliced exon promotes its inclusion[71]. With respect to TDP-43 binding to 3'UTR sequences, the great majority has been detected in the cytoplasm, indicating that this RBP also regulates post-splicing events such as stabilization and/or transport[71]. This view is reinforced by earlier studies showing that (1) TDP-43 enhances the stability of several mRNAs[72, 73], (2) it is present in RNA-transporting granules[74], and (3) it affects motoneuron terminal synapses in animal models[75]. *Tdp-43*-null mice are embryonic lethal due to peri-implantation defects, while hemizygotes exhibit motor defects[75, 76].

FUS

Like TDP-43, mutations in the fused in sarcoma (FUS) gene cause familial ALS and FTLD-FUS. FUS is a ubiquitously-expressed RNA-processing protein and is predominantly localized in the nucleus. It contains a single RRM, a Gly-rich domain, three RGG domains that are also implicated in RNA binding, and a zinc-finger domain that binds GGUG RNA sequences[77, 78]. The vast majority of mutations associated with ALS are missense, occurring in the Gly-rich and nuclear localization signal (NLS) motifs. NLS mutations disrupt the nuclear import of FUS, resulting in a reduction of nuclear function and an increase in the cytoplasmic portion rendering it prone to aggregation, which is likely the first step in the pathophysiological cascade that leads to FUS-associated neurodegeneration[79-82]. Like TDP-43, FUS is thought to be an important component of the spliceosome machinery[83, 84]. It also regulates transcription by binding to RNA pol II, affecting its phosphorylation[85]. Because transcription and pre-mRNA splicing are tightly coupled[86], FUS may function like HU to integrate these processes through RNA-protein and protein-protein interactions.

High-throughput studies have revealed that some 60% of FUS binding occurs in distal intronic regions, ~30% in proximal introns, and ~10% in 3'UTRs[87]. Further, ~30% of literature-curated lncRNAs contain FUS binding sites[87]. Interestingly, FUS is often bound to the antisense RNA strand at the promoter regions and downregulates sense-strand transcription[88]. Importantly, FUS tags most often cluster in alternative splice sites rather than constitutively-spliced splice sites, also suggesting a role in alternative splicing[88]. Gene ontology analysis revealed that FUS splice targets are predominantly involved in axonogenesis, axon guidance, cell adhesion, and other cytoskeleton-associated pathways[87, 89, 90]. Finally, comparison of TDP-43 and FUS targets detected only a few RNAs bound by both proteins[87]. Inbred *fus*-null mice die perinatally[91] and exhibit dendritic spine defects compatible with its role in local mRNA transport[92] and translation[92]. Transgenic *fus* mice succumb to progressive paralysis and die after ~12 weeks. These mice show FUS-positive inclusions in spinal motor neurons and therefore replicate some aspects of human pathology[93].

RBPs and Local mRNA Translation: Spatiotemporal Control of Protein Expression

Neurons develop and maintain not only elaborate but also distinct types of processes, the axon and the dendrites, that extend to great distances. These processes or compartments are then engaged in synapses with hundreds to thousands of counterparts in other neurons. Such synaptic contacts, which represent the minimal storage unit of information in the nervous system, are maintained through structural and functional coupling of a repertoire of the same and different proteins in these distinct compartments[94]. Many of these proteins are transported to terminals on kinesin motors, particularly during the initiation phase of synapse formation, while a great number of other proteins are locally translated during differentiation and maturation[95, 96]. In the latter case, the asymmetric localization of mRNAs helps to limit protein expression to these compartments. Stimulus-induced remodeling of synaptic strength, also known as synaptic plasticity, occurs at each individual synaptic terminal, in part as a result of rapid translation of these localized mRNAs. Consequently, dynamic regulatory mechanisms for transport and the quantitative and qualitative translation of these mRNAs are in place with RBPs playing a central role. Here, we focus on two relevant RBPs, fragile-X mental retardation protein (FMRP) and cytoplasmic polyadenylation element-binding (CPEB) protein.

FMRP

FMRP is an RBP that is highly expressed in the brain and critically contributes to mRNA transport as well as translational control at the synapse[97, 98]. As the name suggests, it is responsible for fragile-X syndrome (FXS), the first neurological disease clearly linked to a dysfunction of RNA metabolism. FXS is caused by a CGG triplet repeat expansion within the 5'UTR of the FMR1 gene, resulting in abnormal DNA methylation and transcriptional silencing[99, 100]. FXS patients suffer from intellectual disability and autism. In neurons, FMRP is associated with polyribosomes in the cytosol and dendrites (no polyribosomes have been detected at presynaptic terminals as yet) and has also been detected in axons and growth cones[98, 101-106]. FMRP is a multi-domain protein harboring two KH domains and a single Arg-Gly-Gly-rich (RGG-type) RNA-binding domain[107, 108] of which KH2 is perhaps the most critical for function[109, 110]. In marked contrast to other RBPs, FMRP preferentially binds to coding sequences with no discernible preference for sequence or structural motif[103] despite earlier reports[107, 111]. HITS-CLIP analysis revealed that its mRNA targets are highly enriched in both pre- and post-synaptic terminals and some 30 of these targets have been linked to autism spectrum disorders, possibly explaining the etiology of FXS. Nearly all investigations have shown that FMRP represses translation by causing ribosome stalling[103] and trapping mRNAs in cytoplasmic granules[112]. This mechanism appears to be selective and reversible, involving the phosphorylation of FMRP and its subsequent interaction with the miRISC complex. Thus, it has been delineated that phosphorylation of FMRP at serine 499 suppresses translation, while activity-dependent dephosphorylation by protein phosphatase 2A allows translation of bound mRNAs[8]. Mechanistically, when FMRP is phosphorylated it recruits Argonaute 2 miRISC complexes loaded with miRs to repress translation, while it releases miRISC from target mRNAs upon its dephosphorylation, allowing their translation to occur[113]. An additional function of FMRP

was recently proposed by Dictenberg *et al*., who showed that FMRP directly associates with kinesin motors and likely serves as an adaptor for microtubule-based mRNA transport in an activity-dependent manner in dendrites[114].

CPEB

The CPEB protein family is comprised of four paralogous members, CPEB1–4, all of which are widely expressed, sometimes with overlapping patterns[115]. CPEB1, the best characterized member, binds to cytoplasmic polyadenylation element (CPE) sites (UUUUAU or UUUUAAU) in the 3' UTR of target mRNAs and modulates poly(A) tail length *via* interaction with other proteins[116]. On the other hand, CPEB2–4 do not bind CPE or regulate polyadenylation[117]. CPEBs harbor two RRM and two zinc finger motifs by which they exert their effects[118]. The mechanism of action of CPEB1 was originally delineated in *Xenopus* oocytes[119], but more recently, most of the auxiliary components have been identified in neuronal dendrites too[120]. Following transcription, most mRNAs acquire long poly(A) tails of 200–250 nucleotides. After export to the cytoplasm, however, the CPE-containing mRNAs are bound by CPEB1 and its interacting partners that include the poly(A)-specific ribonuclease (PARN) and the poly(A) polymerase germ-line development 2 (GLD2) proteins. When both are bound to CPEB1, PARN activity predominates resulting in a shortened poly(A) tail of 20–40 nucleotides[121, 122]. Stimuli that promote CPEB phosphorylation lead to the expulsion of PARN from the RNP complex and allow an increase in the poly(A) tail by GLD2 polymerase. The elongated poly(A) tail then serves as a platform for the poly(A)-binding protein (PABP) to recruit the 40S ribosomal subunit to the 5'UTR of the mRNA and start translation. Phosphorylation of CPEB1 in dendrites is thought to be mediated by aurora kinase A and/or calcium/calmodulin-dependent protein kinase type II alpha[120, 123-125]. CPEB1 has, in addition, been shown to repress translation by recruiting the 4E-BP protein neuroguidin that interacts with the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) to prevent its association with eIF4G to initiate translation[6]. Of note, CPEB is highly enriched at post-synaptic densities, indicating that it is important for local translation[116, 124]. Accordingly, *cpeb1*-null mice have memory deficits and reduced long-term potentiation (LTP)[126-128]. In addition, *cpeb1* mice mutated at phosphorylation sites T171 and S177 in cerebellar Purkinje neurons display significant impairment of motor coordination and motor learning delay, reinforcing the overall importance of CPEB1 for synaptic function[129].

RBPs and Alternative Polyadenylation: Fine-tuning mRNA Translation

Polyadenylation is a two-step process that involves endonucleolytic cleavage of the pre-mRNA, followed by the synthesis of a polyadenylation tail at the 3' end. The target selectivity of cleavage is mediated by four sequence elements in the 3'UTR. Foremost is a polyadenylation signal containing the canonical AAUAAA or AUUAAA sequence (also known as poly(A) signals or PAS), located 10–35 nucleotides upstream of the cleavage site, and serves as the binding site for the cleavage and polyadenylation specificity factor (CPSF1). Downstream of PAS is a less well-defined region rich in U or GU nucleotides referred to as downstream element (DSE) that constitutes the binding site for cleavage-stimulating factor (CSTF). The interaction of CPSF1 and CSTF proteins is thought to be the most important determining factor for the selection of a cleavage site. Then, there is an element upstream of PAS that contains U(G/A)UA nucleotides and is the binding site of the cleavage factor I complex (CPSF5 plus CPSF6 or 7). This element is thought to promote recognition of the cleavage site. A fourth sequence rich in G nucleotides downstream of DSE has also been proposed to play a role in polyadenylation (pA), but the protein(s) that bind it has yet to be identified.

The presence of non-canonical PAS sequences together with the tissue-specific distribution of auxiliary RBPs that recognize/compete for binding onto the polyadenylation elements is thought to determine alternative polyadenylation (APA). Generally, two types of APA are distinguished. One in which APA sites are located in introns/internal exons, resulting in the production of different protein isoforms (qualitative change), and one in which APA sites are located in the 3'UTR region, giving rise to transcripts encoding the same protein isoform but with different 3'UTR lengths. Given that 3'UTRs are the main targets of miRs and regulatory RBPs, APA is expected to modify gene expression quantitatively in the latter case. In this regard, Legendre *et al*., (2006) carried out a systematic examination of 3'UTRs produced by APA and found that 52% of miR target sites are located downstream of the

main PAS site[130]. It is estimated that half of human genes undergo alternative cleavage and polyadenylation to generate transcripts with variable 3'UTR lengths[131]. A close connection between gene transcription and pA site choice has been demonstrated, in which highly-expressed genes transcribe mRNAs with shorter 3'UTRs, while transcripts that are expressed at lower levels are associated with longer 3'UTR isoforms[132]. Along with this, higher gene expression is tightly linked to cell division, where short 3' UTR isoforms with fewer miR sites are abundant in proliferating cells[133]. In contrast, differentiated cells possess longer 3'UTRs[132]. With respect to the nervous system, 3'UTR analysis of the longest and shortest human mRNA transcripts revealed that pre-synaptic mRNAs have significantly longer 3'UTRs compared to all other transcripts, including post-synaptic ones. The tendency of pre-synaptic mRNAs to have relatively longer 3'UTRs remained when analysis of the shortest 3'UTR isoforms was carried out. In contrast, post-synaptic transcripts revealed a significant drop in 3'UTR length between the longest and shortest 3'UTR isoforms. These results indicated that pre-synaptic mRNAs maintain a relatively long 3'UTR for enhanced *trans* regulation, irrespective of 3'UTR length fluctuations, while post-synaptic proteins possess a broader spectrum of 3'UTR lengths to avert *trans* regulation under specific conditions[134].

Several examples illustrate the role of APA in mRNA localization. Perhaps the best-studied molecule is brain-derived neurotrophic factor (BDNF) that is processed to two transcripts with either a long or a short 3'UTR, both encoding the same protein. The short 3'UTR mRNA is restricted to the soma whereas the long 3'UTR mRNA is preferentially targeted to dendrites. Mutant mice lacking the long 3'UTR isoform show little expression of BDNF in dendrites, despite normal levels of total BDNF. As a result, these mice exhibit deficits in the pruning and enlargement of spines, as well as impairment in LTP in dendrites but not in the soma of hippocampal neurons[135]. Phenotypically, they develop severe hyperphagic obesity[136]. Furthermore, BDNF transcripts are differentially regulated, with the long 3'UTR isoform being translated under stimulation with pilocarpine, insulin, or leptin. The short BDNF 3'UTR isoform on the other hand, displays constitutive translation[136, 137]. Dendritic targeting of BDNF is thought to be in part mediated by the binding of CPEB1 to a CPE-like element in the 3'UTR after KCl-induced depolarization in hippocampal neurons[138]. Further, the stability of BDNF long 3'UTR mRNA is mediated by HuD binding to a highly conserved AU-rich element, specifically located in the long 3'UTR[139].

Computational predictions have indicated that variations of the canonical PAS sequence are relatively frequent, occurring in >30% of the ends[140]. Interestingly, while the canonical sequence predominates in genes with a unique PAS site, the less-conserved variant PAS sites occur with higher frequency in genes with multiple PAS sites. Moreover, these variant sites tend to be located upstream of the more canonical PAS site, indicating that APA is regulated by the abundance of polyadenylation complex proteins or the existence of cell-type specific *trans* auxiliary proteins[141]. Indeed, evidence exists for both mechanisms. Ji and Tian have revealed that CPSF and CSTF components are strongly upregulated during the generation of induced pluripotent stem cells from different tissues and this is associated with the usage of proximal PAS sites, while longer 3'UTR isoforms appear with aging as a result of weakened mRNA polyadenylation activity[132, 142]. Moreover, genome-wide analysis of existing mRNA-sequencing data revealed that a third of non-canonical proximal PAS sites tend to possess a higher frequency of U and GU nucleotides downstream of the pA site compared with canonical pA signals, implying that a strong CSTF binding site might compensate for the absence of a consensus hexanucleotide[143]. Interestingly, these U/GU sequences are also prime binding sites for HU proteins. Hence, Zhu *et al*. have shown that all HU proteins selectively block both cleavage and poly(A) addition at these sites, possibly by interfering with CSTF[144]. More recently, further support to this came from the study of transgenic ELAV flies that display ectopic synthesis of long mRNAs, indicating that ELAV binds directly to proximal PAS sites to suppress cleavage and pA in the brain[145]. Remarkably, the mRNAs of HU proteins also code for different APA variants displaying both differential expression and stability mediated by family members, indicating that HU proteins also have auto-regulatory functions[146-148].

Recently, a link between RBP and miRs sites has emerged. Initially, it was reported that destabilization mediated by a transfected miR is generally attenuated by the presence of destabilizing AU-rich motifs and augmented

by stabilizing U-rich motifs, the binding sites of translation- and turnover-associated RBPs such as HU, AU-binding factor 1, and tristetrapolin (TTP)[149, 150]. Subsequently, transcriptome-wide analysis of HuR revealed that most miR sites are in the immediate vicinity of HuR sites[4, 50, 151]. The authors elaborated that when miR and HU sites overlap the transcripts are preferentially regulated by HU proteins, but when they do not overlap the transcripts are regulated by miRs. Interestingly, *hu* transcripts are themselves direct targets of miRs and concurrently, directly regulate the stability and/or maturation of other miRs, pointing to a vast repertoire of different regulatory loops[4, 50, 152-154].

Like HU proteins, NOVA proteins appear to be an important component of APA in the brain. HITS-CLIP analysis of the genomic position of NOVA clusters revealed that 23% of tags map to intergenic regions that likely correspond to previously-undescribed isoforms of RefSeq genes with alternative terminal exons[63]. To further delineate this, the same group used exon array screening of altered 3'UTR length between NOVA-2 wild-type and null brains to identify ~300 mRNA transcripts with such differences. The data suggested that NOVAs bind YCAY elements flanking regulated pA sites, and that the position of NOVA binding may determine whether it acts to promote or inhibit pA site use. In transcripts in which NOVA enhances the use of a pA site, it binds to more distal elements and may antagonize the action of auxiliary factors. In cases where NOVA suppresses pA site use, binding sites are located within 30 nucleotides of the pA site and overlap with the canonical CPSF and/or CSTF binding sites, likely interfering with the formation of the cleavage complex[63].

Another example of a splicing factor multi-tasking at the 3'UTR of mRNAs is PTBP. PTBP-1 has been shown to either compete with CSTF for recognition of the pA signal's pyrimidine-rich DSE reducing 3'end cleavage[155] or induce 3' processing and polyadenylation by directly recruiting the splicing factor heterogeneous nuclear ribonucleoprotein H to G-rich sequences, which then stimulates pA through direct interaction with CSTF[156].

RBPs and Neurodegeneration: Nucleation Centers for Neurodegenerative Processes in the Aging Brain

The molecular and cellular bases of neurodegenerative diseases are poorly understood. Traditionally, they are described as protein disorders in which misfolded monomeric proteins initially oligomerize and then aggregate to form fibrils[157, 158]. These processes are largely thought to be unidirectional and detrimental, with no biological function. Their kinetics is dependent on the amount of starting proteins, their aggregation propensity or hydrophobicity, and the ability of the mechanisms of cell clearance - chaperones, proteasomes, and autophagy - to minimize their rate of assembly. The recent finding that the pathological redistribution of some RBPs from nucleus to cytoplasm is a hallmark feature of a wide spectrum of neurological disorders, however, has highlighted the involvement of the very dynamic RBPs and/or RNAs in the development of these processes. The importance of RNAs is underlined by the fact that mutations that disrupt the RNA binding ability of RBPs, like in the case of FUS or TDP-43, reduce or prevent their toxicity[159-162]. Further evidence comes from the finding that some RNAs, such as the products of mutated *c9orf72* and *fmr1* genes, lead to neurodegeneration by a poorly-characterized mechanism that is likely to involve the accumulation and sequestration of RBPs to nuclear foci[163-166].

The recently appreciated importance of RBPs in neurodegeneration is reflected in their highly-conserved protein structure. Apart from containing RNA recognition motifs, they all possess a glycine-rich hydrophobic domain that mediates self-dimerization and non-self protein-protein interactions. In some RBPs, like T-cell intracellular antigen-1 (TIA-1), the hydrophobic domain further shares homology with prion proteins[167]. The protein-protein interactions mediated by RBPs are normally reversible and tightly linked to the fate of their mRNA target. In general, when mRNAs are not engaged in translation, they assemble with RBPs in RNA granules. These granules are of three main types: (1) ribonucleoprotein particles (RNPs) that function in mRNA nucleocytoplasmic shuttling and axonal and dendritic transport, (2) processing bodies that also contain translation repressors and components of the mRNA decay machinery, and (3) stress granules (SGs) that sequester non-essential capped mRNAs in response to stress, promoting the translation of essential stress response proteins such as HSP70. As expected, RNA granules are highly dynamic structures constantly

exchanging mRNA transcripts and proteins through all stages of mRNA processing[168, 169]. Of these, SGs have recently received particular attention as most if not all of the RBPs linked to neurodegeneration associate with them in cell culture. Thus, TDP-43, FUS, ataxin-2 (ATXN2), survival motor neuron 1, and angiogenin have all been shown to co-localize with classic SG markers (TIA-1, TIAR, TTP, and G3BP) in cells undergoing stress. Further, disease-linked mutations of *tdp-43*, *fus*, and *atxn2* genes promote agglomeration in SGs, either by directly increasing the tendency of the protein to aggregate or by preventing nuclear translocation[79, 160, 170-175]. Of interest, examination of the brains of boxers and head injury patients also revealed accumulation of TDP-43 in cytoplasmic aggregates that may be remnants of trauma-induced SGs[176, 177]. Moreover, SG proteins such as TIA-1 and PABP co-localize with neuropathology in the brains of patients with Alzheimer's disease, FTDP-17, FTLD-TDP, and ALS[175, 178].

Thus, an alternative view of the neurodegeneration processes could be the formation of prolonged assemblies of RNPs in cytoplasmic granules, particularly SGs, that lose their dynamic disassembly over time and can only be cleared by autophagic mechanisms[179]. Since autophagy declines with aging[180], the clearance of these assemblies slows down, allowing some to be retained or grow by sequestering incoming RBPs. This ultimately alters mRNA metabolism, resulting in the production of aberrant mRNAs that either further potentiate RBP assembly or are mis-expressed, disrupting protein homeostasis and leading to cell death. Extracellularly released RBP complexes could then be endocytosed by surrounding cells, perpetuating the toxic effect[181].

One of the most puzzling questions that arise from neurodegeneration studies is why neurons are so profoundly affected by aging. Moreover, it is intriguing that pathology is only detected in distinct populations of neurons in the brain, despite the fact that the RBPs associated with neurodegeneration are ubiquitously expressed. A possible answer may lie in the unique features of neurons that include longevity, poor supply of progenitors, polarization, and degree of interconnection. Neurons are the longest-living cells in the body that, in time, could accumulate toxic protein aggregates that subsequently derail homeostatic mechanisms and drive cell death. The neurogenic niches supplying progenitors are limited and located in distinct areas of the brain, thus, most neurons are never replaced, allowing deficits to persist and grow over time. Further, by being highly polarized, neurons are greatly dependent on RBPs for function and are therefore highly vulnerable to RBP defects, whether these are inherited or caused by environmental input. It is known that the entire translation machinery is present at synaptic terminals, and at least in dendrites, all three types of RNA granules have been detected[182, 183]. Confined by space and the need to rapidly respond to synaptic stimuli, the different types of granules and their constituting RBPs are in close physical proximity, allowing enhanced ribonucleoprotein interactions. It is possible that over time or under stress, cycling of RBPs between these granules may start to lose coherence and initiate the formation of aggregates with other RBPs and mRNAs/proteins to disrupt synaptic function. It is conceivable that neurodegenerative pathology is initiated at these sites, a view supported by findings showing that some synaptic degeneration precedes neuronal loss[184-186]. Finally, because neurons are highly interconnected and spread out, there is a greater chance of receiving toxic RNP assemblies by endosomes from neighboring derailed cells. These toxic RNPs disrupt host RNA metabolism and disperse the defect in a manner resembling prion propagation.

Conclusion

RBPs are important mediators of qualitative and quantitative protein expression in neurons. Their role spans all stages of neuronal development including neurogenesis, differentiation, and synaptic plasticity. Deficits in RBP expression and/or distribution disrupt mRNA metabolism, leading to intellectual disabilities, motor impairments, and neurodegeneration. High-throughput sequencing studies have greatly advanced our understanding of their interactions with mRNA targets. In future, a major challenge is to better discern their roles in development and disease and how they fine-tune the expression of key neuronal proteins, given the multitude of RBP-mRNA and RBP-RBP interactions in the different RNP-processing granules.

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·Review·

Mechanisms of neuronal membrane sealing following mechanical trauma

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Membrane integrity is crucial for maintaining the intricate signaling and chemically-isolated intracellular environment of neurons; disruption risks deleterious effects, such as unregulated ionic flux, neuronal apoptosis, and oxidative radical damage as observed in spinal cord injury and traumatic brain injury. This paper, in addition to a discussion of the current understanding of cellular tactics to seal membranes, describes two major factors involved in membrane repair. These are line tension, the hydrophobic attractive force between two lipid free-edges, and membrane tension, the rigidity of the lipid bilayer with respect to the tethered cortical cytoskeleton. Ca^{2+}, a major mechanistic trigger for repair processes, increases following flux through a membrane injury site, and activates phospholipase enzymes, calpain-mediated cortical cytoskeletal proteolysis, protein kinase cascades, and lipid bilayer microdomain modification. The membrane tension appears to be largely modulated through vesicle dynamics, cytoskeletal organization, membrane curvature, and phospholipase manipulation. Dehydration of the phospholipid gap edge and modification of membrane packaging, as in temperature variation, experimentally impact line tension. Due to the time-sensitive nature of axonal sealing, increasing the efficacy of axolemmal sealing through therapeutic modification would be of great clinical value, to deter secondary neurodegenerative effects. Better therapeutic enhancement of membrane sealing requires a complete understanding of its intricate underlying neuronal mechanism.

Keywords: axolemmal sealing; membrane tension; line tension; phospholipase; calpain; poly-ethylene glycol; patch model

Introduction

The integrity of the plasma membrane is critical to the cell as it protects and maintains the functionality of the isolated intracellular environment. Situations involving trauma to the plasma membrane result in disrupted integrity and subsequent permeability to ions and molecules. Of principal interest is Ca^{2+} influx into the intracellular space, activating proteases, disrupting mitochondrial function, and activating apoptotic pathways[1, 2]. Thus, effective and efficient repair of plasma membrane integrity is essential for cell survival.

Mechanical disruption of the neuronal membrane has been extensively studied *in vitro*, *in vivo*, *ex vivo*, and after traumatic brain injury (TBI) or spinal cord injury (SCI)[3-6]. Membrane healing within the nervous system has been studied in a number of model organisms: sea slug (*Aplysia californica*)[7], cockroach (*Periplaneta americana*)[8], guinea pig[5], earthworm (*Lumbricus terrestris*)[9], squid (*Loligo pealei*)[10], rat[11], and snail (*Helisoma trivolvis*)[12]. Mechanical disruption of neurons has focused primarily on axonal transection, stretch, and compression[13-17]. Such injuries can produce a breach in membrane integrity from the moment of injury, and this is termed “mechanoporation”[3].

Experimental data on axonal trauma indicate that repair of the initial membrane breach is the necessary first step to allow growth-cone formation and subsequent axonal regeneration[18]. Thus, establishment of this membrane sealing is key to the successful recovery of the neuron, highlighting the value of understanding the sealing process. Well-established quantitative techniques in membrane integrity analysis include dye staining or fluorescent marker influx through a membrane breach[5,17,19,20], and de-staining of fluorescent markers localized intracellularly before the trauma[21]. The magnitude of the labeling through these techniques is a function of the applied mechanical load to the membrane[22].

While neurons differ from other cell types in morphology, environmental exposure, mitotic status, motility, and function, a consideration of different cell types is essential to better understand the healing of the neuronal membrane upon mechanical disruption. The cytoskeletal and structural distinctness between somal membrane and axolemma may cause differences in membrane sealing; however, the processes may have much in common due to the presence of cell-type specific enzymes involved in the repair process. One of the largest discrepancies in membrane sealing between neurons and other cell types is the time course, exemplified by neuronal membranes requiring minutes to hours[13] compared to Swiss 3T3 fibroblasts and sea urchin eggs requiring seconds to minutes[23, 24]. These discrepancies in physiological mechanisms could stem from an evolutionary lack of preparedness within the mechanically-protected neuron population compared to the relatively exposed fibroblast population. So, caution must be used when considering broad claims of applicability between these very different populations of cells.

Proposed Models of Membrane Sealing

Based on *in vitro* analysis of cellular and axolemmal membrane repair, several models have emerged to explain the cellular mechanics involved in membrane sealing. The factors of interest in this discussion are line tension and membrane tension, which are regulated by the dynamics of intracellular vesicles and cytoskeletal remodeling. Line tension refers to the thermodynamic force at the free phospholipid edge of a membrane lesion that favors hydrophobic interactions between adjacent phospholipids, thereby promoting spontaneous membrane sealing. The membrane tension is opposing membrane sealing based on the rigidity of the underlying tethered cortical cytoskeleton[25,26], which prevents the progression to a lower entropic state in which phospholipids interact between the lipid free edges during a membrane breach.

Considering the implications of tension, McNeil and Terasaki constructed a framework to illustrate the mechanism of membrane resealing[19]. Line tension has been proposed to dominate in situations of small disruptions less than 1 μm in diameter, thereby promoting membrane sealing[19] (Fig. 1C); above this diameter, membrane tension would overcome the energy for thermodynamic distortion of the free membrane edge, so it is necessary to reduce membrane tension to facilitate resealing[27] (Fig. 1A). Thus, a logical mechanism of sealing would involve reducing membrane tension to decrease the gap in the membrane to <1 μm, to facilitate association between adjacent phospholipids on each side of the gap (Fig. 1C). The reduction in membrane tension essential for sealing has been quantified using the laser tweezer method, and has been shown to correspond with vesicle exocytosis, which increases the membrane surface area and consequently decreases the tension[21]. For even larger membrane disruptions, Ca^{2+}-dependent intervesicular fusion forms a membrane patch to seal the larger gap more efficiently[24, 28, 29]. The cellular commitment to one of these two mechanisms of repair may be based on the magnitude of Ca^{2+} influx as determined by the disruption size, mediating the occurrence of vesicle-vesicle or vesicle-membrane fusion events based on the resulting level of intracellular Ca^{2+}. Soluble NSF attachment protein receptors (SNAREs) are implicated in this Ca^{2+}-dependent membrane repair process, as synaptotagamin, SNAP-25, synaptobrevin, and syntaxin mediate the Ca^{2+}-dependent vesicular fusion[10, 30]. An alternative idea to the membrane patch has recently been proposed, implicating Ca^{2+}-dependent transglutaminases in the cross-linking of intracellular proteins to form a proteinaceous clot that decreases the membrane permeability, similar to the principle of the membrane patch[31, 32]. In addition, recent studies with streptolysin O pores and electroporation have suggested that endocytosis helps remove lesions from

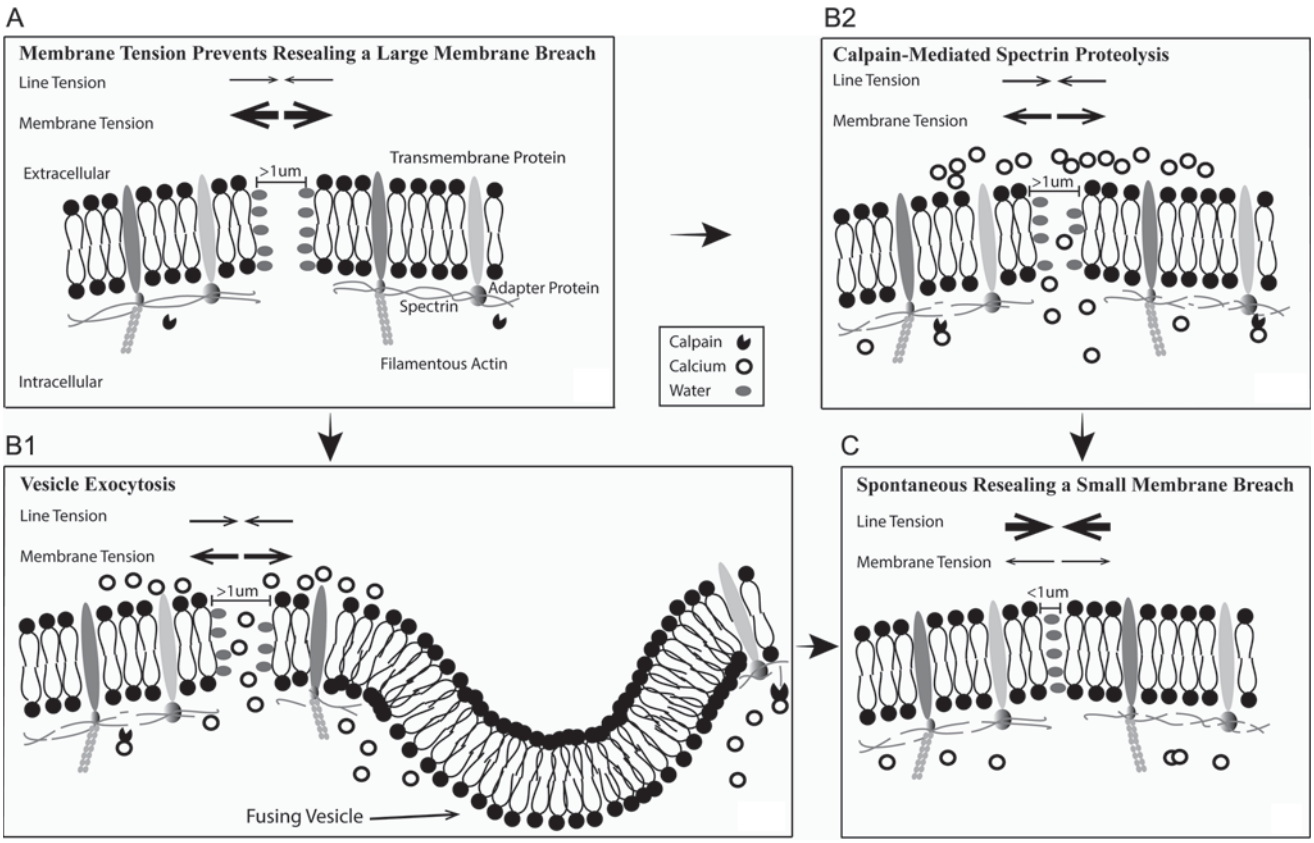

Fig. 1. Forces influencing membrane sealing. The membrane sealing process is governed by a balance between line tension and membrane tension at the axolemma. Line tension promotes membrane sealing through the attractive force between adjacent hydrophobic regions of the membrane, and membrane tension generated from tethers to the underlying cortical cytoskeleton hinders resealing. A: When the membrane breach is >1 μm, membrane tension is the dominant force and membrane sealing is prevented. However, when the breach is reduced by membrane-vesicle fusion (B1) or calpain-mediated cortical cytoskeletal proteolysis of targets such as spectrin (B2), line tension becomes the dominant force and spontaneous membrane sealing occurs (C). The magnitudes of these forces are represented by the sizes of the corresponding arrows in each diagram.

the plasma membrane, thereby facilitating membrane sealing[31,33,34]. Such a mechanism would first involve an exocytosis-dependent reduction in membrane tension, which is conducive to subsequent endocytosis of the membrane lesion.

Role of Line Tension in Axolemmal Sealing

Line tension, as described previously[35], promotes spontaneous membrane sealing through the thermodynamic force of attraction between hydrophobic phospholipid regions along the membrane site of disruption following injury. This force opposes the membrane tension; a predominance of line tension would result in spontaneous membrane sealing (Fig. 1C). Line tension relates largely to the packing ability within the membrane such that the efficient packing of lipid bilayer components results in decreased line tension[36]. Decreased packaging efficiency through cholesterol incorporation has been proposed to limit the free rotation of fatty acyl tails in the membrane, resulting in increased line tension[36]. Line tension is inversely proportional to the membrane disruption radius, as depicted by poration of liposomes and through the derived pore free energy equation[36].

Temperature Dependence of Line Tension

Hypothermic treatment following SCI and TBI has been implemented in clinical settings due to its proposed benefits of reducing intracranial pressure and providing neuroprotection. However, mechanistically, these benefits

have not been experimentally shown. The decreased temperature, based on elementary membrane dynamics, would induce decreased membrane fluidity and increased packing efficiency. As described above, increased packing efficiency would decrease line tension; thus, hypothermia would decrease the rate of membrane sealing through regulating thermodynamic potential energy alone. This proposition is supported by studies using the double sucrose-gap recording chamber, which showed that sealing efficiency *ex vivo* decreases dramatically at 25°C compared to that at 31°C and 37°C[13], indicating a need for reconsideration of the hypothermic treatment protocol. In addition, although there are no significant differences in membrane sealing between 31°C and 37°C[13], hypoxia-induced injury recovers significantly better at mildly hypothermic temperatures *in vitro*[37, 38]. Thus, the mild hypothermic condition of 31°C appears to be the most conducive for healing from mechanically-induced TBI or SCI.

Viral protein-mediated fusion, an experimental model for studying membrane fusion mechanics, has shown dependence on temperature, such that decreasing the temperature results in slowing of the fusion process[39]. Phospholipase C (PLC) is also temperature-dependent such that activation of PLC, vesicle aggregation, and vesicle fusion increase with increasing temperature[40]. Thus, the data further contraindicate the use of intense hypothermia in treating SCI and TBI during the early stages when membrane repair is the priority. Based on studies of axolemmal repair *in vitro*, the time course of sealing appears to be 45 min–1 h, using the presence of resting membrane potential and horseradish peroxidase exclusion as indicators of successful sealing[13]. More conclusive studies on the time course of mammalian neuronal membrane sealing *in vivo* are needed to further direct clinical care for patients with neurological trauma.

Role of Membrane Tension in Neuronal Membrane Sealing

Lowering the membrane tension following liposome pore formation is known to facilitate repair[36, 41]. Artificial decreases in membrane tension by the surfactants Pluronic F68 NF[27] and polyethylene glycol (PEG) also facilitate repair[17]. Further studies have shown that deposition of membrane vesicles at the lesion site decreases plasma membrane tension[21, 42] (Fig. 1B1). The implications for membrane tension in mammalian plasma membrane repair and the Ca^{2+}-dependent nature of the repair have been reported, using the laser-tweezer method of membrane tension quantification[43], within Swiss 3T3 fibroblasts[21]. Laser scissors or glass needle methods of injury (5 μm in diameter) revealed that the membrane repair process largely involves a quantifiable Ca^{2+}-dependent reduction in membrane tension following vesicle fusion to the plasma membrane[21]. Also, membrane tension increases proportionally to the square of the radius of the membrane pore[36], suggesting that reducing the pore radius is necessary to achieve a membrane seal. Moreover, using atomic force microscopy, Nehrt *et al.* found that PEG reduces the neuronal membrane tension[17]. Based on the small pore-diameter necessary for effective line tension-mediated sealing[19], a reduction in the pore diameter facilitated by a less rigid membrane would better facilitate repair of the injury (Fig. 1B2). Reduction in membrane tension has a complex group of potential causes that interplay simultaneously to produce the cytoskeletal and membrane changes necessary to facilitate repair.

Cytoskeletal Modification

The membrane tension of cells is largely derived from adhesion to the underlying cytoskeleton[44], implying the importance of cytoskeletal rearrangement in membrane sealing. Cytoskeletal modification is essential for membrane sealing in a number of cell types, including neurons, based on the finding that inhibition of the Ca^{2+}-activated cysteine protease calpain leads to incomplete membrane sealing[5,25,45]. Cytoskeletal modification is also critical for growth-cone formation following axolemmal sealing[7, 46]. Investigation into calpain has also linked its activation to cell mortality[47-49]. Thus, based on the apparently contradictory data, there must be an optimal level of activity to facilitate Ca^{2+}-dependent spontaneous membrane sealing without inducing the deleterious intracellular effects. It has been proposed that mechanisms of increasing membrane permeability can induce excessive calpain activation in the presence of high intracellular Ca^{2+} concentrations[2]. Therefore, calpain may effectively help membrane sealing in a threshold range of intracellular Ca^{2+} concentrations to facilitate optimal cytoskeletal reorganization.

The cysteine protease calpain has been implicated in cytoskeletal modification. Spectrin[50], part of the cortical cytoskeleton, has been suggested to act as a barrier against vesicle exocytosis, such that subsequent Ca^{2+}-dependent activation of calpain and proteolytic cleavage of the spectrin intracellular meshwork would facilitate vesicle exocytosis[51]. Calpain facilitates replenishment of the releasable vesicle pool in healthy neurons. This is hypothesized to be due to the mobilization of vesicles into a readily-releasable state[52], supporting the idea that calpain-mediated proteolysis also promotes vesicle exocytosis through its role in vesicle dynamics. Recent research into the cortical cytoskeleton of axons has shown the presence of a 180–190 nm periodic arrangement of circumferential actin rings, composed of short actin filaments, interconnected by spectrin tetramers[53]. This arrangement of the actin-spectrin network would give rigidity to the axolemma and following membrane injury may be the basis of the majority of membrane tension; as the spectrin recoils toward the circumferential actin, the membrane is pulled away from the injury site, thereby opposing axolemmal sealing.

Anchoring proteins in the cortical cytoskeleton participating in membrane-cytoskeletal interaction are also proposed to be calpain substrates. Protein 4.1R and G homologs identified in rat brain neurons play a modulatory role in spectrin and filamentous-actin (F-actin) association in the cortical cytoskeleton[54]. Data have shown the Ca^{2+}-dependent calpain cleavage of a protein 4.1A and B homolog present in erythrocytes[55, 56]; thus, calpain may mediate the cleavage of R and G homologs in neurons following Ca^{2+}-dependent activation. The actin cytoskeletal network also participates in this cortical skeleton[53] and is hypothesized to be a major modulator of microtubule polymerization[57]. This effect on cytoskeletal architecture by actin would thereby regulate the plasma membrane surface area in neurons[57]. Microtubule reorganization is an integral step in axolemmal sealing, facilitating subsequent growth-cone formation[51]. Microtubule-associated proteins (MAPs) are known targets of calpain-mediated proteolysis[48, 50]; by MAP cleavage, calpain participates in Ca^{2+}-dependent microtubule reorganization following axolemmal trauma, thereby altering cytoskeletal organization. Actin also has implications in membrane repair through its association with integrins, thereby forming focal adhesions *via* talin, which is a calpain substrate in fibroblasts[58]. Many of the examples of calpain-mediated cleavage of substrates described above can lead to a looser membrane association with the cortical cytoskeleton, thereby decreasing membrane surface tension (Fig. 1B2). It is also possible that the cleavage of talin and other focal adhesion mediators facilitates proximal axonal stump retraction, allowing for a greater plasma membrane surface area relative to the underlying cortical cytoskeleton, which further decreases the membrane tension and facilitates membrane sealing.

Exocytotic Vesicle Dynamics Relating to Membrane Sealing

Intracellular vesicle exocytosis, as well as vesicle formation *via* endocytosis, can largely impact membrane tension[59, 60]. The vesicle source for membrane repair has been reported to be Golgi-derived vesicles[51], lysosome vesicles[61, 62], vesicles formed by membrane endocytosis adjacent to the lesion[63], and myelin membrane delamination[9]. Kinesin and myosin-V through both filament systems have been implicated in the dynamics of the intracellular vesicle pool[64] and vesicle localization to the site of trauma, facilitating Ca^{2+}-dependent exocytosis and membrane resealing[65]. Multiple mechanisms exist for controlling the magnitude of the contribution of each of these vesicle sources to the membrane repair process.

Phospholipase enzymes following membrane trauma

The phospholipase enzymes act in pathways to modify the cytoskeleton, vesicle dynamics, and the phospholipid population. Phospholipases A2 (PLA2), D (PLD), and C (PLC) are involved in vesicle exocytosis[66] and thereby axolemmal sealing. PLA2 activity has been implicated in membrane sealing in some experimental systems: cockroach[8, 67], frog[68], mouse[69], and snail[18]. The influx of Ca^{2+} is a good candidate for the initiation of signal transduction in the phospholipase pathways (Figs. 2 and 3). In fact, Ca^{2+} has been hypothesized to mediate the membrane localization of specific isozymes of PLC and cytosolic PLA2 (cPLA2)[70-72]. PLCδ1, PLCη and cPLA2 present in neurons[73, 74] contain a C2 Ca^{2+}-sensing domain which facilitates Ca^{2+}-dependent phospholipase membrane localization[40, 70, 75-78] and subsequent recognition of substrates and catalytic activity. Specifically, PLA2 activity produces arachidonic acid and lysophophatidic acid (LPA)[75, 79].

All PLC isoforms contain C2 domains and EF-hands that classically function as Ca^{2+}-binding motifs, but their role in the broad Ca^{2+}-binding capacity among PLC isoforms needs to be clarified[80]. In contrast, the PLD C2 domains have only been identified in plant isozymes[81]; thus, the direct Ca^{2+}-dependent activation of PLD has not been implicated in mammalian models.

A subsequent step in the sealing pathway involves protein kinase C (PKC). PKC isozymes have been categorized into three major classes: conventional (Ca^{2+}/diacylglycerol (DAG)-activated), novel (DAG activated, Ca^{2+}-insensitive), and atypical (Ca^{2+}/DAG-insensitive)[75]. Arachidonic acid is a potent stimulator of novel PKC (nPKC) and synergistically enhances conventional PKC (cPKC) activity[75]. Thus, following mechanical membrane trauma, both cPKC and nPKC isoforms may be activated. cPKCα, cPKCβ, and nPKCδ would then activate PLD[82, 83]. Localization of nPKCδ in rat CNS neurons[84] and cPKCα and cPKCβ in rat sensory neurons[85] has been reported. Neuronal localization is suggestive of the potential relevance to axolemmal sealing. In addition, competitive inhibition studies have shown that the novel PKC subspecies nPKCη and nPKCθ are critical in sealing B104 cells[86, 87]. The downstream pathways for PKC isozymes are vast and have yet to be elucidated, but the sealing-relevant pathways have been identified.

PKC plays a role in the generation of a membrane source for repair processes in the intracellular Golgi-derived vesicle pool[27]. cPKC and nPKC are also implicated in the phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) which has been hypothesized to mask the interaction site for phosphatidylinositol 4,5-bisphosphate (PIP2), leading to the release of PIP2 microdomain clusters from the lipid bilayer[88]. In addition, phosphorylation of MARCKS by PKC is potentiated by arachidonic acid[75]. The proposed PKC activation (Fig. 2) initially occurs in response to both Ca^{2+} and arachidonic acid, hence the cycle of PKC activation is amplified following the production of DAG by PLC.

The model next includes the activity of PLD (Fig. 2), shown to be regulated by a Rho-family member, ADP-ribosylation factor (Arf) 1, and PKC[82]. The role of PLD in membrane sealing is hypothesized to involve the release of phosphatidic acid (PA) from phosphatidylcholine (PC) by cleavage[89]. PA stimulates phosphatidylinositol-4-phosphate 5-kinase (PI(4)P 5-kinase), thereby increasing PIP2 formation[90]. In addition, PA following cleavage by PA hydrolase forms DAG[90] which is not capable of enhancing membrane translocation of any PKC isoform[91]. Enhanced PA, through PLD, allows for PLA2 processing of PA to form LPA[90]. It has been noted that lysophospholipids have detergent-like properties[92], which may aid in sealing a membrane breach or dehydrating the lipid free edges to increase line tension. LPA, although not being well understood as a chemical messenger, has some hypothesized functions in the fusion and fission dynamics of vesicles[93]. LPA overexpression causes Rho-dependent cellular changes in focal adhesion, cell motility, the cytoskeleton, process retraction, and cell survival[94], implying its importance in cytoskeletal remodeling, which may reduce membrane tension. Moreover, it has been proposed that exogenous PLD acts through G-protein coupled LPA-receptors to activate Rho signaling pathways[95, 96]. These findings suggest that LPA activates Rho signaling.

Rho-family GTPase signaling has multiple roles in the membrane response to trauma. These include Rho-dependent cytoskeletal modification as described above, and inhibition of receptor-mediated endocytosis *in vivo* and clathrin-mediated endocytosis *in vitro* by Rho[97]. Therefore an increase in the intracellular vesicle stores would occur secondary to a continued Golgi-derived supply of vesicles. This is of importance to the membrane patch hypothesis due to the need for an enhanced presence of membrane vesicles to facilitate patch formation. A study in PC-12 and N1E-115 cells reported that Rho signaling results in neurite retraction following contraction of the cortical actomyosin cytoskeleton[98]. This corresponds well to the morphological response observed following axonal trauma[99] and would further reduce the membrane tension. This membrane retraction likely occurs with simultaneous endocytosis; thus, to fit temporally with the inhibition of endocytosis by Rho, it may occur as a later step of membrane repair following phospholipase activity. The Rho-family of GTPases has been implicated in PLCβ and PLCε activation, although the complete isozyme-specific pathways have yet to be elucidated[40, 80]. GTPase signaling has numerous possibilities for generating cellular changes, which need to be investigated in a cell-type isoform-specific manner

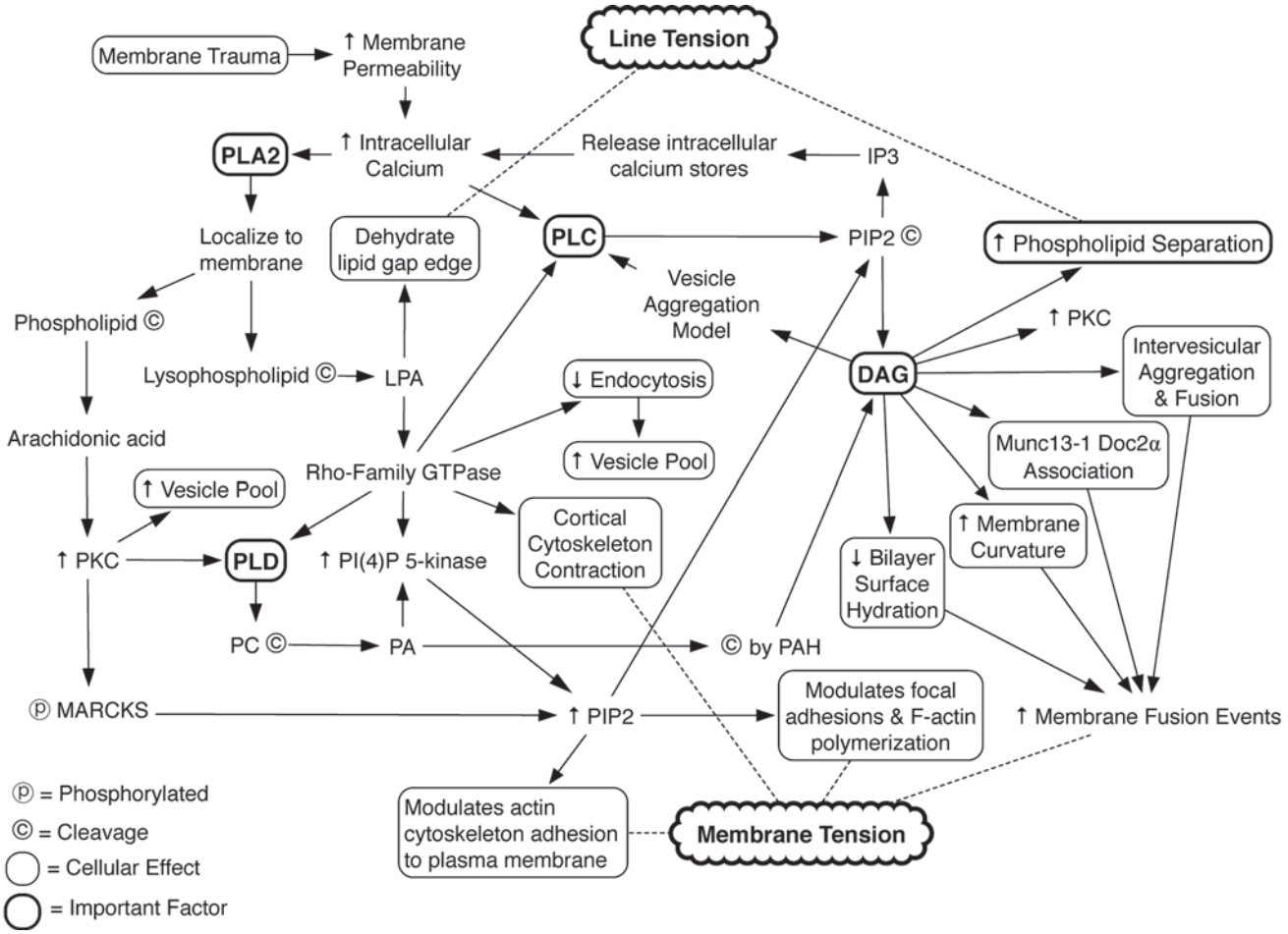

Fig. 2. Diagram of phospholipase enzyme activity and membrane sealing. Phospholipase enzyme activity in response to membrane trauma serves to amplify the magnitude of changes in cytoskeletal and membrane composition to promote membrane sealing. The major phospholipase enzymes involved are PLA2, PLC, and PLD. DAG, upon deposition primarily through PLC catalysis, amplifies membrane fusion events through proposed methods of membrane monolayer modification. DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PAH: phosphatidic acid hydrolase; PC, phosphatidylcholine; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C.

to ensure a correct hypothesis regarding its application to membrane sealing.

The activation of PLC is a key step in the phospholipase cascade to deposit DAG, which is known to modulate membrane characteristics. Activation of PLC may involve Ca^{2+}-dependent localization to the plasma membrane, or Rho-family signaling. An alternative hypothesis for activation of PLC is a vesicle aggregation model in which generation of DAG at a threshold level through PLC catalysis and subsequent vesicle aggregation induces full enzyme activity[40]. This mode has relevance considering the vesicle aggregation necessary in the patch hypothesis of membrane sealing[19]. PLC activation causes cleavage of PIP2 into inositol 1,4,5-trisphosphate (IP3) and DAG[40,75]. PLC through IP3 participates in Ca^{2+} signal amplification through the release of intracellular Ca^{2+} stores[40] (Fig. 2).

PLC cleavage of PIP2 is important in neurite membrane sealing through the production of DAG[87]. PIP2 also plays a role in cytoskeletal dynamics and cell signaling; it modulates the adhesion between the actin cortical cytoskeleton and the plasma membrane, and cleavage of PIP2 results in decreased adhesion energy[100]. The

decreased adhesion energy then decreases membrane tension, promoting membrane resealing. Further, PIP2 modifies actin-associated proteins such as profilin, gelsolin, α-actinin, and vinculin, causing alterations of the actin cytoskeleton[101-103]. Vinculin is modified by PIP2 such that dissociation of its head-tail configuration unmasks its talin and actin-binding sites[102]. Therefore, as PIP2 increases the cortical cytoskeletal association, cleavage of PIP2 by PLC would decrease protein component association and subsequently reduce membrane tension. However, as seen in the proposed phospholipase cascade (Fig. 2), Rho family GTPases act to stimulate the production of PIP2 by enhancing the activity of PI(4)P 5-kinase[102], and thus stimulate tighter cortical cytoskeletal adhesion. As vinculin is a key structural component within the cytoskeleton through talin- and actin-binding[104], exposure of its binding sites following PIP2 stimulation may allow for more effective calpain protease cleavage following axolemmal trauma. Vinculin cleavage by calpain within neurons has not been studied, but there is evidence for vinculin susceptibility to calpain-mediated proteolysis in other cell types[58,105]. Thus, early PIP2 deposition following membrane trauma may serve to increase the susceptibility to Ca^{2+}-dependent calpain cleavage in the cortical cytoskeleton through cytoskeletal conformational changes followed by cleavage of PIP2 after the full activation of PLC to mediate a decrease in membrane tension.

DAG is generated after PIP2 cleavage by PLC, and this has many implications in membrane sealing[40]. DAG decreases bilayer surface hydration and increases the separation between adjacent phospholipids[106]. This would increase membrane fluidity, thereby decreasing membrane tension, and the decreased packaging efficiency would increase line tension (Fig. 2). Certain membrane components can impart negative membrane curvature on a lipid monolayer based on their focal concentration; DAG has been shown to convey these properties on a monolayer[107]. DAG does not mix homogenously within the phospholipid bilayer resulting in islands of DAG-rich regions[106], and this has implications for imparting maximum membrane curvature within a localized region of the monolayer favoring membrane fusion. DAG has also been shown to promote intervesicular aggregation and vesicle fusion following production by PLC[106], a very relevant step for promoting the formation of a membrane patch and full PLC activation by the vesicle aggregation model. DAG is known to induce an association between Munc13-1 and Doc2α, a step involved in Ca^{2+}-dependent vesicle exocytosis[108]. DAG plays a well-established role in activation of the cPKC and nPKC subfamilies through DAG interaction with the C1 domain of PKC proteins[40, 75]. Overall, DAG is implicated in membrane dynamics directly by promoting vesicle fusion and indirectly through activation of other signaling cascades, both of which could promote axolemmal repair following trauma.

In sum, the complex interplay between the PLA2, PLC, and PLD families modulates plasma membrane and vesicle dynamics (Fig. 2), facilitating a reduction in membrane tension through increased fluidity and final membrane fusion. Full activation of the cascade would support the hypothesized membrane patch formation adjacent to the site of membrane trauma in response to Ca^{2+} influx.

SNARE protein involvement in exocytosis-mediated repair Ca^{2+}-dependent SNARE protein association participates in axolemmal sealing[10, 30] and membrane sealing in other invertebrate and mammalian cell types[10,109-111]. The interaction between Ca^{2+}-dependent vesicle-localized synaptotagamin and membrane-localized syntaxin, facilitated by C2A and C2B domains on synaptotagmin[112], mediates vesicle fusion[113-115]. Thus, Ca^{2+} influx at the site of membrane injury would facilitate vesicle-membrane fusion.

Synapsin, a synaptic vesicle-associated phosphoprotein, participates in vesicle dynamics and, following dissociation, enhances SNARE-mediated vesicle exocytosis through its role in vesicle-actin tethering[116-118]. Synapsin is also implicated in axonal regrowth and growth-cone dynamics based on the time course[119] and sites[120] of intracellular accumulation. Synapsin phosphorylation *via* protein kinase A (PKA) is hypothesized to regulate synapsin dissociation from the vesicle membrane, thereby trafficking the vesicle pool from the reserve to the readily releasable state[121]. Synapsin proteins enhance neurite outgrowth *via* cAMP-dependent PKA, hypothetically determining the rate of membrane insertion[122]. There are also phosphorylation sites on synapsin I for calcium/calmodulin-dependent protein kinase (CaMK)I, CaMKII, and CaMKVI[123, 124]. Data suggest that, following influx, Ca^{2+} activates CaMKII, which

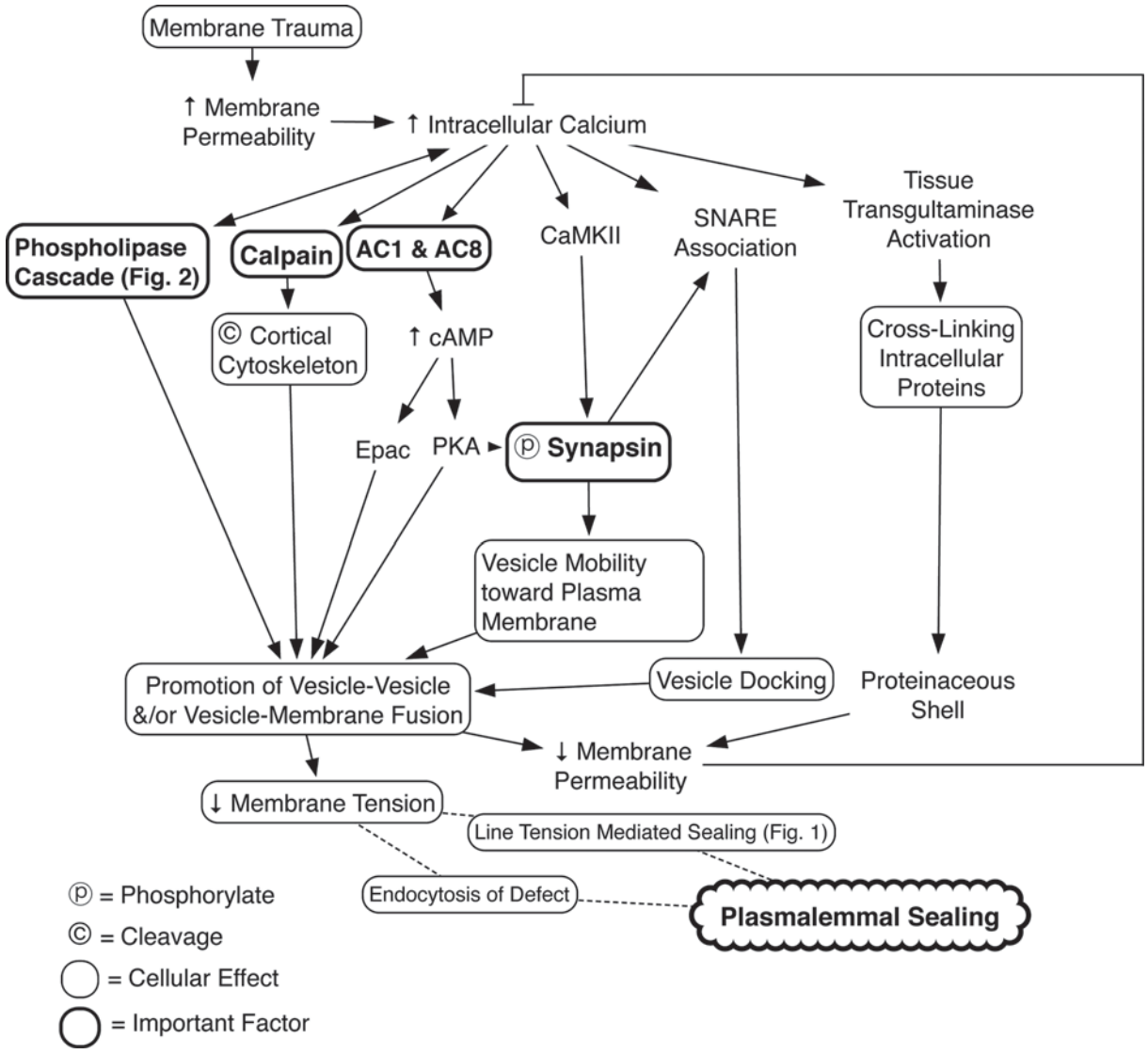

Fig. 3. Diagram of the overall neuronal membrane sealing mechanism. The major trigger for the signaling cascades that precipitate membrane sealing is the influx of Ca^{2+} through the membrane disruption following trauma. The major pathways involved in subsequent signaling are phospholipase enzymes, calpain proteases, the cAMP cascade, SNARE-mediated vesicle deposition, and the proposed formation of an intracellular proteinaceous shell. The resulting decrease in membrane permeability as the membrane seals serves as feedback on the signaling pathways to return to homeostasis following plasmalemmal sealing.

then phosphorylates synapsin I, thereby reducing its binding to vesicles[124], similar to the PKA-mediated phosphorylation noted above. CaMKII inhibition also reduces the slow and fast phases of vesicle recruitment during Ca^{2+}-mediated exocytosis[65]. These findings justify the inclusion of CaM kinases in the model of vesicle recruitment and fusion dynamics during membrane sealing (Fig. 3). In summary, a logical result of synapsin phosphorylation by CaMKII or cAMP-mediated PKA, which both increase as a result of Ca^{2+} influx (Fig. 3), would be an increase in vesicle exocytosis likely facilitated by SNARE proteins[116, 122].

Parallel cAMP axolemmal sealing pathways Ca^{2+}-dependent exocytosis has been reported to involve cAMP-dependent signaling pathways in pancreatic β-cells[125].

For neuron membrane sealing, the presence of adenylyl cyclase (AC)1 and AC8, the major Ca^{2+}-sensitive isoforms within neurons[126], could be the starting point for a cAMP-signaling cascade to generate vesicle exocytosis. These Ca^{2+}-dependent exocytotic events have been found in B104 cells[86]. cAMP mediates two parallel pathways of vesicle dynamics through interaction with PKA and exchange proteins activated by cAMP (Epac)[127-129], which has been suggested to be the mechanism of action in injured B104 cells investigated using small molecular inhibitors of PKA and Epac[86].

PKA has also been implicated in the potentiation of Ca^{2+}-dependent exocytosis and membrane resealing following an initial membrane injury in Swiss 3T3 fibroblasts[23]. Involvement of PKA in the signaling that promotes membrane sealing may be based on its regulation of synapsin, increasing the number of readily-releasable vesicles[122]. Overall, the cAMP pathway is another important Ca^{2+}-dependent mechanism to deposit membrane at the site of injury, and decrease the membrane gap and surface tension.

Membrane curvature and vesicle mechanics The spontaneous curvature imparted on the membrane by microdomains is a key characteristic in defining the fusibility of lipid membranes. Some agents that impart this negative spontaneous monolayer curvature are cholesterol, DAG, phosphatidylethanolamine[107], and α-tocopherol[130]. It is believed that agents imposing negative curvature have a small hydrophilic head group relative to a larger hydrophobic domain, leading to a concave structural conformation that imparts negative spontaneous curvature to the membrane[107]. Cholesterol-induced spontaneous membrane curvature has a threshold level for facilitating vesicle fusion[131], such that other substances must induce a comparable or more negative curvature to effectively induce fusion[107].

Following the current understanding of the stalk-pore model for membrane fusion, a point-like protrusion initially forms, followed by the stalk structure, then the transmonolayer contact forms as a hemifusion diaphragm, which decays into the fusion pore[39, 132, 133]. The energy barrier of these fusion intermediates is the rate-limiting step for membrane fusion[134]. The major energy barrier that the negative spontaneous membrane curvature aids in overcoming is the short-range hydration repulsion existent on the point-like protrusion structure between the fusing membranes[133] and development of the hemifusion diaphragm[134]. Such microdomains enriched in cholesterol, sphingomyelin, DAG, α-tocopherol, and phosphatidylethanolamine result in an increased capacity for membrane fusion by inducing membrane curvature[107, 131, 135]. The membrane components that convey spontaneous curvature also likely affect the local protein and lipid organization[107], altering enzymatic activity and binding specificity. In relation to membrane repair, deposition of negative curvature-inducing component DAG into local microdomains surrounding injured membrane following the phospholipase cascade (Fig. 2), could facilitate vesicle-vesicle and vesicle-membrane fusion events to restore plasma membrane integrity.

Endocytotic Vesicle Dynamics Relating to Membrane Sealing

Endocytosis is a mechanism of absorbing the membrane breach defect[31]. Application of the pore-forming protein streptolysin O and mechanical membrane disruption in NRK, HEK-293, and HeLa cells result in endosomes adjacent to the injury, representing endocytosed membrane[33]. During this process, actin cytoskeletal disruption is suggested to enhance endocytosis and thereby wound repair. In addition, exocytosis of adjacent lysosomal vesicles[61, 62], which is hypothesized to precede endocytosis of the membrane gap, would decrease membrane tension to a threshold level that facilitates subsequent endocytosis[31]. This is consistent with the phospholipase model (Fig. 2), in which inhibition of endocytosis by Rho-family GTPases occurs following membrane trauma. However, exocytosis would then serve to decrease the membrane tension, concurrent with decreasing Ca^{2+} influx as the membrane gap closes, which decreases Rho-GTPase signaling, both of which alleviate the inhibition of endocytosis. This would allow for a membrane breach to subsequently be absorbed into an endosomal vesicle and restore plasma membrane integrity, without the need for completion of a line tension-dependent sealing process (Fig. 1).

Endocytosis plays additional cellular roles during neuron injury. The retrograde axonal transport of endosomes has been implicated in a trauma-induced signaling mechanism to communicate injury signals to the

cell body[111]. Lastly, endocytosis is also a contributor to the vesicle supply for membrane sealing[63]. This emerging role of endocytosis in membrane repair is relatively recent and requires further studies to better understand the interplay between endocytosis and exocytosis, the two seemingly opposite vesicle processes in membrane sealing.

Role of Environmental Factors

Extracellular Ca^{2+} Concentration

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is essential for successful sealing of the axolemma following trauma[10, 12, 110, 136, 137]. The extracellular Ca^{2+} ($[Ca^{2+}]_o$) is also critical, in that the optimal efficiency of sealing occurs with 2 mmol/L $[Ca^{2+}]_o$, and an effective block on sealing occurs with $[Ca^{2+}]_o$ <0.5 mmol/L[5]. This may be due to the necessity of a steep gradient between the extracellular and intracellular $[Ca^{2+}]$ to promote influx and the subsequent activation of repair pathways (Figs. 2 and 3). This entry of Ca^{2+} would activate the pathways for membrane repair in the neuron, but the simultaneous uptake of Ca^{2+} into multiple damaged axons at the site of injury *in vivo* could focally deplete the extracellular gradient and prohibit sufficient influx of Ca^{2+} into the repairing axons. Such focal *in vivo* $[Ca^{2+}]_o$ depletion, of one to two orders of magnitude, has been found following mechanical injury and persists for several hours[137-139]. The lack of a corresponding $[Ca^{2+}]_i$ could prevent the effective activation of the sealing mechanism, leading to excessive axonal dieback or retrograde cell death[5].

Role of Reactive Oxidative Species in Membrane Sealing

Oxidative damage is a factor of interest concerning membrane sealing due to the lack of flux control at the membrane breach. With regard to studies of reactive oxidative species (ROS), highly noteworthy in the field of membrane repair has been the therapeutic application of the free-radical scavenger α-tocopherol[140], a physiologically-relevant form of vitamin E in humans, based on its lipophilic nature[141]. Experimental data have shown that when an oxidative challenge is presented to a resealing membrane, membrane repair is inhibited, but α-tocopherol blocks this[142]. However, this rescue only occurs in the presence of extracellular Ca^{2+}, so α-tocopherol enhances Ca^{2+}-dependent repair pathways and the antioxidant properties are necessary but not sufficient to promote repair[142]. These antioxidant properties may be effective in preventing the formation of the toxic metabolites of lipid peroxidation, such as acrolein, generated by lipid peroxidation and feed-forward ROS production[143].

Studies within vascular smooth muscle cells implicate α-tocopherol in the localization of protein phosphatase 2A to the membrane, and this facilitates PKCα dephosphorylation and subsequent inhibition[144,145]. This inhibition of PKC inhibits the formation of the membrane-bound enzyme complex NADPH-oxidase[146], thereby decreasing the amount of superoxide produced. However, in regard to membrane sealing, PKC inhibition appears counter-productive because according to the model proposed (Fig. 2) and as previous studies have shown[27,86,87], inhibition of PKC decreases plasma membrane sealing. Further studies are needed to better understand the cellular pathways that are altered during α-tocopherol application and the isozyme-specific PKC pathways involved after membrane trauma.

As discussed above, α-tocopherol is known to impart negative membrane curvature on phospholipid membranes, thereby enhancing the formation of fusion intermediates[107,130]. X-ray diffraction studies of α-tocopherol incorporation into plasma membrane do not show any direct binding of α-tocopherol to substrates[130], which would normally allude to the mechanism of action. It is hypothesized that the membrane curvature induced by the lipophilic domain of α-tocopherol causes stress in the membrane and distorts the lipid-protein interaction, thus, it may alter the protein conformation to a level sufficient to affect cellular activity[130]. This also illustrates similar effects that would be experienced by the membrane immediately following trauma, such that the membrane protein activity would be altered in response to the lipid bilayer conformation induced by mechanical trauma. Elucidating the mechanism of the impact of α-tocopherol on membrane sealing could prove clinically effective due to the feasibility of vitamin E supplementation in patients following trauma to enhance membrane repair.

Further studies of antioxidants in relation to membrane trauma have led to the postulation of another activation mechanism for sealing. Melatonin, a powerful antioxidant[147], has been tested for its impact on axolemmal sealing at high and low concentrations in B104 cells following axotomy[86]. The results showed that melatonin

application decreases sealing, probably due to the lack of activation of oxidative-induced membrane sealing mechanisms. This appears to contraindicate the application of anti-oxidants to enhance membrane sealing, which as described with α-tocopherol, was shown to promote sealing in otherwise sealing-incompetent cells[142]. In support of this oxidative trigger for membrane sealing, oxidative stress appears to give similar results through a novel repair protein present in skeletal muscle (MG53), which has been proposed to become activated and thereby promote membrane sealing through oxidation-induced mutation in the protein[148]. This protein is a member of the muscle-specific tripartite motif (TRIM)72 family and orthologous TRIM proteins have been identified in molluscan neurons[149]. This provides support for the potential involvement of TRIM family proteins in axolemmal sealing. This mechanism is important because it marks a pathway that may act upstream of Ca^{2+} to induce pathways for the promotion of membrane repair[148].

Mechanical Factors of Injury

During a study of mechanisms of membrane sealing, the model systems are often manipulated, for the sake of control, in a monolayer setting *in vitro*. While this controls variables during manipulation, it does not simulate the three-dimensional aspect of mechanical properties and the environment experienced by the system *in vivo*, pertaining to axon receding, sealing, growth-cone generation, and target localization. An effective balance between the *in vitro* and *in vivo* settings during experimentation is through the use of *ex vivo* experimental designs, such as the double sucrose-gap device[5, 13, 17, 150]. This device allows for the control of an *in vitro* setting while permitting the use of spinal cord segments that provide a three-dimensional native tissue environment conducive for monitoring the recovery of membrane potential in real time, which serves as a functional indicator of membrane resealing. These favorable conditions for tissue culture and reliable data acquisition support the pursuit of future *ex vivo* sealing experiments to further clarify the physiology of axolemmal sealing.

Axon caliber is also an important variable in successful membrane sealing, especially when considering a transection injury. It has been shown that axons with smaller diameters exhibit faster spontaneous resealing following transection[11]. Under conditions of therapeutic application of PEG[17] and warming from 25°C to 37°C[13] following axonal transection, faster sealing occurs in the smaller caliber axons. While the underlying mechanism remains unclear, it has been speculated that the sealing rates are based on the size of the axonal defect. The axon caliber prior to transection dictates the size of the corresponding membrane lesion following transection. A larger axolemmal defect will result in a lower line tension between the free lipid edges (see Section 3). Without the favorable effects of line tension to promote axolemmal sealing, a larger vesicle deposition will be required to close the membrane gap thereby allowing the thermodynamic force between the lipid free edges to facilitate sealing of the membrane or subsequent endocytosis of the membrane defect. Further exploration of the differential rates of sealing related to axonal caliber and effective manipulation of environmental conditions to promote resealing for all axon calibers has clinical significance, in that nervous system injuries involve axons ranging in caliber.

The method of injury must also be considered regarding the effectiveness of therapeutics and the timescale of resealing. It is evident from previous studies involving axonal transection that membrane disruptions exclude dye markers within 60 min post-injury[5, 13]. The time required to achieve competent membrane integrity, measured by dye exclusion, is much greater in compression injury, in that only 50% of the axons have measurable membrane integrity at the 60-min time point[151]. This contrast exemplifies the potential difference in cellular mechanisms between membrane sealing in a single plane, as in transection injury, and in a segmental out-of-plane region, as in compression injury. Also, compression injury may have the potential to generate multiple membrane breaches, requiring sealing of each to re-establish full integrity. Based on the high clinical prevalence of compression injuries, compared to transection injuries, further understanding into the membrane biophysics of compression injury is of great relevance to the clinical treatment of SCI and TBI.

Methods to Enhance Integrity and Repair of the Neuronal Membrane

Understanding the mechanism axons use to seal

membrane breaches, as well as capitalizing on novel pathways, will facilitate the discovery of agents that enhance natural membrane sealing. As described above, application of α-tocopherol enhances membrane sealing, likely on the basis of preventing lipid peroxidation and inducing spontaneous membrane curvature[142]. Temperature is implicated as a variable with potential therapeutic application through the evident clinical benefits of mild hypothermia[38]. A growing cohort of research has shown that beneficial therapeutic effects on axonal membrane sealing are achieved by application of the synthetic polymer PEG[6,17,151]. To date, there is no established mechanism of action for PEG-mediated membrane sealing. However, the characteristics of PEG-coated surfaces have been shown to affect membrane properties. These include a large excluded volume, high PEG chain mobility, a high degree of hydration, and low interfacial energy between PEG and water molecules[152,153]. Also, PEG *in vivo* has been shown to localize at sites of trauma[154,155] by an unknown mechanism, promoting its therapeutical use as a direct treatment or as a drug delivery vessel. This, in conjunction with the properties of PEG-coated surfaces, suggests that PEG acts like a chemical sponge at the site of trauma. So, PEG may be able to associate with the lipid-free edges because of the low interfacial energy between PEG and water molecules, facilitating a more favorable thermodynamic state of the lipid-PEG-water configuration. PEG may thereby be able to dehydrate the area surrounding the free lipid edge. By removing the water within the membrane gap, it may decrease the gap diameter thereby increasing the line tension[6]. This conceivably complex structural configuration necessary to associate with the lipid free edge is facilitated by the high chain mobility of PEG[152, 153]. PEG may also dehydrate the membrane bilayer surface hydration layer, similar to DAG, promoting the formation of vesicle fusion intermediates. This would enhance the membrane-sealing rate through increased efficiency of vesicle exocytosis to decrease membrane surface tension and promote vesicle-vesicle fusion as seen in the membrane patch model.

Experimental support for PEG shows a quantitative reduction in the membrane surface tension through measurements made by atomic force microscopy[17]. This effect has been reported in other surfactants, such as Pluronic F68 NF in Swiss 3T3 fibroblasts[27] and P-188 in muscle cell membranes[156], as well as in solvents such as dimethylsulfoxide[26, 157], which improves guinea-pig spinal cord axolemmal sealing[158]. Thus, surfactants such as PEG likely mediate enhanced membrane sealing through increasing line tension and decreasing membrane tension, thereby altering the membrane properties to promote membrane healing. Based on the proposed model (Fig. 3) centering on achieving the same results through cellular physiology, PEG appears to be a valuable therapeutic to supplement native membrane sealing mechanics.

Conclusion

In summary, many pathways are implicated in the complex cellular process of membrane sealing. To best understand the interplay between different proposed mechanisms, experimentation on membrane sealing must maintain a focus on each finding being a part of the whole process. The extent to which mechanisms of repair seen in fibroblasts, erythrocytes, myocytes, and even invertebrate giant axons overlap with those in the mammalian CNS is unclear. Despite the knowledge gap for the overlap between various experimental systems, investigation needs to continue into the mammalian neuronal sealing mechanism, with the current knowledge serving as a framework, to better understand recovery from neurological trauma and have additional implications for neurodegenerative disease.

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·Review·

Innate immune responses regulate morphogenesis and degeneration: roles of Toll-like receptors and Sarm1 in neurons

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The central nervous system is recognized as an immunoprivileged site because peripheral immune cells do not typically enter it. Microglial cells are thought to be the main immune cells in brain. However, recent reports have indicated that neurons express the key players of innate immunity, including Toll-like receptors (TLRs) and their adaptor proteins (Sarm1, Myd88, and Trif), and may produce cytokines in response to pathogen infection. In the absence of an immune challenge, neuronal TLRs can detect intrinsic danger signals and modulate neuronal morphology and function. In this article, we review the recent findings on the involvement of TLRs and Sarm1 in controlling neuronal morphogenesis and neurodegeneration. Abnormal behaviors in TLR- and Sarm1-deficient mice are also discussed.

Keywords: axon; cytokines; dendrite; innate immunity; interleukin-6; Sarm1; toll-like receptor

Introduction

The innate immune system recognizes pathogenic molecules derived from bacteria and viruses and activates the expression of various antiviral and inflammatory cytokines, the complement cascade, and phagocytosis to eliminate foreign pathogens. Distinct from adaptive immunity, innate immunity lacks antigen specificity. It uses pattern recognition receptors to identify pathogen-associated molecular patterns, including lipopolysaccharide, lipopeptides, flagellin, and single- and double-stranded RNA and DNA[1]. In addition to foreign molecules, these pattern recognition receptors can also recognize endogenous ligands, which are released from cells and tissues undergoing stress or injury[2]. This results in either chronic or acute inflammatory responses in the absence of pathogen infection. Thus, innate immunity serves as an alarm system that responds to both exogenous pathogens and endogenous damage signals.

Toll-like receptors (TLRs), the most well-studied pattern-recognition receptors, play critical roles in the initiation of innate immune responses. At least 13 TLRs have been identified in mammals. Different TLRs recognize distinct molecular patterns. Based on their subcellular localization, TLRs can be separated into two categories. The first group, containing TLR1, TLR2, TLR4, TLR5, and TLR6, is expressed on the cell surface. The second category, containing TLR3, TLR7, TLR8, TLR9, and TLR13, is localized to the intracellular endosomal compartment[3].

TLRs are widely expressed in various types of cells. Microglia, the specialized immune cells in the brain, constitutively express a broad array of TLRs[4, 5]. The most well-studied TLRs in microglia are TLR2 and TLR4 that are key players in neuroinflammation in CNS trauma and neurodegenerative disease[6]. TLR2 and TLR4 signaling induces microglia activation after brain injury or pathogen infection, and this produces various pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor α (TNFα), type I interferon (IFN), and IL-1β. The exacerbated inflammation in brain causes neuronal loss and brain damage[7]. In the past decade, the accumulated evidence

suggests that neurons do have innate immunity. The importance and biological meaning of the neuronal innate immune responses have recently been investigated. In this review, we focus on the function of neuronal TLRs and their downstream effectors in neuronal development and neurodegeneration.

TLRs and Toll/interleukin-1 Receptor (TIR) Domain-containing Adaptors in Neurons

TLR expression and activation have been reported in both neuronal cell lines and primary cultured neurons. The human NT2-N neuronal cell line expresses TLR1, TLR2, TLR3, and TLR4[8]. Activation of TLR3 using poly I:C, a synthetic double-stranded RNA (dsRNA), induces the expression of antiviral and inflammatory cytokines, including IFN-β, CCL-5, CCL-10, TNFα, and IL-6 in NT2-N cells[8]. Similarly, rodent neurons express a variety of TLRs and their downstream effectors. Activation of TLR4 using lipopolysaccharide (LPS) induces CCL5, CXCL1, TNFα, and IL-6 production in mouse cortical neurons[9]. Furthermore, Kaul and colleagues performed quantitative PCR to examine the expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 in developing brains and found that the levels of TLR7 and TLR9 correlated particularly well with brain development[10]. Our recent study also indicated that activation of neuronal TLR7 induces both mRNA and protein expression of IL-6 and TNFα[11]. In addition to TLRs, neurons also express the critical TIR domain-containing adaptors, which transduce the downstream signals of TLRs, including myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adapter-inducing IFN-β (Trif)[10] and sterile alpha and HEAT/Armadillo motif-containing 1 (Sarm1)[12]. Notably, Sarm1 is predominantly expressed in neurons rather than astrocytes, microglia, or the peripheral immune system[12-14]. This characteristic is unique to Sarm1 among all of the known TLRs and TIR domain-containing adaptors. Indeed, Sarm1 plays multiple roles in the nervous system, and this will be discussed in following sections. Based on these lines of evidence, it is clear that neurons express various TLRs and TIR domain-containing adaptors and that activation of neuronal TLRs regulates the expression of various cytokines.

Endosomal TLRs and Their Ligands

In addition to sensing foreign pathogens, TLRs respond to intrinsic damage signals[15]. The exogenous and endogenous ligands specific to the various TLRs have been summarized in several reviews[3, 16]. Here, we are particularly interested in the endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) because they recognize nucleic acids. TLR3 recognizes dsRNA, TLR7 and TLR8 are activated by single-stranded RNA (ssRNA), and TLR9 binds to unmethylated CpG DNA. All four are localized to the endosomal pathway, by which they interact with their ligands. Foreign bacteria and viruses are internalized and digested *via* the endosomal pathway. The bacterial and viral nucleic acids then interact with the endosomal TLRs in the intracellular vesicular compartments and activate innate immune responses, including the expression of anti-viral and inflammatory cytokines. Several studies suggest that self nucleic acids can be ligands of TLR3, TLR7, TLR8, and TLR9[2]. For instance, heterologous RNAs released from necrotic cells or generated by *in vitro* transcription activate the TLR3 signaling pathway and induce IL-8 secretion[17]. *In vivo*, TLR3 is required for injury-induced acute inflammatory responses. During experimental polymicrobial septic peritonitis and ischemic gut injury, the levels of inflammatory cytokines quickly drop to baseline in TLR3-deficient mice[18]. Thus, dying cells (both apoptotic and necrotic) are sources that provide self nucleic acids to activate endosomal TLRs.

Some reports have specified the types of endogenous ligands for TLR3 and TLR7 binding. RNAs containing a high degree of self-complementarity target TLR3, whereas TLR7 is activated by uridine-rich RNAs[19]. Moreover, TLR7 recognizes microRNAs (miRNAs), particularly let-7[20], miR-21, and miR-29a[21]. Because microRNAs are present in exosomes[22,23], it has been suggested that cells release miRNA into the environment *via* exosomes, activating TLR7 in other cells[20, 21]. Interestingly, in this model, the effect of miRNA on other cells is not *via* the canonical pathway, in which miRNAs complementarily bind to mRNA and reduce the expression of the targeted mRNA. Instead, the internalized exosomal miRNAs enter the endosomal pathway, are consequently released from exosomes, and activate TLR7 in the intracellular vesicular compartments (Fig. 1). Through this mechanism, TLR7 may receive

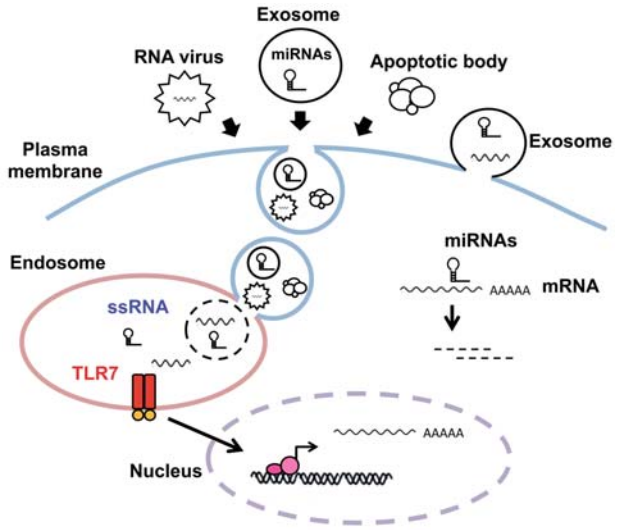

Fig. 1. Exogenous and endogenous ligands of TLR7. Both exogenous and endogenous ligands are recognized by TLR7 *via* the endosomal pathway. Endogenous ligands can be delivered *via* apoptotic bodies or exosomes. Exosomes containing proteins, mRNAs, and miRNAs are released from cells and travel either a short or long distance to influence the activity of target cells. All viruses, bacteria, apoptotic bodies, and exosomes can be internalized and enter the endosomal pathway. In endosomes, partially-digested ssRNAs, including mRNAs and miRNAs, are recognized by TLR7, thus triggering innate immune responses. In this model, miRNAs perform a novel function, the activation of the TLR7 signaling pathway, rather than directly silencing gene expression.

signals from distant cells and trigger an innate immune response.

The known pathogenic, synthetic, and endogenous ligands for TLR3, TLR7, TLR8, and TLR9 are summarized in Table 1. It should be noted that although imiquimod (termed R837, an imidazoquinoline compound), CL075 (a thiazoquinoline compound) and loxoribine (a guanosine analog) have all been commonly used as TLR7 agonists[24-27], several studies have indicated non-specific effects of imiquimod on neurons. We have shown that imiquimod, CL075, and loxoribine restrict dendrite growth in wild-type rodent cortical and hippocampal neurons[11]. However, CL075 and loxoribine lose their effects in TLR7-knockout neurons, suggesting an essential role of TLR7 in the efficacy of CL075 and loxoribine with respect to neuronal morphology. In contrast, the ability of imiquimod to restrict dendrite growth is not affected by TLR7-knockout[11]. This result suggests that TLR7 is not the only target of imiquimod in cortical and hippocampal neurons. In dorsal root ganglion neurons, it has been shown that imiquimod treatment results in the activation of transient receptor potential vanilloid 1 and the inhibition of background and voltage-gated K^+ channels, which are TLR7-independent[28,29]. These independent results indicate that the specificity of imiquimod for TLR7 is a concern, at least regarding neurons. Our study suggested that CL075 and loxoribine are more specific to TLR7[11]. Thus, to study the function of TLR7 in neurons, imiquimod (R837) should be avoided to minimize non-specific effects.

TLRs and Neuronal Morphogenesis

In neurons, the activation of TLR pathways likely performs multiple functions. Similar to other types of cells, the activation of TLRs in neurons induces the expression of cytokines, as described above. Moreover, evidence indicates that TLR activation is also critical for neuronal morphogenesis. In cultured dorsal root ganglion, cortical, and hippocampal neurons, treatment with poly I:C, a synthetic dsRNA, induces growth-cone collapse and inhibits neurite outgrowth[30]. The effect of poly I:C is mediated *via* TLR3 because neurons lacking functional TLR3 do

Table 1. Summary of the pathogenic, synthetic, and endogenous ligands of mouse TLR3, TLR7, TLR8, and TLR9

	Pathogenic ligands	Synthetic ligands	Endogenous ligands
TLR3	dsRNA	Poly IC	mRNA[30, 61]
TLR7	ssRNA	CL075, Loxoribine, R848[11, 20, 21, 32]	let-7, miR-21, miR-29a[20, 21, 32]
TLR8		R848[31]	?
TLR9	Unmethylated CpG DNA	CpG ODN	DNA

not respond to poly I:C[30]. This study indicates that TLR3 activation plays a negative role in neurite outgrowth.

In addition to TLR3, TLR7 and TLR8 have also been suggested to negatively regulate neurite outgrowth in mouse cortical neurons[11, 31]. Ma and colleagues showed that R848, an imidazoquinoline compound, inhibits neurite outgrowth and triggers apoptosis in cortical neurons[31]. Because R848 can activate TLR7 as well as TLR8, Ma and colleagues then used antibodies to examine the expression of TLR7 and TLR8 in cortical neurons, and their data suggested that only TLR8, and not TLR7, is expressed. Thus, they concluded that the negative effect of R848 is mediated *via* TLR8 but not TLR7[31]. However, their results are in conflict to many recent studies from various laboratories regarding the expression of TLR7 in neurons[10, 11, 20, 32], as evidenced by the results of *in situ* hybridization, quantitative polymerase chain reaction (Q-PCR), and immunostaining using TLR7 antibodies[10, 11, 20, 32]. Actually, the expression level of TLR7 in neurons is even higher than that of TLR8[11]. Therefore, the R848 treatment in Ma's study likely activates both TLR7 and TLR8 in neurons to inhibit neurite growth. To determine the role of TLR8 in neuronal morphogenesis, additional investigations using specific agonists and genetic manipulations (such as knockout mice and RNAi-mediated knockdown) are needed. As for the role of TLR7 in neuronal morphogenesis, we have demonstrated that TLR7-knockout neurons have longer axons and dendrites, and reintroduction of TLR7 into TLR7-deficient neurons rescues this overextension[11]. Moreover, activation of TLR7 by TLR7-specific agonists (CL075 and loxoribine) negatively regulates dendritic growth in wild-type but not in TLR7-deficient neurons. In Ma's study, they applied 500 μmol/L loxoribine to cultured neurons and did not detect a negative effect on neurite outgrowth[31]. We used 1 mmol/L loxoribine and did find a reduction of dendritic length[11]. Possibly, loxoribine is relatively inefficient, and a higher concentration may be required to activate TLR7. Both genetic manipulation and pharmaceutical treatment support a function of TLR7 in neuronal morphogenesis.

TLR Downstream Signaling in Regulation of Neuronal Morphology

To trigger innate immune responses, two key TIR domain-containing adaptors, MyD88 and Trif, are involved in the canonical TLR pathways. TLR3 transduces signals *via* Trif, TLR4 uses both Myd88 and Trif to activate downstream signals, and the remaining TLRs use MyD88 as their adaptor. The signals may go through NF-κB, interferon regulatory factors (IRFs), and the AP-1 family to induce the expression of inflammatory cytokines and interferons. The detailed signaling pathways of TLRs are available in previous reviews[3, 16, 33, 34].

With respect to the negative regulation of neuronal morphogenesis, only TLR7 signaling is well understood. TLR7 uses a canonical pathway, namely the MyD88-dependent pathway, to induce IL-6 expression in cultured cortical and hippocampal neurons[11]. MyD88 is essential for the capacity of TLR7 to regulate dendrite growth, as MyD88 knockout neurons do not respond to CL075 stimulation. Interestingly, in addition to IL-6 activation, TLR7 activation in neurons also induces the mRNA expression of TNFα and IL-1β, but not IFNβ. At the protein level, only IL-6 and TNFα are detectable in the culture supernatant[11]. Because IL-1β requires a second signal to activate the inflammasome and caspase 1 to cleave pro-IL-1β to IL-1β[35, 36], the undetectable level of IL-1β in the supernatant is likely due to the lack of a second stimulus to activate the inflammasome. Between IL-6 and TNFα, only IL-6 is critical for restricting the dendritic outgrowth of cultured neurons because IL-6 knockout neurons lose their response to TLR7 activation[11]. In contrast, TNFα-knockout neurons remain sensitive to TLR7 activation of dendrite outgrowth[11]. This result suggests that TNFα is not required for the effect of TLR7 on neuronal morphology (Fig. 2). Notably, a previous study indicated that adding exogenous IL-6 or TNFα is sufficient to inhibit dendrite development in cultured cortical neurons[37]. It is not clear what is responsible for this contrasting result. One possibility is the doses used. In the exogenous experiment, 100 U TNFα was added, which is ~2000 pg/mL[37]. When cultured neurons were treated with CL075 to activate TLR7, only 6–8 pg/mL of TNFα was detected in the supernatant[11], which is ~0.4% of the concentration applied exogenously. Interestingly, the concentration of IL-6 in the supernatant of TLR7-activated neurons was even lower (0.6–0.9 pg/mL), which is ~0.001% that of the exogenously-applied IL-6 (~80 000 pg/mL)[11, 37]. It is unclear whether IL-6 synthesized *de novo* in cultured neurons results in more potent activation of the IL-6 receptor. Additional investigations are needed to address this issue.

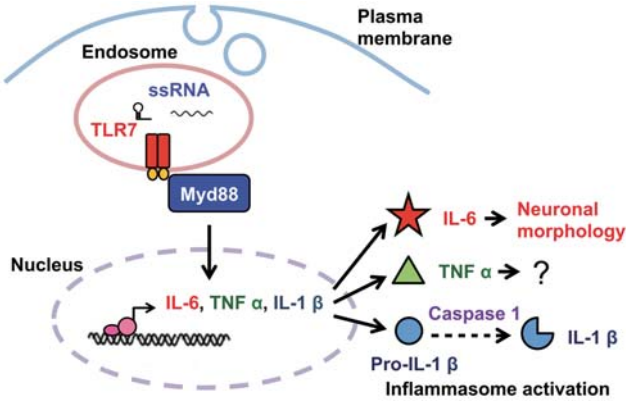

Fig. 2. The signaling pathway downstream of TLR7 in neurons. In cultured cortical and hippocampal neurons, activation of TLR7 induces IL-6, TNFα and pro-IL-1β expression *via* a MyD88-dependent mechanism. Due to the lack of a secondary signal, pro-IL-1β cannot be processed to IL-1β. Both IL-6 and TNFα are released into the culture medium. However, only IL-6 negatively regulates dendrite outgrowth. The role of TNFα in this process is unclear.

In contrast to TLR7, the signaling pathway downstream of TLR3 that controls neurite outgrowth remains to be elucidated[30]. Poly I:C treatment inhibits neurite outgrowth in a TLR3-dependent but NF-κB-independent manner[30]. In this study, MyD88 knockout neurons were also used to demonstrate that MyD88 is not involved in the TLR3 pathway[30]. However, because TLR3 delivers its signals using Trif but not MyD88, it appears to be more appropriate to examine the role of Trif rather than MyD88 in the TLR3 pathway to control neurite outgrowth. It is also unclear whether cytokines are involved downstream of TLR3 in neurons.

Role of TLR7 in Neurodegeneration

In addition to morphogenesis, the expression of TLR may also play a role in neurodegeneration. The studies contributed by Dr. Seija Lehnardt's laboratory unexpectedly revealed that TLR7 recognizes the miRNA let-7, consequently resulting in neurodegeneration[20, 32]. They reported that let-7 released from dying cells activates TLR7 expression in neurons, triggering neuronal death *in vitro* and *in vivo*[20, 32]. Similar to the findings regarding neuronal morphogenesis, MyD88 is required for the function of TLR7 in neurodegeneration, as MyD88 knockout neurons do not respond to let-7[20]. However, it is not clear whether cytokines, such as IL-6 and TNFα, are involved. Interestingly, microglia are not involved in the effect of let-7 on neurodegeneration, as depletion of microglia *via* the expression of thymidine kinase of *Herpes simplex* virus under the control of the CD11 promoter does not influence the effect of let-7 on neuronal death[20]. This result suggests that neuronal TLR7 plays a predominant role in the response to let-7. Related to neurodegenerative disease, they further found that patients with Alzheimer's disease exhibit a higher copy number of let-7 in the cerebrospinal fluid[20]. This was the first study to demonstrate that the recognition of self miRNA by neuronal TLR7 is critical for triggering neurodegeneration.

Innate Immune Responses of CNS Cells: Neurons *versus* Glia

As described above, both neurons and microglia express various TLRs and produce cytokines after their activation. However, the efficiency of cytokine production is much reduced in neurons as compared with microglial cells. Activation of TLRs in neurons produces a very low level of pro-inflammatory cytokines[11, 38, 39], while microglia and astrocytes secret large amounts of cytokines[40, 41]. The low level of cytokine production by neurons seems unlikely to induce a global innate immune response in the brain. Therefore, this raises the question of why neurons need their own innate immunity. Based on current knowledge, we propose that TLRs in neurons function as chemorepulsive sensors. During development, programmed cell death occurs frequently but locally while neurons extend their axons and dendrites[42, 43]. Activation of neuronal TLRs by RNA and/or DNA derived from dead cells may prevent axon and dendrite growth into an unhealthy area through a cell-autonomous mechanism or paracrine signaling[11, 30]. The cytokines produced by neurons may be just enough to recruit and activate local microglia, which in turn engulf the debris of dead cells and do not cause global inflammation. Axons and dendrites then may grow into or pass through the cleaned-up area and establish proper neuronal circuits with healthy neurons. Therefore, the biological meaning and function of TLR activation in neurons could be distinct from that in microglia during development.

TLRs and Mouse Behaviors

Several studies using mouse genetic models have explored the roles of TLRs in learning/memory and sensory and motor behaviors. The first example is TLR3-knockout mice[44]. In the Morris water maze, novel object recognition, and contextual fear conditioning, TLR3-deficient mice exhibit enhanced hippocampus-dependent memory. Interestingly, amygdala-dependent learning and memory are impaired in these mice. Anxiety-related behaviors, which are strongly associated with the amygdala, are also reduced in TLR3-deficient mice[44]. It is not clear why TLR3 deficiency enhances hippocampus-dependent performance but impairs amygdala-dependent behaviors. Additional studies are needed to elucidate the roles of TLR3 in different brain regions.

The behaviors of TLR4-knockout mice have also been analyzed [45]. In the Morris water maze, these mice travel a much shorter distance to locate the hidden platform, suggesting that deletion of TLR4 enhances the acquisition of hippocampus-dependent spatial learning and memory. However, these mice exhibit a lower frequency of the freezing response in contextual fear conditioning, suggesting an impairment of contextual fear conditioning, another hippocampus-dependent spatial learning paradigm. It is unclear why TLR4 deletion has opposite effects on two hippocampus-dependent behavioral paradigms. One result that must be taken into consideration is the improved motor activity of TLR4-knockout mice. These mice exhibit a higher swimming speed in the Morris water maze and enhanced motor performance on the rotarod test[45]. The higher locomotor activity of TLR4-knockout mice may account for the reduced freezing response rate in the fear conditioning task. Thus, it is difficult to conclude whether TLR4 deficiency indeed impairs contextual fear conditioning. Another spatial learning/memory paradigm that is less sensitive to locomotor activity is needed to further evaluate the function of TLR4 in hippocampus-dependent learning and memory.

Although the role of TLR9 in neurodevelopment and neurodegeneration has yet to be investigated, the behaviors of TLR9-knockout mice have been described[46]. In contrast to TLR3- and TLR4-knockout mice, TLR9-knockout mice do not exhibit any phenotype in the Morris water maze. However, they exhibit hyperactive sensory responses and motor behaviors. TLR9 mutant mice are more sensitive to thermal stimuli in response to a hot plate. Moreover, motor responsiveness under anxiety-provoking conditions in an open field test is enhanced in these mice; similarly, prepulse inhibition of the acoustic startle response is also enhanced[46]. This study indicates that TLR9 is important for sensory and motor behaviors in mice.

Interestingly, although TLRs share similar signaling pathways and downstream mediators to trigger innate immune responses, the behavioral phenotypes of TLR3-, TLR4- and TLR9-deficient mice are distinct. These findings suggest that each TLR likely performs a unique function in the brain. It is not clear whether these distinctions are related to the expression levels or patterns of these TLRs in the brain. It is also possible that TLRs use unique downstream pathways in neurons, thus resulting in distinct functions in the brain. The detailed signaling pathway of each TLR in neurons needs to be investigated to address this possibility.

Notably, all of the knockout mice used in the studies discussed above are conventional knockout mice. Thus, TLRs are missing from both the nervous system and peripheral tissue. Although neuronal TLRs have been shown to regulate neurodevelopment and neurodegeneration, it cannot be ruled out that TLRs in peripheral tissues may indirectly influence brain function by modulating peripheral innate immunity. Neuron-specific knockout mice are required to conclusively determine the roles of neuronal TLRs in cognition and behaviors.

Function of Sarm1 in Brain

The predominantly neuronal expression of Sarm1 distinguishes it from all other TIR domain-containing adaptors involved in TLR signaling[12, 14]. Consistent with the original finding regarding the involvement of Sarm1 in innate immunity[47], Sarm1 knockdown in the mouse brain disrupts the expression levels of inflammatory and antiviral cytokines. At the embryonic stage, Sarm1 knockdown increases IL-6 and IFNβ expression. In the adult Sarm1 knockdown brain, IL-1β, IL-12 and CCL5 are upregulated, while TNFα and IFNβ are downregulated[13]. Interestingly, Sarm1 is only expressed in neurons but not glia in the brain[13]. The aberrant cytokine expression profiles found in Sarm1-knockdown brains suggest the critical role of

neurons in controlling innate immune responses in the brain.

Similar to TLR3 and TLR7, Sarm1 also controls neuronal morphology and function. The first study revealing the role of Sarm1 in neurons used *Caenorhabditis elegans*. Tir-1, the *C. elegans* ortholog of Sarm1, is critical for synaptic signaling to the nucleus, and is involved in the left-right asymmetric expression of the odorant receptor in olfactory neurons[48]. Tir-1 receives a Ca^{2+} signal *via* CaMK and transduces its signals *via* the ASK1-MEK4/7-JNK pathway consequently regulating gene expression[48]. The synaptic localization of Tir-1 is microtubule-dependent[49]. Because Tir-1 influences the JNK complex in *C. elegans* neurons, the effect of Sarm1 on JNK was also investigated in mammalian neurons. The data indicate that Sarm1 associates with JNK3, recruits JNK3 to mitochondria, and regulates cell death after deprivation of glucose and oxygen[14]. This discovery has recently been confirmed by other studies[50, 51].

In addition to cell death, Sarm1 is actually important for neuronal morphology. Using GST-syndecan-2 fusion protein as bait, Sarm1 has been identified as a novel syndecan-2-interacting protein in the mouse brain[12]. Syndecan-2, a synaptic heparan sulfate proteoglycan, regulates synapse formation and dendritic arborization through various downstream mediators[12, 52]. For syndecan-2-mediated synapse formation and maintenance, both neurofibromin and CASK protein complexes are required[52-54]. Sarm1 is essential for syndecan-2-regulated dendritic arborization. Sarm1 receives signals from synaptic syndecan-2 and acts through the ASK1-MEK4/7-JNK pathway to modulate dendritic arborization[12]. Interestingly, Sarm1 expression in neurons is detectable far earlier than that of syndecan-2. Therefore, Sarm1 also controls syndecan-2-independent events, such as axonal outgrowth and the establishment of neuronal polarity[12]. Thus far, the upstream signal of Sarm1 in regulating axonal outgrowth and neuronal polarity is still unclear. Since Sarm1 functions as an adaptor molecule, the identification of additional Sarm1-interacting proteins would provide clues to the mechanism underlying its role in axonal outgrowth and neuronal polarity.

Sarm1 is also critical for axon degeneration during injury. In both flies and mice, deletion of Sarm1 effectively prevents Wallerian degeneration for weeks after axotomy[55]. The TIR domain of Sarm1 is important for activation of the downstream destruction pathway, while multimerization mediated by the SAM domain of Sarm1 is also essential for the function of Sarm1 to trigger axon degeneration[56]. Despite the involvement of its SAM and TIR domains, it is completely unclear which pathway Sarm1 uses to trigger axon degeneration. Association with mitochondria is clearly not required for Sarm1-dependent axon degeneration[56]. There is also no evidence regarding whether the ASK1-MEK4/7-JNK pathway plays a role in Sarm1-mediated axon degeneration. It is puzzling that Sarm1 appears to play both positive and negative roles in neuronal morphology. Sarm1 is required for neuronal morphogenesis during development, but it triggers axon degeneration after injury. Because Sarm1 is widely distributed throughout the various subcellular compartments of neurons, it is possible that it associates with various proteins at distinct subcellular regions consequently regulating different events. The studies of the functions of Sarm1 are summarized in Figure 3.

The influence of Sarm1 on neurons also results in abnormal behaviors and electrophysiological responses in Sarm1-knockdown mice[57]. Although Sarm1-deficient mice show normal locomotor activity and anxiety behaviors, they exhibit several autism-like behaviors, including reduced cognitive flexibility and greatly decreased social interactions. Besides, Sarm1-knockdown transgenic mice are defective in both contextual and auditory fear conditioning[57]. Echoing the defects in associative memory, these mice have hyper-NMDAR-dependent long-term potentiation and impaired mGluR-dependent long-term depression (LTD)[58]. Treatment with CDPPB, a positive mGluR allosteric modulator, effectively ameliorates the mGluR-dependent LTD, associative memory, and social interaction[58]. Because Sarm1 regulates neuronal innate immunity, morphogenesis, and activation, the behavioral defects of Sarm1-knockdown mice support the hypothesis that immune challenge during early development increases the risk of psychiatric disorders later on. Although direct evidence of mutations in the human Sarm1 gene in patients suffering from psychiatric disorders is lacking, several independent studies suggest an association of Sarm1 with autism spectrum disorders. First of all, the human Sarm1 gene is located at chromosome 17q11 (17:26,698,987–26,728,065), which is within the locus

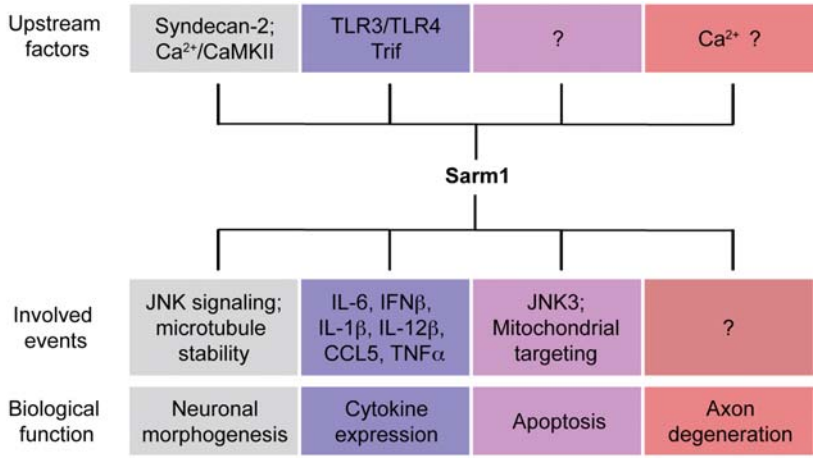

Fig. 3. Summary of Sarm1 function in neurons. See detailed description in the main text.

of autism, susceptibility to, 6 (Auts6, OMIM%609378, 17:24,000,000–31,800,000). Second, a comparison of the protein expression profiles of control individuals and patients suffering from autism revealed that the Sarm1 protein levels in the mid-frontal cortex are decreased in autistic patients[59]. Finally, Sarm1 mRNA has been predicted to be recognized by Fragile X mental retardation protein (FMRP)[60], which is encoded by the Fragile X mental retardation 1 gene. Because Fragile X syndrome is a well-known monogenic disorder associated with autism, recognition of Sarm1 by FMRP also implies an association of Sarm1 with autism. It is likely that Sarm1 plays a critical role in linking the innate immune response to neuronal morphogenesis and psychiatric disorders.

Conclusion

Neuronal TLRs and TIR domain-containing adaptor molecules not only regulate the innate immune responses of neurons but also play critical roles in controlling neuronal morphogenesis and function. These new findings impact the hypotheses regarding crosstalk between the nervous and immune systems. Although the detailed signaling pathways and the molecular regulation of TLRs and TIR domain-containing adaptors in neurons are largely unknown, the cell-autonomous innate immune responses likely play crucial roles. Neuron-specific knockout mice should be used in the future to further evaluate the contribution of neuronal innate immune responses to the regulation of neuronal development, degeneration, and function.

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·Original Article·

Clustering of surface NMDA receptors is mainly mediated by the C-terminus of GluN2A in cultured rat hippocampal neurons

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ABSTRACT

N-methyl-D-aspartate receptors (NMDARs) containing different GluN2 subunits play distinct roles in synaptic plasticity. Such differences may not only be determined by the channel properties, but also by differential surface distribution and synaptic localization. In the present study, using a Cy3-conjugated Fab fragment of the GFP antibody to label surface-located GluN2 subunits tagged with GFP at the N-terminus, we observed the membrane distribution patterns of GluN2A- or GluN2B-containing NMDARs in cultured rat hippocampal neurons. We found that surface NMDARs containing GluN2A, but not those containing GluN2B, were inclined to cluster at DIV7. Swapping the carboxyl termini of the GluN2 subunits completely reversed these distribution patterns. In addition, surface NMDARs containing GluN2A were preferentially associated with PSD-95. Taken together, the results of our study suggest that the clustering distribution of GluN2A-containing NMDARs is determined by the GluN2A C-terminus, and its interaction with PSD-95 plays an important role in this process.

Keywords: NMDA receptors; GluN2A; GluN2B; PSD-95; receptor clustering

INTRODUCTION

N-methyl-D-aspartate-type ionotropic glutamate receptors (NMDARs) in the central nervous system play critical roles in synaptic plasticity, synaptogenesis, and excitotoxicity[1,2,3]. Functional NMDARs are believed to be tetrameric complexes assembled from two GluN1 and two GluN2 (GluN2A–2D) subunits[4,5]. Different NMDAR subtypes have distinct channel properties, such as open probability and time-course of currents[6]. Moreover, the surface expression and synaptic localization of different NMDAR subtypes are distinct and differentially regulated during development and in response to neuronal activity and sensory experience. At nascent synapses, NMDARs predominantly contain GluN2B. During postnatal development, there is an increase in the expression and subsequent surface localization of GluN2A-containing NMDARs[7]. Neuronal activity may bidirectionally remodel the synaptic localization of NMDAR subtypes. Chronic activity enhances the levels of GluN2A-containing NMDARs at synaptic sites, while blockade of activity promotes the surface expression of those containing GluN2B[8].

The GluN2 subunit plays critical roles in controlling the surface expression and synaptic localization of NMDARs. It has an intracellular C-terminus which may interact directly with other scaffolding proteins, adaptor proteins, or downstream signaling proteins. The PDZ-binding motif at the distal end of the C-terminus directly interacts with PSD-MAGUK proteins, such as PSD-95 and SAP102[9,10] and this interaction promotes NMDAR clustering[11], surface expression[12], and the targeting of GluN2A *versus* GluN2B to synapses[13]. Furthermore, the C-terminus of the

GluN2 subunit contains several sites for post-translational modification such as phosphorylation and palmitoylation, which may contribute to the distinct regulation of NMDAR subtypes[14].

In this study, by imaging surface NMDARs using a Cy3-conjugated Fab fragment of GFP antibody, we found that the GluN2A-containing NMDARs were more clustered, while those containing GluN2B were more diffuse in both immature and mature hippocampal neurons. And the clustering distribution of the GluN2A-containing NMDARs was determined by the subunit C-terminus.

MATERIALS AND METHODS

DNA Constructs

Construction of EGFP-GluN2B, EGFP-GluN2A, ECFP-GluN2A, and ECFP-GluN2B was as described previously[5,15]. GFP or CFP was tagged to GluN2B or GluN2A at the extracellular N-terminus. EGFP-PSD-95 and EGFP-SAP102 were gifts from S. Visini (Georgetown University, Washington, DC). To generate the GFP-GluN2A-Δ7 or CFP-GluN2A-Δ7 construct, the first two primers, (5'-TGTAGCGATGTTGACCGCACCTACA-3' and 5'-AGGCAGATCTTACTTGTACACTCGTCTATTGCT GCAGG -3'), were designed and used in PCR cloning of the cDNA sequence encoding the C-terminal tail of GluN2A lacking the PDZ binding domain (PSIESDV)[9], using the original EGFP-GluN2A or ECFP-GluN2A construct as template. Then the BglII-treated original GFP-GluN2A or CFP-GluN2A construct and the PCR fragments were ligated with T4 ligase. The construction of GFP/CFP-GluN2A-Mut3 was similar to that of GFP/CFP-GluN2A-Δ7 and subcloned with PCR products encoding the C-terminal tail of GluN2A which had 11 amino acids identical to GluN2B. GFP/CFP-GluN2B-C_{GluN2A} was constructed to replace the complete C-terminal of GFP/CFP-GluN2B with the complete C-terminal of GluN2A. GFP/CFP-GluN2A-C_{GluN2B} was constructed to replace the complete C-terminal of GFP/CFP-GluN2A with that of GluN2B. All constructs were verified by DNA sequencing.

Neuron Culture and Transfection

Primary hippocampal cultures were prepared from one-day postnatal Sprague-Dawley rats as described previously[15]. Briefly, the hippocampi were chopped and digested in 0.25% trypsin (Sigma, St. Louis, MO) for 15 min at 37°C. Dissociated cells were plated at a density of 1×10^6 in 35-mm dishes with poly-L-lysine-coated coverslips in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 10% horse serum, and 2 mmol/L glutamine (all from Invitrogen). The culture medium was changed to Neurobasal medium plus B27 (Invitrogen) the next day. The neurons were routinely transfected after 5 days *in vitro* (DIV5) by adding 3 to 4.5 μg of total DNA and 4 μL Lipofectamine 2000 (Invitrogen) in a final volume of 500 μl OPTI-MEM to the 35-mm dish containing neurons and 1.5 mL Neurobasal medium, and incubated for 3 h at 37°C. The cells were then rinsed in Neurobasal medium and the original medium was added.

Generation of Cy3-conjugated Anti-GFP Fab Fragment

Glutathione *S*-transferase (GST) and histamine (HIS) fusion GFP proteins were cloned, expressed, and purified using conventional methods. A polyclonal antibody to GFP was generated by immunizing rabbits with GST-GFP fusion protein, then affinity-purified on nitrocellulose strips containing the HIS-GFP fusion protein. The Fab fragment was generated by papain cleavage of anti-GFP polyclonal antibodies. The Fab fragment was conjugated to the Cy3 fluorophore with the Cy3 mAb labelling kit following the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). The purity of the Fab fragment was confirmed by SDS-PAGE.

Surface Staining and Immunocytochemistry

Anti-GFP surface staining was performed as previously described[15]. Briefly, coverslips were incubated with rabbit polyclonal antibody against GFP for 7 min at room temperature, then, after washes, neurons were incubated with Alexa546-conjugated goat anti-rabbit antibody (Molecular Probes, Grand island, NY) for another 7 min at room temperature, then viewed directly under a fluorescence microscope. For anti-GFP Fab surface staining, transfected neurons were incubated only with Cy3-conjugated anti-GFP Fab fragment at 2 μg/mL for 8 min at room temperature before imaging.

For immunocytochemical studies, neurons were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.2% Triton X-100/PBS, blocked with

10% normal goat serum in PBS for 30 min, washed with PBS, incubated with primary antibody for 1 h at room temperature, and stained with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes, Grand island, NY) for 1 h at room temperature. Anti-PSD-95 antibody (Upstate Biotechnology, Lake Placid, NY) was used at 1:200 dilution, and anti-synaptophysin antibody (Abcam, Cambridge, MA) was used at 1:200 dilution.

Image Acquisition and Data Analysis

Neurons that appeared healthy and morphologically intact were imaged by fluorescence microscopy. Fluorescent labeling was imaged with an Olympus FLUO1000 confocal microscope (Tokyo, Japan) with a 40× PlanApo oil-immersion objective (0.65 NA). Images for each fluorophore were acquired sequentially and averaged over three scans. The image data were analyzed and quantified using MetaMorph software (Universal Imaging Corp., West Chester, PA). For surface receptor analysis, clusters were determined by a threshold set at twice the average dendritic gray value, and the number of clusters from at least 5 dendrites extending at least 100 μm was measured. Average total intensity per 10 μm of surface staining was analyzed with MetaMorph software. Five dendritic sections were measured and averaged to give a value for each cell included. Co-localization with PSD-95, SAP102, and synaptophysin was defined as having overlapping or adjacent pixels. All data were analyzed using SPSS version 13 (SPSS, Chicago, IL). Statistics were calculated with Student's *t* test, and significance was set at $P < 0.05$. Data are expressed as mean ± SEM.

RESULTS

Distribution Pattern of Surface NMDARs in Cultured Hippocampal Neurons during Development

To explore the distribution patterns of surface NMDARs, we transfected GFP-tagged GluN2 plasmids (GFP-GluN2A or GFP-GluN2B) into cultured hippocampal neurons. Since GFP labeled the N-terminus of the GluN2 subunit, live cell-surface staining with anti-GFP antibody was used to detect the surface GFP-GluN2 subunits at different times after transfection[15]. To exclude the cascade reaction of primary and secondary antibodies and shorten the staining time, we generated a Cy3-conjugated Fab fragment of GFP antibody (Fab-Cy3) for surface staining of the GFP-GluN2 subunits. We found that, at DIV7, more clusters were observed when surface GFP-GluN2B was stained with polyclonal anti-GFP antibody (Fig. 1A, upper panels) compared with Fab-Cy3 staining (Fig. 1A, lower panels). This indicated that the cascade reaction of primary and secondary antibodies and a longer staining time may induce clustering of surface receptors. Therefore, we used Fab-Cy3 in the subsequent experiments, rather than polyclonal anti-GFP antibody, to assess the distribution pattern of surface GFP-GluN2.

To ensure comparability of surface staining, equal amounts of GFP-GluN2B or GFP-GluN2A cDNA were transfected into cultured hippocampal neurons at DIV5. First, we observed the distribution patterns of the surface GFP-GluN2B and GFP-GluN2A 2 days after transfection (DIV7) and found that most of the surface GFP-GluN2B was diffusely distributed throughout the soma and dendrites with rare clusters (Fig. 1B, upper panels). In contrast, the surface GFP-GluN2A was distributed in a clustered pattern. Quantitative analysis showed that the density of surface GFP-GluN2A clusters was statistically higher than that of GFP-GluN2B clusters (Fig. 1C). These results indicated that the surface distribution pattern of NMDARs containing GluN2B is distinct from those containing GluN2A during the early stage of hippocampal neuron development. Then, we assessed the synaptic localization of surface GFP-GluN2A clusters and found that, at DIV7, the density of clusters of synaptophysin, a presynaptic marker, was much lower than that of surface GFP-GluN2A clusters, although most of synaptophysin was co-localized with GFP-GluN2A. This indicated that the surface GFP-GluN2A clusters were located not only in the synapses, but also in the dendritic shaft and soma (Fig. 1D).

Next, we examined the surface distribution of GFP-GluN2A and GFP-GluN2B at DIV14 and found that, although the density of surface GFP-GluN2B clusters increased significantly (Fig. 2A, upper panels), it was still statistically lower than that of surface GFP-GluN2A clusters (Fig. 2A, lower panels). We further analyzed the ratio of average immunofluorescence intensity between clustered receptors and diffuse receptors, and found that this ratio for GFP-GluN2A was statistically higher than that for GFP-GluN2B. These results indicated that surface NMDARs containing GluN2A form more clusters than those containing GluN2B in mature hippocampal neurons.

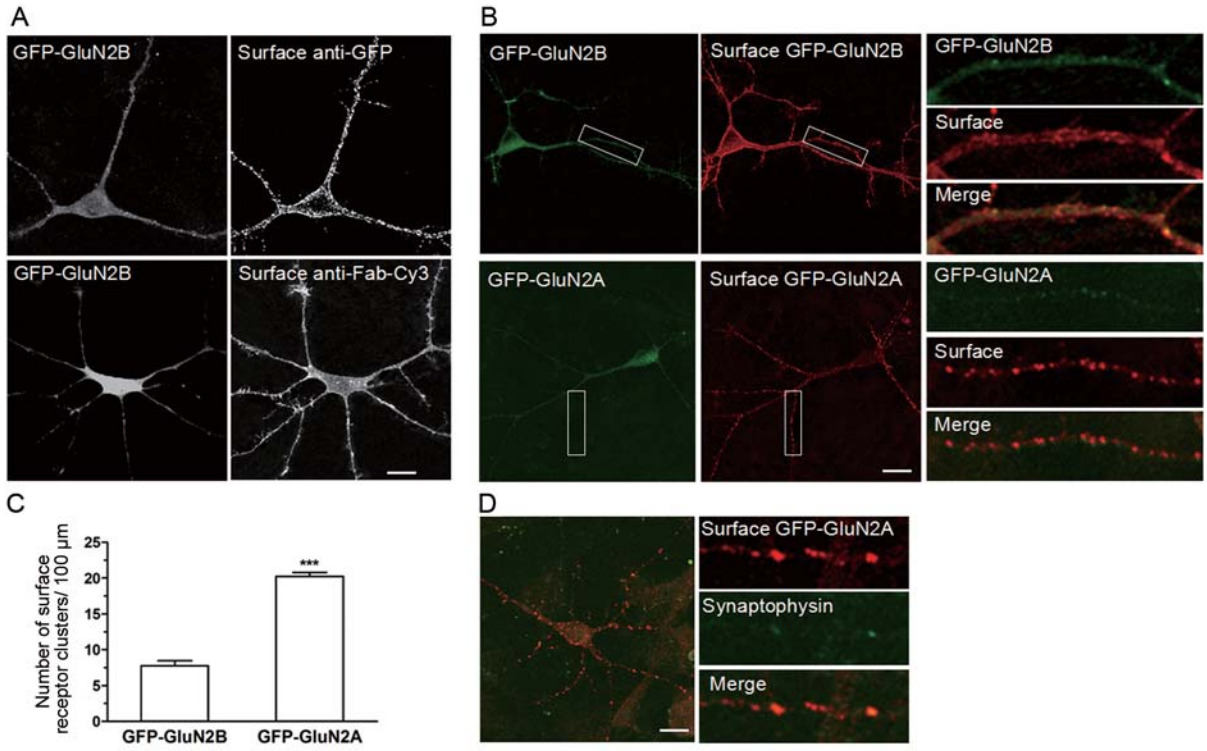

Fig. 1. Surface NMDARs containing GluN2A are more inclined to cluster than those containing GluN2B at DIV7. A: GFP-tagged GluN2B construct was transfected into cultured hippocampal neurons at DIV5. Then surface-expressed receptors were detected at DIV7 by conventional anti-GFP antibody (upper panels) or Cy3-conjugated anti-GFP antibody Fab fragment (Fab-Cy3, lower panels) (scale bar, 10 μm). B: Live cultured hippocampal neurons transfected with GFP-GluN2A or GFP-GluN2B at DIV5 were surface-stained with Fab-Cy3 at DIV7 (scale bar, 10 μm). In each panel, insets show segments of dendrites with GFP-GluN2 fluorescence (green), surface staining with Cy3-conjugated anti-GFP Fab (red), and their merged images. C: Quantitative analysis of the number of surface-distributed GluN2A-containing or GluN2B-containing NMDAR clusters per 100 μm dendrite at DIV7 after transfection with GFP-GluN2A or GFP-GluN2B at DIV5 (*P <0.01, Student's t test; error bars represent mean ± SEM). D: Co-localization of surface GluN2A-NMDAR clusters (red) with the presynaptic marker synaptophysin (green) at DIV7 after transfection with GFP-GluN2A at DIV5 (scale bar, 10 μm).**

In addition, the surface GFP-GluN2A clusters partially co-localized with synaptophysin at DIV14 (Fig. 2D). Taken together, our data suggested that, compared with surface NMDARs containing GluN2B, those containing GluN2A are more inclined to cluster in both premature and mature hippocampal neurons.

The C-Terminus of the GluN2 Subunit Determines the Distribution Pattern of Surface NMDARs

The GluN2 subunit has a long, intracellular C-terminus which mediates the intracellular trafficking and synaptic targeting of NMDARs[16,17]. To assess whether it contributes to the distribution patterns of surface NMDARs, we constructed the chimeric mutants GFP-GluN2B-C_{GluN2A} and GFP-GluN2A-C_{GluN2B}, in which the C-termini of GluN2A and GluN2B were completely exchanged (Fig. 3A). We found that the surface density of GFP-GluN2B-C_{GluN2A} clusters at DIV7 was significantly increased and did not statistically differ from that of GFP-GluN2A clusters (Fig. 3B). In contrast, the surface GFP-GluN2A-C_{GluN2B} was distributed in a more diffuse pattern, similar to that of GFP-GluN2B. The surface density of GFP-GluN2A-C_{GluN2B} clusters was significantly lower than that of GFP-GluN2A. These results indicated that the surface distribution pattern of NMDARs depends on the C-terminus of GluN2.

The last four amino-acids (ESDV) of GluN2 form the PDZ-binding domain, which directly interacts with proteins of the PSD-MAGUK family and mediates the clustering and

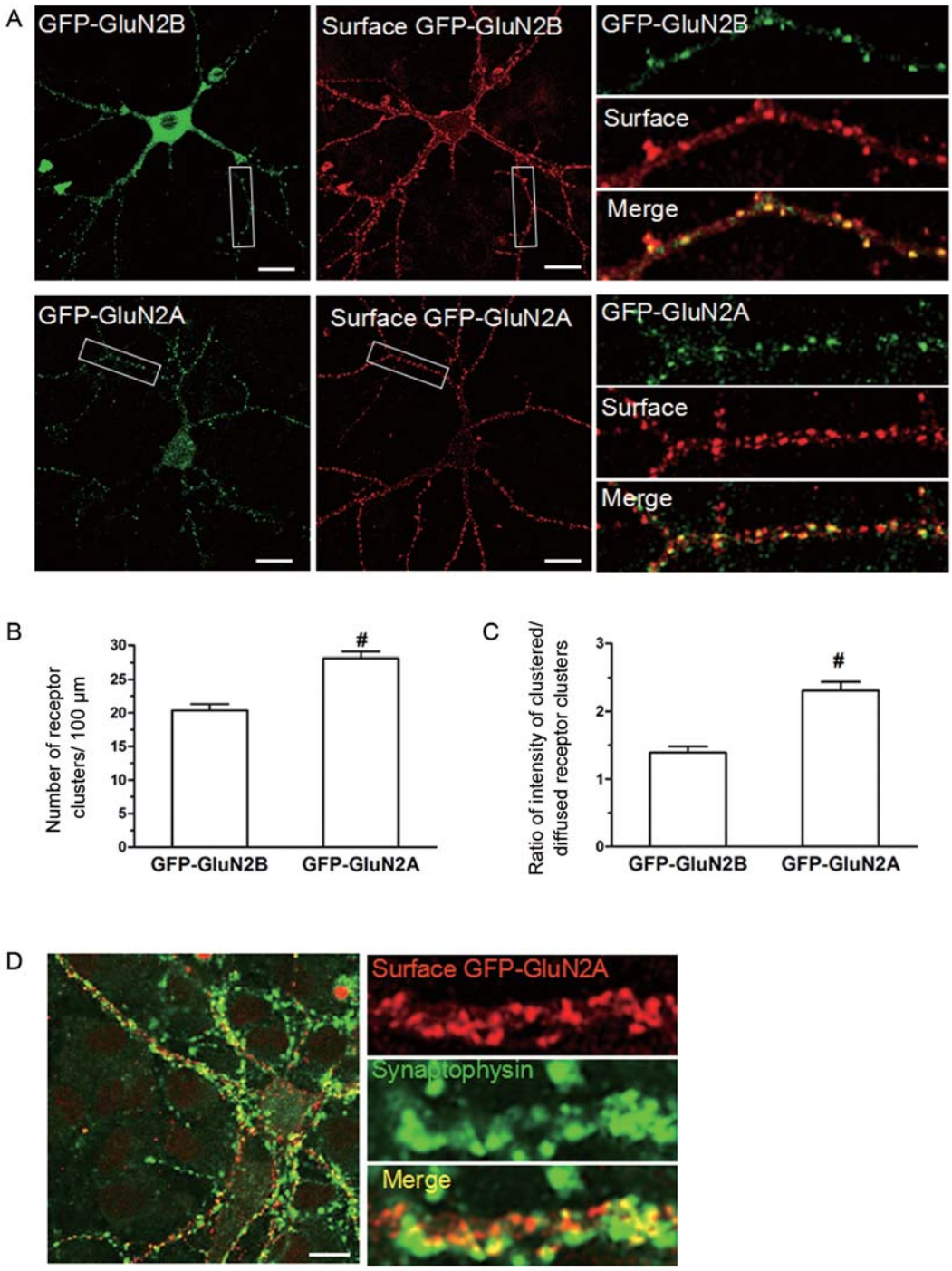

Fig. 2. Distribution patterns of GluN2A- and GluN2B-containing NMDARs at DIV14. A: Neurons were transfected with GFP-GluN2A (lower panels) or GFP-GluN2B (upper panels) at DIV5, and then live cell-surface stained with Fab-Cy3 at DIV14 (scale bars, 10 μm). B: Density of the surface clusters of GFP-GluN2A or GFP-GluN2B per 100 μm dendrite ($^{\#}P$ <0.01, Student's *t* test; mean ± SEM). C: Ratio of average immunofluorescence intensity between the surface clustered and diffuse receptors. The surface NMDARs containing GluN2A or GluN2B were divided into clustered and diffuse pools, and then the ratio of average immunofluorescence intensity of the two pools was measured ($^{\#}P$ <0.01, Student's *t* test; mean ± SEM). D: Co-localization of surface NMDARs containing GluN2A (red) and the presynaptic marker protein, synaptophysin (green) at DIV14 after transfection with GFP-GluN2A at DIV5. Most of the surface GluN2A-containing NMDAR clusters were synaptically located at DIV14 (77.0 ± 1.9%; *n* = 20; scale bar, 10 μm).

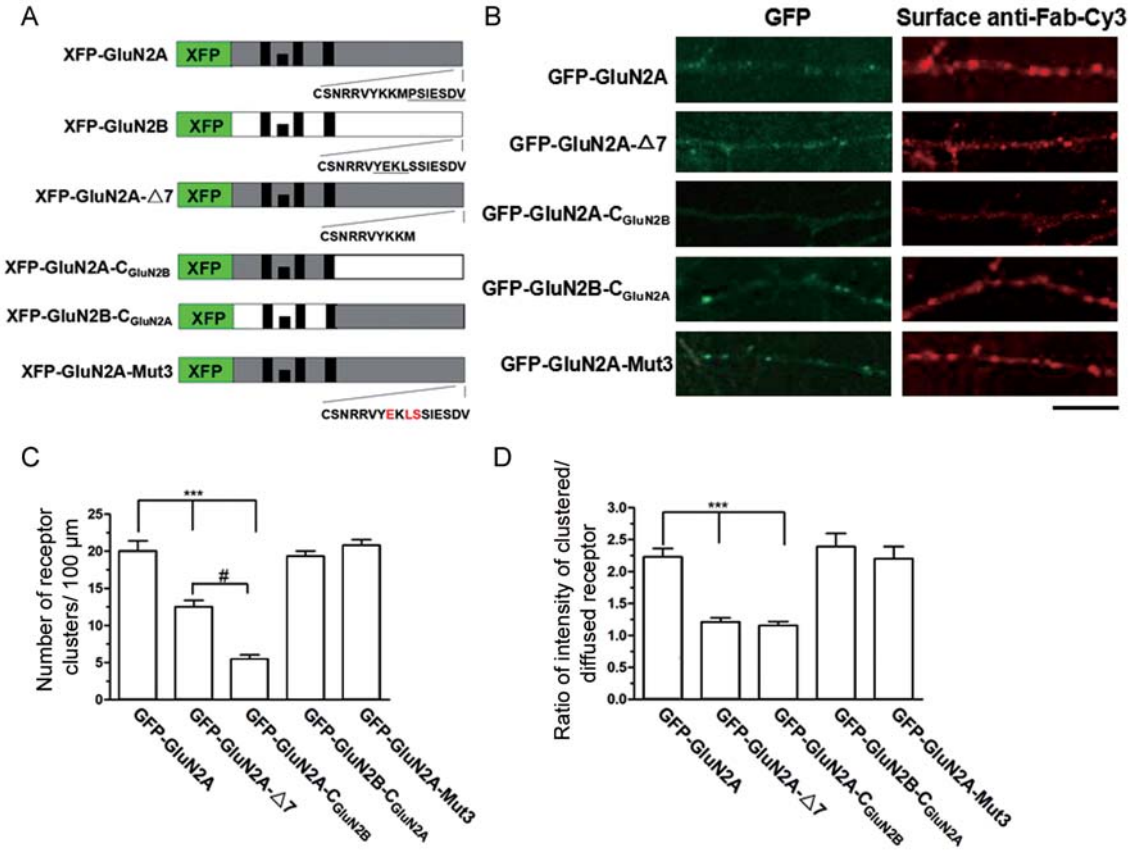

Fig. 3. Surface distribution pattern of GluN2A-containing NMDARs depends on the C-terminus. A: Schematic representation of the mutant proteins used in this experiment. XFP indicates GFP or CFP. XFP-GluN2A-Δ7 was a mutation of GluN2A that lacked the last 7 amino-acids (including the PDZ-binding domain). XFP-GluN2A-Mut3 was a mutation of GluN2A, in which the last 11 amino-acids were converted to the corresponding amino-acids in GluN2B (three amino-acids were mutated: K1455E, M1457L, and P1458S). B: Hippocampal neurons were transfected with GFP-tagged GluN2A constructs at DIV5, and then were surface-stained with Fab-Cy3 at DIV7 (scale bar, 10 μm). C: Number of the surface GluN2A-containing NMDAR clusters per 100 μm dendrite after transfection with different GFP-tagged GluN2A constructs. Compared with full-length GFP-GluN2A, GFP-GluN2A-Δ7 and GFP-GluN2A-C_{GluN2B} showed a decrease in cluster density, while GFP-GluN2B-C_{GluN2A} and GFP-GluN2A-Mut3 did not show a significant difference (*P <0.01, Student's t test). More receptor clusters were observed in neurons expressing GFP-GluN2A-Δ7 than in those expressing GFP-GluN2A-C_{GluN2B} ($^{\#}P$ <0.05, Student's t test; mean ± SEM). D: Ratio of average immunofluorescence intensity between clustered and diffuse receptors. Compared with full-length GFP-GluN2A, GFP-GluN2A-Δ7 and GFP-GluN2A-C_{GluN2B} showed a decrease in the ratio, while GFP-GluN2B-C_{GluN2A} and GFP-GluN2A-Mut3 did not show a significant difference (***P <0.01, Student's t test; mean ± SEM).**

synaptic targeting of NMDARs[9,18-20]. To assess the role of this domain in the distribution pattern of different NMDAR subtypes, we generated a mutant construct of GluN2A with the last seven amino-acids deleted (GFP-GluN2A-Δ7) (Fig. 3A) and found that the surface density of GFP-GluN2A-Δ7 clusters was significantly lower than that of surface GFP-GluN2A clusters. However, the surface density of GFP-GluN2A-Δ7 clusters was still higher than that of GFP-GluN2A-C_{GluN2B} clusters (Fig. 3C). This indicated that the PDZ-binding domain of the GluN2A subunit partially determines the distribution pattern of GluN2A-containing NMDARs.

Previous work suggests that YEKL in the distal C-terminus of the GluN2B subunit is a binding site for AP-2, which is pivotal in determining the synaptic localization of GluN2B-containing NMDARs (Fig. 3A). Interestingly, the

GluN2A subunit has a similar motif (YKKM), but this motif is not a substrate for AP-2 binding[12,19,21]. To determine the role of this motif in the distribution patterns of different NMDAR subtypes, we generated a construct, GFP-GluN2A-Mut3, in which the GluN2A YKKM motif was mutated to YEKL (Fig. 3A). We found that the surface density of GFP-GluN2A-Mut3 clusters did not differ from that of GFP-GluN2A clusters (Fig. 3C), suggesting that the YEKL motif is not important in the determination of NMDAR distribution patterns.

PSD-95 Specifically Associates with Surface GluN2A-containing NMDAR Clusters in Hippocampal Neurons

Our results above indicated that the C-terminus of GluN2 mediates the differential surface distribution pattern between GluN2A- and GluN2B-containing NMDARs. MAGUKs family proteins, including PSD-95 and SAP102, are the major postsynaptic proteins that bind to NMDARs *via* the cytoplasmic tail of the GluN2 subunit. To determine whether association between surface NMDARs and MAGUKs also occurs in a GluN2-dependent manner, we tested the co-localization of MAGUKs (PSD-95 and SAP102) with surface GluN2 subunits (GluN2A and GluN2B). We first co-transfected hippocampal neurons at DIV5 with ECFP-GluN2A or ECFP-GluN2B and PSD-95-GFP and analyzed the co-localization of surface GluN2 clusters with PSD-95-GFP at DIV14 (Fig. 4A, B). We found that most of the surface GluN2B clusters were not concentrated at the sites of PSD-95 puncta, while the surface GluN2A clusters were highly co-localized with PSD-95 puncta (Fig. 4E). Next, we co-transfected cultured hippocampal neurons with SAP102-GFP and ECFP-GluN2A or ECFP-GluN2B, and found that both the surface GluN2B clusters and the surface GluN2A clusters co-localized well with SAP102-GFP (Fig. 4C-E). These data showed that PSD-95, but not SAP102, is specifically associated with surface NMDARs containing GluN2A, indicating that PSD-95 is involved in determining the distribution pattern of different NMDAR subtypes.

Interestingly, the surface density of ECFP-GluN2B clusters was significantly increased when co-expressed with SAP102-GFP (Fig. 4F), suggesting that overexpression of SAP102 induces the clustering of GluN2B-containing NMDARs.

The C-Terminus of GluN2A Determines the Specific Association of Surface GluN2A-containing NMDARs with PSD-95

To identify the structural basis of the specific association of GluN2A with PSD-95, we co-transfected neurons with CFP-GluN2B-C_{GluN2A} or CFP-GluN2A-C_{GluN2B} and PSD-95-GFP (Fig. 5A) and analyzed the co-localization ratios of the surface CFP-GluN2B-C_{GluN2A} or CFP-GluN2A-C_{GluN2B} clusters with PSD-95. We found that the surface GluN2A-C_{GluN2B} was distributed more diffusely and showed little co-localization with PSD-95 puncta. In contrast, surface GluN2B-$C_{GLIuN2A}$ clusters were highly co-localized with PSD-95 puncta (Fig. 5B). These results indicated that the C-terminus of GluN2 is critical to the different association between NMDAR subtypes and MAGUKs proteins.

Next, we co-transfected neurons with ECFP-GluN2A-Δ7 and PSD-95-GFP, and found that the co-localization level of surface ECFP-GluN2A-Δ7 clusters with PSD-95 was significantly decreased compared to that of surface ECFP-GluN2A with PSD-95 (Fig. 5A). However, it was still higher than the co-localization level of surface GluN2A-C_{GluN2B} with PSD-95 (Fig. 5B). When ECFP-GluN2A-Mut3 and PSD-95-GFP were co-transfected into hippocampal neurons, the surface ECFP-GluN2A-Mut3 clusters co-localized with PSD-95, and did not differ from that of ECFP-GluN2A. Taken together, these data indicated that the PDZ-binding domain of the GluN2A subunit partially determines the specific association of GluN2A-containing NMDARs with PSD-95.

Expression of the GluN2A Subunit Promotes Clustering of PSD-95 in Cultured Hippocampal Neurons

Previous work has shown that the distribution of both endogenous and exogenous PSD-95 protein changes from a diffuse to a clustered pattern in cultured neurons during development[22]. Here, we also found that PSD-95-GFP was diffusely distributed at DIV7 when transfected alone into cultured hippocampal neurons (Fig. 6A). Interestingly, the density of PSD-95-GFP puncta significantly increased at DIV7 when co-expressed with ECFP-GluN2A, compared with expression alone or co-expression with ECFP-GluN2B (Fig. 6B). Furthermore, PSD-95 puncta were highly co-localized with surface GluN2A-containing NMDAR clusters (Fig. 6C). Together with our finding that overexpression

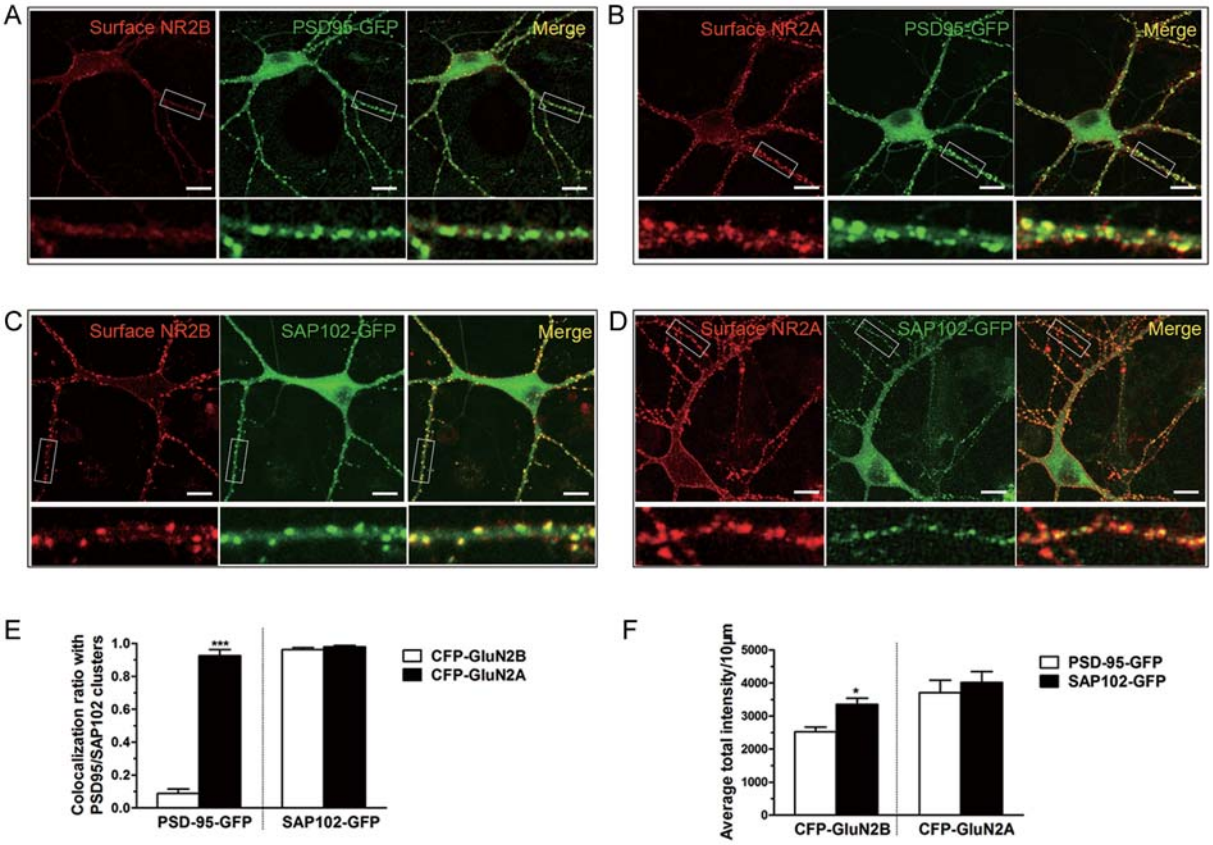

Fig. 4. PSD-95 specifically associates with surface GluN2A-containing NMDARs in hippocampal neurons. A–D: Cultured hippocampal neurons were co-transfected with ECFP-GluN2B/PSD-95-GFP, ECFP-GluN2A/PSD-95-GFP, ECFP-GluN2B/SAP102-GFP, or ECFP-GluN2a/SAP102-GFP at DIV5, and then surface-stained with Cy3-conjugated anti-GFP Fab fragment at DIV14 (scale bars, 10 μm). E: Percentage co-localization of surface-stained GluN2B or GluN2A clusters with PSD-95 or SAP102 puncta. Compared with SAP102, few PSD-95 puncta co-localized with GluN2B-NMDAR clusters (****P* <0.01, Student's *t* test). As for GluN2A, there were no significant differences in the co-localization ratio with PSD-95 or SAP102 puncta (mean ± SEM). F: Surface expression levels of GluN2A- or GluN2B-containing NMDARs in neurons co-transfected with PSD-95 or SAP102. The surface expression level of GluN2B-containing NMDARs co-transfected with PSD-95 was lower than after co-transfection with SAP102 (**P* <0.05, Student's *t* test). There were no significant differences in the intensity of surface GluN2A-containing NMDARs in neurons co-transfected with PSD-95 or SAP102 (mean ± SEM).**

of SAP102 induced the clustering of surface GluN2B-containing NMDARs, these results suggested that the distribution pattern of MAGUK proteins or GluN2 subunits is tightly controlled by their expression levels.

DISCUSSION

Previous studies have shown that the GluN2 subunit determines many of the biophysical and pharmacological properties of NMDARs, and also influences NMDAR assembly, downstream signaling, receptor trafficking, and synaptic localization[23-25]. In this study, we found that the GluN2 subunit is also responsible for the distinct surface distribution patterns of different NMDAR subtypes. Our results showed that surface NMDARs containing GluN2A were inclined to cluster, while those containing GluN2B were much more diffusely distributed along the dendrites in both immature and mature hippocampal neurons. However, the functional difference between the clustered and the diffuse receptors remains unclear. It is known that receptor clustering is an active process that includes the interaction of receptors with intracellular scaffold proteins,

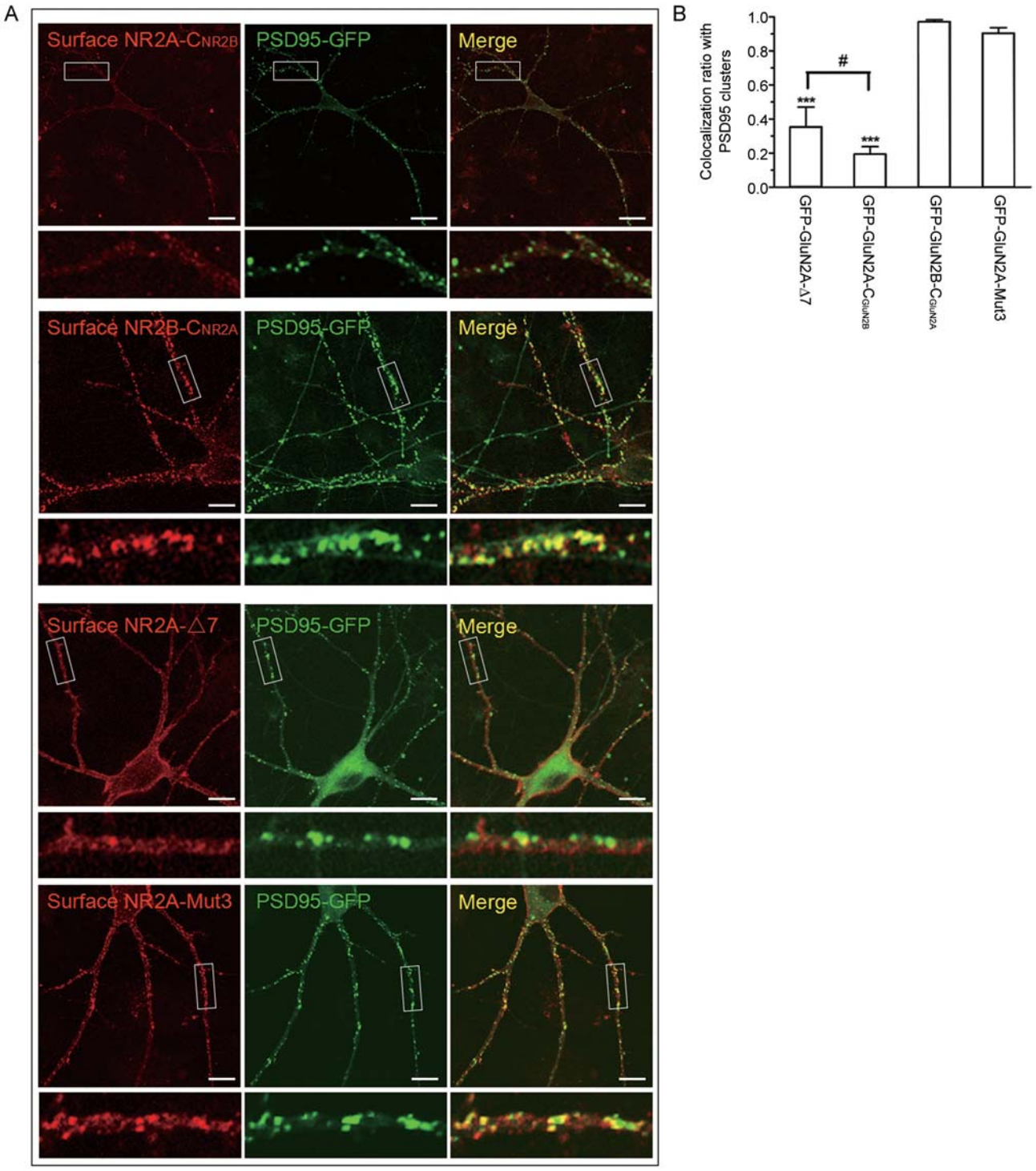

Fig. 5. The PDZ-binding domain partially determines the co-localization of surface GluN2A-containing NMDARs with PSD-95. A: Neurons were co-transfected with PSD-95-GFP and different GluN2A mutants, and then surface GluN2A-containing NMDARs were detected with Fab-Cy3 at DIV14 (scale bars, 10 μm). B: Co-localization ratio of surface GluN2A mutant clusters to PSD-95 puncta. Compared with GluN2B-C_{GluN2A}, the co-localization of GluN2A-Δ7 and GluN2A-C_{GluN2B} with PSD-95 puncta was decreased, while GluN2A-Mut3 showed no statistical difference (*P <0.01, Student's t test; mean ± SEM).**

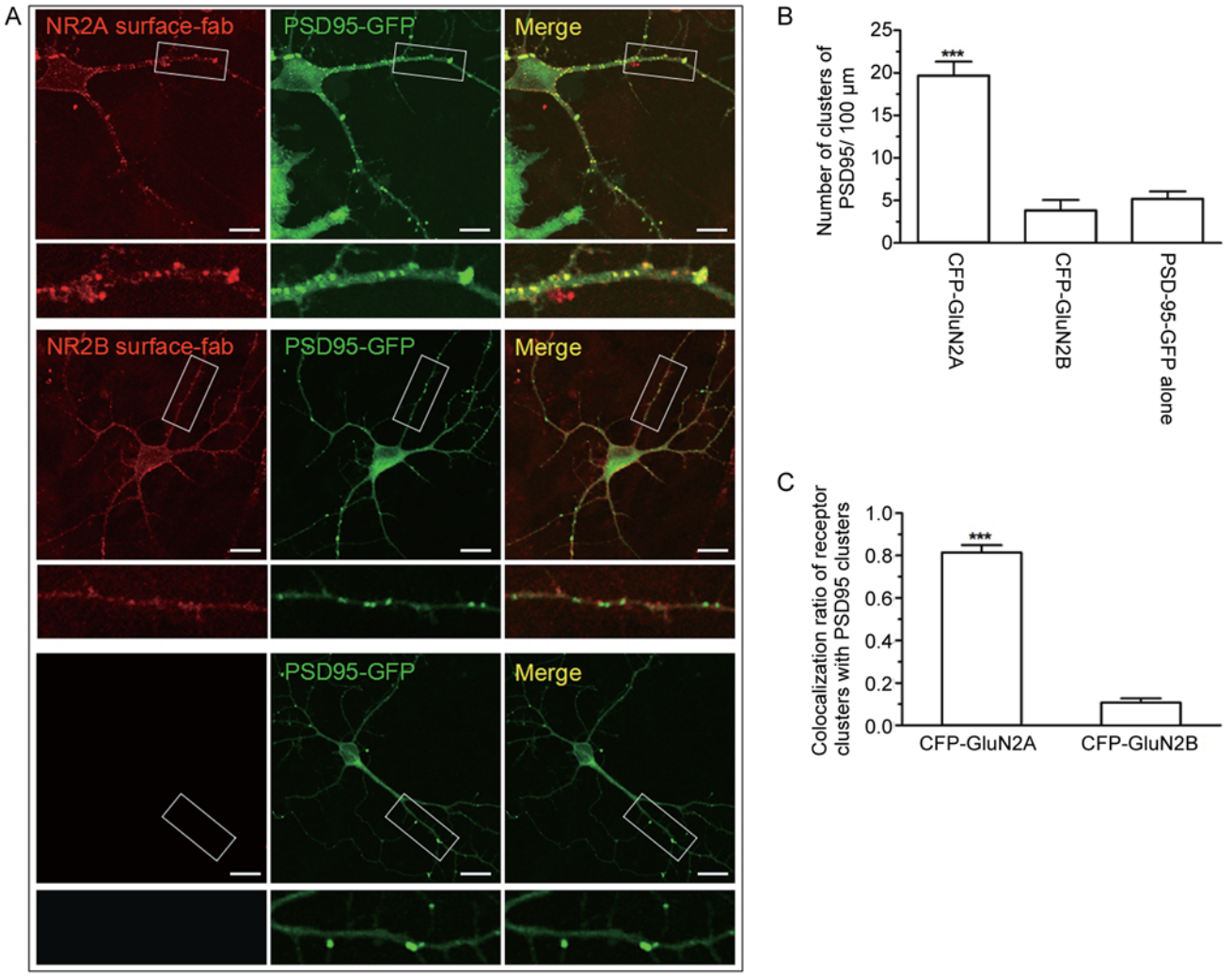

Fig. 6. Overexpression of the GluN2A subunit promotes the clustering of PSD-95 in cultured hippocampal neurons. A: Hippocampal neurons were transfected with PSD-95-GFP/CFP-GluN2A (upper panels), PSD-95-GFP/CFP-GluN2B (middle panels), or PSD-95-GFP alone at DIV5 (lower panels), and the distribution pattern of PSD-95-GFP was observed at DIV7 (scale bars, 10 μm). B: Number of PSD-95-GFP puncta per 100 μm in different groups. The density of PSD-95-GFP puncta increased when co-transfected with CFP-GluN2A, but not with CFP-GluN2B or expressed alone (*P <0.01, Student's t test; mean ± SEM). C: Co-localization ratio of surface receptor clusters with PSD-95 puncta. The co-localization ratio of surface GluN2A-containing receptor clusters with PSD-95 puncta was much higher than that of surface GluN2B-containing receptors (***P <0.01, Student's t test; mean ± SEM).**

adaptor proteins, and signaling proteins to form functional complexes. Therefore, the aggregation of neurotransmitter receptors is a central mechanism in neuronal development, synaptic plasticity, and learning. Here, we found that surface GluN2A-NMDARs were clustered even before mature synapses were formed. It is possible that NMDARs containing GluN2A are more important in synaptogenesis than those containing GluN2B. Accordingly, we found that overexpression of the GluN2A subunit in immature hippocampal neurons induced the clustering of PSD-95, the core component of postsynaptic complexes, which also suggested that expression of the GluN2A subunit promotes the fine-tuning of PSD-95 aggregation. A similar interaction between MAGUK proteins and K^+ channels has been reported. When expressed alone, neuronal MAGUKs or K^+ channels occur diffusely throughout COS cells, while

co-transfection with PSD-95 and $Kv_{1.4}$ results in clustering of both molecules[26]. Together with our study, these results indicate that the interaction between MAGUK and receptors encourages the formation of functional clusters. Some other studies have reported that receptors within clusters are more stable than those outside of clusters[27]. Therefore, another possibility is that the surface stability of different NMDAR subtypes is distinct. It may be that the surface NMDARs containing GluN2A do not readily undergo endocytosis, while those containing GluN2B are dynamically exchanged by endocytosis or exocytosis[28,29].

Our results showed that the entire C-terminus of the GluN2A subunit determines the specific distribution pattern of GluN2A-containing NMDARs, since the patterns were reversed by exchange of the C-termini of the GluN2A and GluN2B subunits. Meanwhile, we found that the PDZ-binding domain of the GluN2A subunit partially, but not completely, determines the clustering of surface NMDARs containing GluN2A. Combined, these data indicate that the interaction of the GluN2 subunits with PSD-MAGUK proteins is one of the key mechanisms for the clustering and synaptic targeting of NMDARs. However, other as yet unknown mechanisms based on the C-terminus of GluN2 are involved in controlling the distribution patterns of surface NMDARs. Recently, research using cultured cortical neurons has shown that the GluN2A and GluN2B subunits have two distinct consensus cysteine clusters in their C-termini. Palmitoylation of these cysteine clusters is involved in the stable expression and constitutive internalization of surface NMDARs[30]. It will be interesting to explore the role of palmitoylation in the surface distribution patterns of NMDARs.

Previous studies indicate that interactions of the PSD-MAGUK family with NMDARs are subtype-dependent. SAP102 preferentially associates with GluN2B-containing NMDARs, while PSD-95 associates with those containing GluN2A. Another study showed that di-heteromeric GluN1/GluN2A and GluN1/GluN2B receptor populations similarly immunoprecipitate PSD-95, SAP102, and PSD-93 in adult rat hippocampus[31]. In this study, we found that PSD-95 specifically co-localized with surface NMDARs containing GluN2A, but not those containing GluN2B, which suggests that the specific association of PSD-95 with GluN2A is important for the surface distribution pattern of GluN2A-containing NMDARs.

In summary, here, we have demonstrated that different NMDAR subtypes have distinct surface distribution patterns, which are mainly determined by the C-terminus of the GluN2 subunit. The specific association of PSD-95 with the GluN2 subunit is also critical for the surface distribution pattern and synaptic localization of NMDARs.

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·**Perspective**·

Non-apoptotic role of caspase-3 in synapse refinement

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Caspases, a family of cysteine proteases, mediate programmed cell death during early neural development and neurodegeneration, as well as following neurotoxic insults. Notably, accumulating lines of evidence have shown non-apoptotic roles of caspases in the structural and functional plasticity of neuronal circuits under physiological conditions, such as growth-cone dynamics and axonal/dendritic pruning, as well as neuronal excitability and plasticity. Here, we summarize recent progress on the roles of caspases in synaptic refinement.

Keywords: caspases; neuronal plasticity; synaptic refinement

Since the discovery of the *Caenorhabditis elegans* caspase gene *ced3*[1], 12 mammalian caspases have been identified, including initiator caspases (caspase-1, -2, -5, -8, -9, -10, -11, and -12) and effector caspases (caspase-3, -6, -7, and -14). Among these, caspase-3 plays a critical role in mediating apoptosis in both the death receptor pathway and mitochondrial pathways[2]. In the nervous system, caspases not only mediate cell death during neural development and neurodegeneration[2], but also play non-apoptotic roles under physiological conditions, e.g., synaptic plasticity[3, 4], dendritic pruning in *Drosophila*[5, 6], and the chemotropic response of axonal growth cones[7]. Recently, we found that caspase-3 plays an important role during synapse refinement at the neuromuscular junction (NMJ)[8] (Fig. 1A). Like other synapses, the development of NMJs involves a complicated refinement process. At early-to-middle embryonic stages, myotubes form spontaneous pre-patterned acetylcholine receptor (AChR) clusters, and after the invasion of motor nerves, the innervated clusters are strengthened and stabilized, while aneural AChR clusters are gradually dispersed[9]. The interplay between positive and negative factors determines the precise matching of presynaptic nerve terminals and postsynaptic structures on the muscle surface. The motoneuron-derived glycoprotein agrin is believed to be the critical positive factor, ablation of which causes severe defects in formation of the NMJ[9]. Interestingly, the neurotransmitter acetylcholine (ACh) has been proposed to be a negative factor[10, 11]. Genetic ablation of *choline acetyltransferase* (*ChAT*) partially rescues AChR clusters in agrin-knockout mice[11] and treatment of cultured muscle cells with carbachol (CCh), a non-hydrolyzable cholinergic agonist, induces the dispersion of AChR clusters[10, 11]. Intriguingly, genetic evidence and results from cultured muscle cells suggest that Cdk5, a cytoplasmic serine/threonine kinase, is an effector in dispersing AChR clusters[11, 12]. More recently, Lee and colleagues reported that the intermediate filament protein nestin is required for ACh-induced association of p35, the co-activator of Cdk5, with the muscle membrane and Cdk5 activation[13]. Similar to the effect of Cdk5 inhibition or ablation, knockdown of nestin in agrin-deficient mice markedly rescues AChR clusters[13]. How does agrin counteract the role of ACh in Cdk5 activation? In a previous study, we showed that CCh stimulation of cultured muscle cells activates the Ca^{2+}-dependent protease calpain, leading to the cleavage of P35 to P25, a more stable and stronger activator of Cdk5[14]. Interestingly, rapsyn, a postsynaptic scaffold protein associated with AChRs, physically interacts with calpain and inhibits its activity. Agrin, by increasing the interaction between calpain and rapsyn, inhibits calpain activity[14]. We

noted that the loss of AChR clusters in agrin-mutant mice is partially rescued by injecting or over-expressing calpain inhibitors in muscle cells[14], to a much lesser extent than that found with *ChAT* ablation. This result prompted us to search for other downstream mediators in the activity-dependent elimination of AChR clusters.

Because caspase-3 is activated at the NMJ in patients and mice with slow-channel syndrome resulting from mutations in certain subunits of AChRs and a sustained elevation of Ca^{2+} concentration in muscles[15], we hypothesized that caspase-3 might be involved in synapse refinement. We found that cholinergic stimulation of cultured muscle cells activates caspase-3 locally in AChR cluster-enriched regions, and notably, active caspase-3 is associated with aneural AChR clusters[8]. Inhibition or genetic ablation of caspase-3 stabilizes AChR clusters *in vitro* and *in vivo*. In line with this notion, the decrease of apoptotic protease activating factor 1 (Apaf-1), an adaptor protein essential for caspase-3 activation in the mitochondrial pathway, also stabilizes AChR clusters. It remains of interests to determine whether and how apoptosomes are recruited to the AChR complex, and are thus involved in cluster dispersion.

We also investigated the mechanism by which caspase-3 functions in the disassembly of AChR clusters and identified Dishevelled1 (Dvl1), a Wnt signaling protein that mediates agrin/MuSK signaling in AChR clustering[16] (Fig. 1A). Blockade of Dvl1 cleavage also stabilizes AChR clusters in culture and *in vivo,* indicating that Dvl1 is a functional substrate of caspase-3[8].

Several lines of evidence support the hypothesis that agrin stabilizes synapses at least partially through counteracting the negative role of ACh during NMJ development. How does agrin limit caspase-3 activation? Interestingly, we found that heat shock protein 90β, which regulates AChR cluster formation and maintenance by stabilizing rapsyn[17], is involved in agrin signaling in restraining caspase-3 activity[8] (Fig. 1A). It has been shown that during dendritic pruning in *Drosophila*[5] and songbird learning[18], caspase-3 activity is strictly controlled by X-linked inhibitor of apoptosis protein (XIAP). Thus, caspase-3 activity is tightly controlled in various physiological conditions during processes of neural development and plasticity.

Does caspase-3 also participate in structural or functional synaptic plasticity in the central nervous

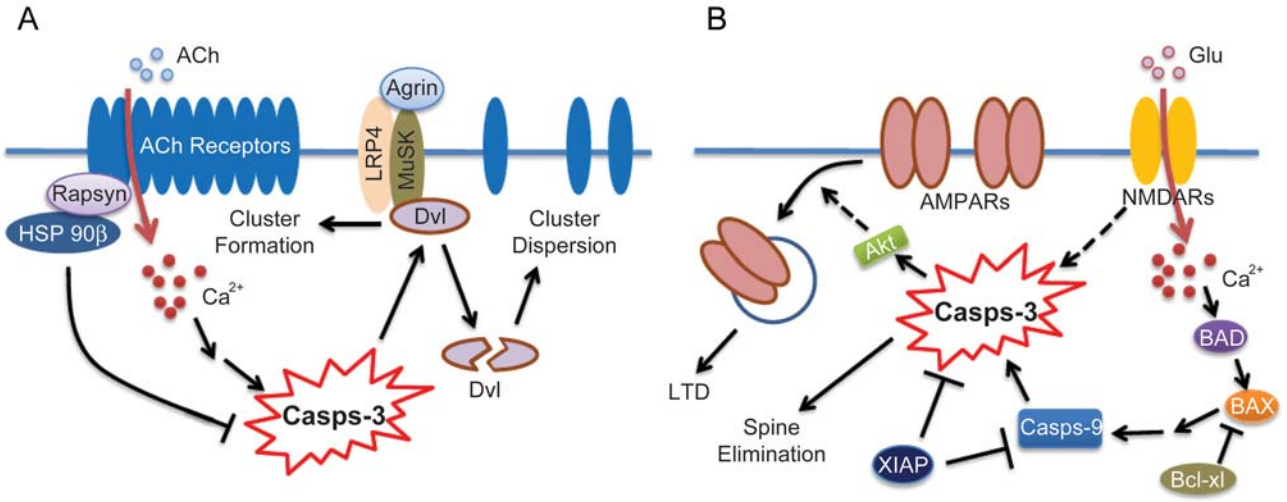

Fig. 1. Non-apoptotic role of caspase-3 in synapse elimination and plasticity. (A) At the NMJ, ACh stimulation increases the intracellular Ca^{2+} concentration, thus activating caspase-3 (casps-3) at postsynaptic sites. Active caspase-3 cleaves Dvl, an adaptor protein that mediates agrin/MuSK signaling, leading to the dispersion of aneural AChR clusters. The rapsyn-associated protein HSP 90β restricts and tightly controls caspase-3 activity at the postsynaptic regions innervated by motor neurons. (B) At the CNS excitatory synapse, NMDA receptor stimulation activates the BAD-BAX-caspase-3 cascade, which causes AMPA receptor internalization and consequently, NMDA-dependent long-term depression (LTD) and spine elimination. AKT acts as the substrate of caspase-3 in LTD induction. The anti-apoptotic proteins XIAP and Bcl-xl inhibit LTD induction by limiting the activation of the apoptotic cascade.

system? Early findings that AMPA receptors are cleaved by caspases during excitotoxic neuronal death suggested this possibility[19]. Notably, an interesting study showed that caspase-3 is involved in AMPA receptor internalization during NMDA receptor-dependent long-term depression (LTD) in hippocampal neurons, and a serine/threonine kinase Akt1 appears to be the substrate of caspase-3 for its action in LTD through controlling glycogen synthase kinase-3β (GSK3β) activity[3] (Fig. 1B). In line with the hypothesis that the mitochondrial pathway is involved in LTD induction, over-expression of the anti-apoptotic protein XIAP or Bcl-xL[3] or down-regulation of the pro-apoptotic protein BAD or BAX inhibits LTD in CA1 neurons[20]. Differential activation of the BAD-BAX-caspase-3 cascade in LTD and apoptosis indicates that fine-tuning of caspase-3 activity during LTD induction may ensure that neurons escape from apoptosis[20]. Amyloid-β_{1-42} (Aβ) is believed to be a critical factor in causing cognitive decline in Alzheimer's disease, presumably by affecting hippocampal long-term potentiation (LTP). Recently, it has been shown that the caspase-3, AKT, and GSK3β pathway is involved in the effects of Aβ on LTP[21]. The failure in LTP-induction manifests in some ways in synapse loss, which occurs normally during development or pathologically during neurodegenerative diseases. Indeed, local activation of caspase-3 by photostimulation of mitochondria-targeted KillerRed, which triggers mitochondrial damage and activates the intrinsic pathway of apoptosis, induces local spine elimination and dendrite retraction in cultured hippocampal neurons, without inducing full apoptosis[22]. In contrast, caspase-3-knockout mice exhibit increased spine density and altered miniature excitatory post-synaptic currents. Taken together, these findings suggest that caspase-3 is involved in the elimination of postsynaptic structures in the CNS and peripheral synapses.

Is it possible that caspases also regulate presynaptic structures or functions? Indeed, we found that genetic ablation of *caspase-3* markedly restores the presynaptic structures of motor nerve terminals in agrin-knockout mice[8]. This phenomenon could be explained by the presence of either retrograde signals expressed in muscle cells or a direct role of caspase-3 in pre-synaptic differentiation. Indeed, some axon guidance factors, e.g., netrin-1 or lysophosphatidic acid, activate caspase-3 in retinal axonal growth cones and caspase-3 activity is essential for the induced chemotropic responses[7]. In addition, local caspase activity has been observed in the branch points of the axonal arbors of young retinal ganglion cells in zebrafish embryos and this pattern correlates with axon-repulsive Slit-Robo signaling[23]. Down-regulation of caspase-3 or caspase-9 increases the stability of arbors and presynaptic sites[23]. Presynaptic differentiation involves several consecutive steps, including biogenesis of synaptic vesicles, transport along axonal microtubules or actin filaments, docking to and fusion with presynaptic axonal membrane, and exocytosis and recycling of synaptic vesicles, and most, if not all, of these steps require the coordination of cytoskeletal structures. The role of caspases in the dynamics of axonal growth cones implies a potential non-apoptotic role of caspases in presynaptic differentiation and remodeling. During maturation of the NMJ, motor nerves shift from multiple innervation to single innervation, while the shape of postsynaptic structures change from plaque to pretzel-like[9]. It would be of interests to determine whether caspases participate in the terminal dynamics of motor nerves as well as the maturation of postsynaptic structures.

In summary, accumulating lines of evidence from various systems have suggested non-apoptotic roles of caspases, in particular caspase-3, in synapse refinement under physiological and pathological conditions. Identification of the mediators responsible for this tightly-controlled local apoptotic pathway is not only helpful for understanding the mechanisms of brain wiring, but is also relevant to understanding brain disorders.

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·Review·

Current status of cell-mediated regenerative therapies for human spinal cord injury

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During the past decade, significant advances have been made in refinements for regenerative therapies following human spinal cord injury (SCI). Positive results have been achieved with different types of cells in various clinical studies of SCI. In this review, we summarize recently-completed clinical trials using cell-mediated regenerative therapies for human SCI, together with ongoing trials using neural stem cells. Specifically, clinical studies published in Chinese journals are included. These studies show that current transplantation therapies are relatively safe, and have provided varying degrees of neurological recovery. However, many obstacles exist, hindering the introduction of a specific clinical therapy, including complications and their causes, selection of the target population, and optimization of transplantation material. Despite these and other challenges, with the collaboration of research groups and strong support from various organizations, cell-mediated regenerative therapies will open new perspectives for SCI treatment.

Keywords: cell-mediated regenerative therapy; spinal cord injury; clinical trials; stem cell

Introduction

A recent literature survey on spinal cord injury (SCI) shows an incidence ranging from 10.4 to 83 cases per million per year (average, 29.5) and a prevalence of 223–755 per million (average, 485)[1]. After SCI, the release of inhibitory molecules, insufficient expression of growth factors, and formation of glial scar at the injury site are negative local consequences that lead to the formation of an impermeable barrier that prevents axons from regenerating across the site of injury[2, 3]. Meanwhile, the capacity of endogenous stem-cell regeneration is limited in the adult central nervous system (CNS). Treatment of SCI poses great challenges to any standard regenerative therapy. Over the past 20 years, great emphasis has been placed on cell-mediated regenerative therapies, and exogenous cell transplantation is thought to be an important means of treating SCI (Fig. 1). Neuronal function can be improved by applying different sources of cells to SCI, and these are not merely restricted to exogenous neural stem cells. Advances have been achieved albeit with considerable challenges. The safety of cell transplantation therapies *via* multiple routes has been widely confirmed. However, their therapeutic efficacy remains unsatisfactory, and the design of studies should be further considered (Fig. 2). In this review, we summarize clinical studies with cell-mediated transplantation for SCI and strategies for further clinical applications. Also, we provide a practical overview of independent clinical studies published in Chinese journals.

Clinical Outcomes of Transplantation Therapy for SCI

Mesenchymal Stem Cell Trials

Mesenchymal stem cells (MSCs) can be obtained from bone marrow, fat, umbilical cord, periosteum, and placenta. These tissues contain small numbers of adult stem cells, which can differentiate into various mesenchymal cells[4].

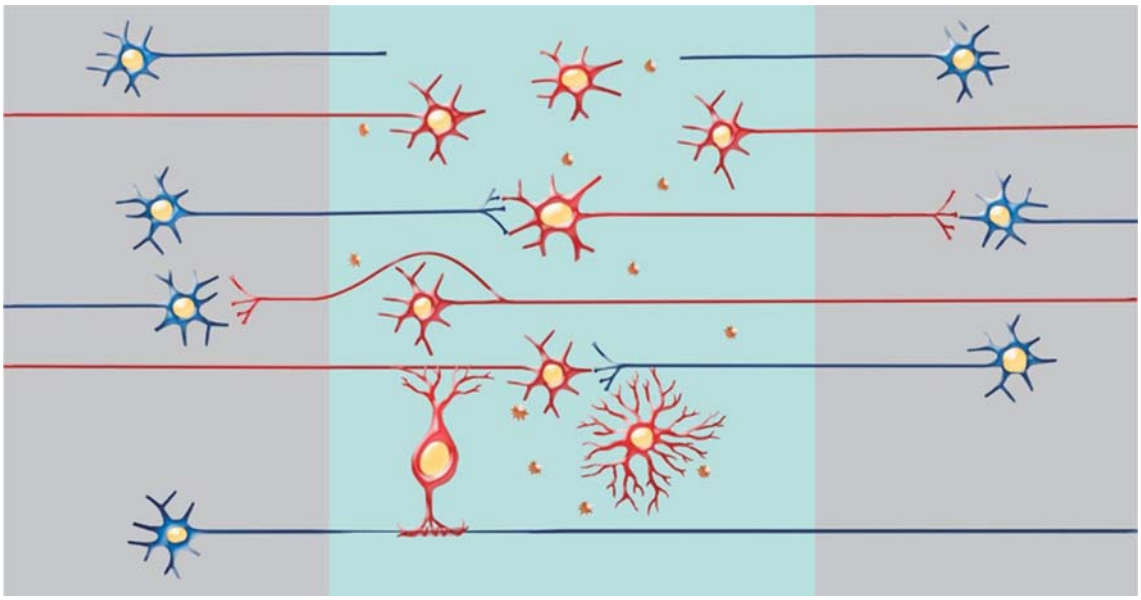

Fig. 1. Schematic diagram of the therapeutic mechanisms, including replacing neurons to reestablish axonal connections, providing a conducive microenviroment for axonal growth (including trophic factors secreted by grafted cells), and remyelinating axons. Red: grafted cells; blue: host cells; brown: trophic factors; aqua blue: supportive matrix.

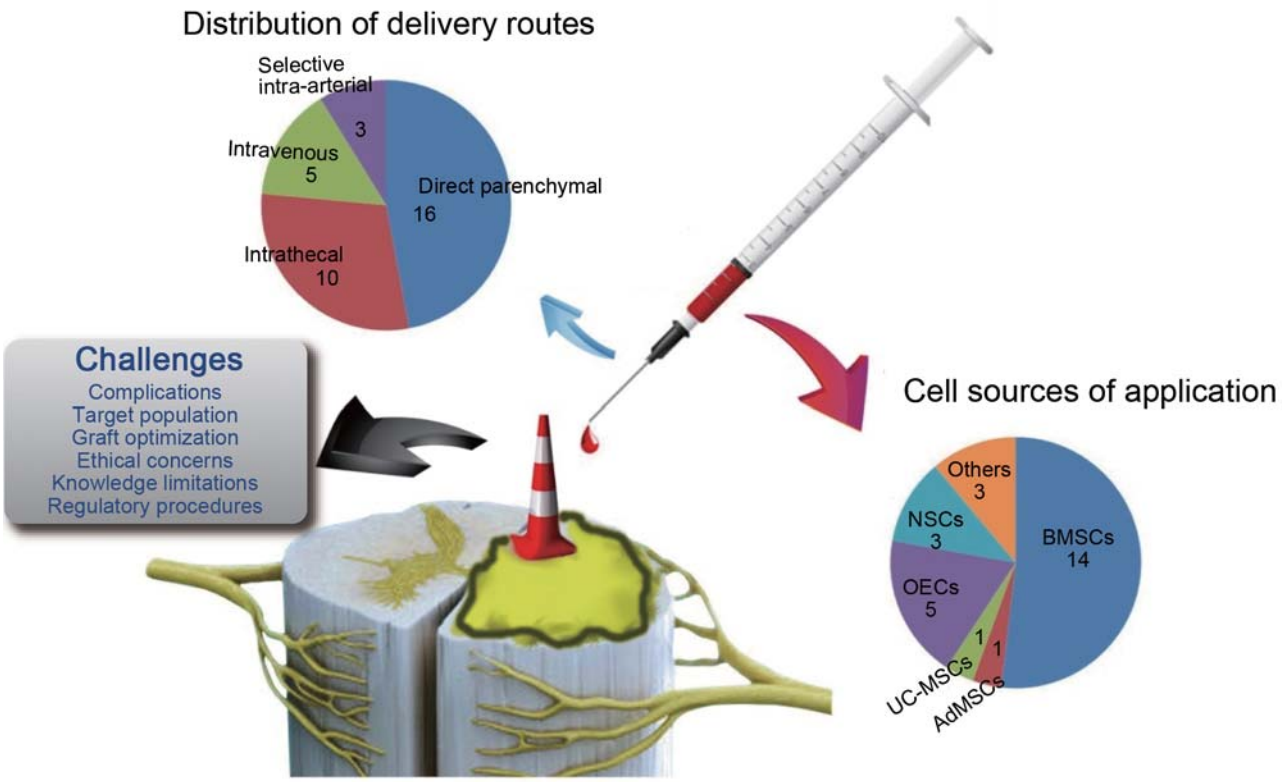

Fig. 2. Cell sources of application and the distribution of delivery routes in reported trials. Challenges of transplantation therapy face scientists and clinicians.

Table 1. Clinical trials for the treatment of SCI using mesenchymal stem cells (MSCs)

Reference	Transplanted cells	Patient group	Evaluation & outcome
Park *et al.*[12]	1.98×10^9 autologous MCPs. DI	6 patients, acute, AIS A, 5 cervical and 1 thoracic	Five patients showed improved neurological function (1 improved from AIS A to B and 4 improved from AIS A to C). No serious complications.
Yoon *et al.*[15]	2×10^8 MCPs. DI	35 patients, 17 acute, 6 subacute, 12 chronic, AIS A	Neurological improvement (29.5% of acute, 33.3% of subacute and 0% of chronic). Fever (62.9%), Neuropathic pain (20%). No tumor formation.
Callera *et al.*[13]	1×10^8 mononuclear cells, 1×10^6 $CD34^+$ cells. IT	10 patients, chronic, 7 paraplegia, 3 tetraplegia	No transplanted cells in CSF after 7 days. No neurological assessments and serious complications were reported.
Moviglia *et al.*[18]	BMMSCs (IA), effector T cells (IV), autologous NSCs (IA), unknown dose	8 patients, chronic, AIS A, 6 cervical and 2 thoracic	Five patients evolved from AIS A to D, 2 remained in the same condition, but exhibited motor and sensitive improvements. No serious complications.
Sykova *et al.*[14]	$104.0\pm55.3\times10^8$ mononuclear cells and $89.7\pm70.7\times10^6$ $CD34^+$ cells. 14 IV, 6 IA	20 patients, 8 subacute, 12 chronic. AIS A-C. 12 cervical and 8 thoracic	Four acute/subacute and 1 chronic patients (IA) showed improved neurological function. 1 acute/subacute patient (IV) showed AIS improvement. No serious complications.
Geffner *et al.*[17]	A total dose of 4×10^8 MCPs. DI, IT and IV	8 patients, 4 acute, 4 chronic. 5 AIS A, 1 AIS B and 2 AIS C. All thoracic	Three acute patients showed improved neurological function (AIS A to C) and 3 chronic patients also improved (1 improved from AIS A to C, 1 from AIS B to C and 1 from AIS C to D). No serious complications.
Mehta *et al.*[63]	AdMSCs, human ESCs-derived hematopoietic SCs, autologous BM–derived hematopoietic SCs, unknown dose. IT	163 patients, chronic, 156 paraplegia, 7 tetraplegia, all were HAI grade 9.	Function improved in 46 patients, 1 grade 4, 3 grade 5, 3 grade 6, and 17 grade 7. 22 patients had improvement in bowel sensations and sweating. 96 patients had postprocedural headache.
Kishk *et al.*[21]	5×10^6 to 10×10^6/kg of MCPs. IT	43 patients, chronic, AIS A	Eighteen patients increased motor scores by 1-2 points and changed from AIS A to B; 24 patients developed neuropathic pain.
Kumar *et al.*[19]	4×10^8 MCPs. IT	297 patients, chronic, AIS A-D	Ninety-seven patients exhibited neurological improvement. 63 patients developed postprocedural headache.
Pal *et al.*[25]	1×10^6 MSC cells/kg. IT	25 patients, 20 AIS A, 5 AIS C, 3 cervical and 22 thoracic	No neurological and electrophysiologic improvements. No serious complications. No tumor formation.
Cristante *et al.*[20]	2.5×10^6 $CD34^+$ MCPs/kg. IA	39 patients, chronic, AIS A	Improvement in SSEP (66.7%). No serious complications.
Chernykh *et al.*[62]	MSCs, Unknown dose. DI and IV	36 patients, chronic, 20 cervical and 16 thoracic	Neurological improvement (66.7%), no serious complications.
Karamouzian *et al.*[22]	7×10^5 to 1.2×10^6 MCPs. IT	11 patients (study group), 20 patients (control group), acute and sub- acute, AIS A thoracic.	Five (study group) and 3 patients (control group) showed marked recovery, but the result was statistically borderline (P=0.095). Eight patients (study group) developed neuropathic pain.
Deda *et al.*[16]	A total of $2.0\text{-}6.7\times10^7$ MCPs. DI, IT and IV	9 patients, AIS A. 6 cervical and 3 thoracic	Neurological and electrophysiological improvements in all patients. One patient improved from AIS A to B and 8 improved from AIS A to C. No serious complications, no tumor formation.
Ra *et al.*[27]	A total of 4×10^8 autologous AdMSCs. IV	8 patients, chronic, male, AIS A-B; 7 quadriplegia and 1 paraplegia	The latency of the left side in leg SSEP increased from 41.93 ± 4.39 to 48.27 ± 3.93 (P <0.05). In one patient, the AIS changed from A to C. No serious complications.
Liu *et al.*[30]	1×10^6 UC-MSCs/kg, once a week, four times as a course. IT	22 patients, subacute to chronic	Treatment was effective in 13 patients. 1 experienced lumbago, and 1 headache. No serious complications.

AdMSCs: adipose tissue-derived mesenchymal stem cells; AIS: American Spinal Injury Association impairment scale; BMMSCs: bone marrow mesenchymal stem cells; CSF: cerebrospinal fluid; DI: direct injection surrounding the lesion; ESCs: embryonic stem cells; HAI: Hauser Ambulation Index; IA: intra-arterial administration; IT: intrathecal administration; IV: intravenous administration; MCPs: mononuclear cells preparations; NSCs: neural stem cells; UC-MSCs: umbilical cord mesenchymal stem cells; SCs: stem cells; SSEP: somatosensory evoked potential.

The leading role of MSCs is believed to be neuroprotective by secreting neurotrophic factors, rather than inducing neural regeneration by transdifferentiation into neurons or glia[5, 6], while the exact mechanisms remain unknown[7]. The immunosuppressive effects of MSCs are considered to be benevolent, particularly as they are thought to ease the characteristic symptoms of SCI by settling the inflammatory response, which in due course reduces cavity formation and demyelination[8]. Under certain conditions, MSCs can be trans-differentiated into neurons and glial cells *in vitro* or *ex vitro*[9, 10], but only an extremely small proportion differentiate and the function of the trans-differentiated cells is not convincing[11] (Table 1).

Bone-marrow Mesenchymal Stem Cells

The majority of stem-cell-based clinical trials for SCI are based on the utilization of bone-marrow mesenchymal stem cells (BMMSCs). MSCs and hematopoietic stem cells (HSCs) are the known types of stem cells in bone marrow, and they are able to differentiate into mesenchymal and hematopoietic cell lineages, respectively. HSCs and MSCs are promising in clinical transplantation as autografts because they are easy to isolate from bone marrow and their effects are reproducible. Whole mononuclear cell preparations (MCPs), including almost all kinds of endothelial and hematopoietic cells, have been used in most clinical studies with bone marrow cells for SCI[12-22]. A comparison between culture-expanded MSCs and human MCPs was made by transplanting them into rodent SCI models, but no differences were found[23]. To date, no clinical study has been reported.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has the ability to guide MCPs to the injured site and improve functional recovery in rodent SCI[24]. In some studies, MCPs have been transplanted in combination with GM-CSF administration, and GM-CSF was found to guide MCP migration to the lesion site, enhance the survival of transplanted cells, and activate the secretion of neurotrophic factors[12, 15, 20]. Park *et al.* first reported combined therapy in acute patients (American Spinal Injury Association Impairment Scale (AIS) A) with direct injection of MCPs into the site of spinal cord damage within seven days post-injury[12]; this produced significant motor improvements, with no immediate worsening of neurological symptoms. The authors further conducted a phase I/II study applying the combined therapy to 35 patients with SCI (17 with acute treatment, 6 subacute, and 12 chronic)[15], with a control group of 13 participants receiving conventional surgery. They found that 5 acute, 2 subacute, and 1 control showed functional improvement during follow-up, while the chronic treatment group did not show any changes[15]. In other studies, only acute and subacute patients have shown functional improvements after intrathecal delivery[19, 21, 22, 25].

However, Deda *et al.* reported mild functional improvements in 9 chronic patients (AIS A) following direct MCP transplantation into multiple areas of the spinal cord[16]. It is noteworthy that these neurological improvements were reported in chronic patients, but a control group essential for evaluating the effectiveness of scar removal was absent[16, 17]. Importantly, after a freeze-thaw cycle, these cells are still able to promote functional recovery[16].

The reported rates of neurological improvement vary greatly. Furthermore, it is difficult to determine whether the small effect is a direct result of the cell-mediated therapy or the aggressive physical therapy program that was simultaneously performed[21]. Sykova *et al.* transplanted BMMSCs intravenously or intra-arterially into 13 chronic patients with complete SCI[14]. Without an aggressive surgical procedure, the improvements in neurologically stable chronic patients are mainly attributed to the effects of cell implantation.

Adipose Tissue-derived Mesenchymal Stem Cells and Umbilical Cord Mesenchymal Stem Cells

As each gram of adipose tissue contains 100,000 MSCs[26], and donor age has little influence on the differential capacity of adipose tissue-derived mesenchymal stem cells (AdMSCs)[27], adipose tissue is a suitable cell source for tissue engineering and regenerative therapy. The isolation of adult stem cells is accessible and reliable. Ra *et al.* applied AdMSCs intravenously to 8 chronic male patients (AIS A-B) suffering SCI for >12 months[27], and no serious adversity related to the transplantation was reported by any patient.

The human umbilical cord mesenchymal stem cell (UC-MSC) is another promising source of stem cells for its property of uniquely prodigious expansion *in vitro*, rapid proliferation, and low immunogenicity[28, 29]. In a clinical

trial by Liu *et al.*[30], UC-MSCs were injected intrathecally into 22 patients with SCI, for 1–3 courses (1×10^6 cells/kg body weight once a week for four weeks as a course), with an average time from injury to participation of 56 months (range, 2–204 months). The treatment was effective in 81.25% of patients with incomplete SCI, but ineffective in all 6 patients with complete SCI.

It is noteworthy that there is no detailed description of rehabilitation therapy in these reported MSCs trials other than "both the groups were given supervised physiotherapy, and it continued throughout the study period"[22]. Physical rehabilitation programs, which have proved their value in the functional recovery of SCI victims, should be described[31].

Neural Stem Cell Trials

Although embryonic stem cells (ESCs) have wide perspectives for clinical application in various kinds of diseases, only one single clinical trial of ESC-derived oligodendrocyte progenitor cell transplantation has been initiated; and that to determine safety and efficacy[32, 33]. In July 2010, the first trial of transplantation therapy for SCI patients finally received approval from the US Food and Drug Administration (FDA). Geron Inc. initiated a phase I trial for patients suffering from subacute complete thoracic spinal cord trauma (AIS A). In late 2011, the company announced cessation of this trial for lack of funding and discouragingly burdensome regulatory procedures. The company reported no serious adverse events.

The preclinical data of fetal human brain-derived stem cells promoted the phase I/II clinical trial of StemCells Inc. in July 2011[34, 35]. In that study, cell grafts were directly transplanted into the injury sites of 12 chronic thoracic SCI patients, with 12-month follow-up for safety and potential improvement. At the end of the study, an individual 4-year observational trial was initiated, and to date no complications have been reported.

In January 2013, Neuralstem Inc. announced that a phase I safety trial of NSCs (NSI-566RSC) in chronic SCI patients received approval from the FDA. NSI-566RSC, the lead cell therapy material of this company, is cultured human fetal spinal cord NSCs. Well-designed experimental studies have demonstrated the survival, migration, neuronal differentiation, and motor circuit integration of these promising cells in rat SCI models[36-38]. In addition to the preclinical data, the safety of cell administration has been demonstrated in an amyotrophic lateral sclerosis clinical trial[39]. This multicentre study recruited eight chronic thoracic SCI (T2-T12) patients (AIS A). To evaluate the safety of transplantation is the primary objective; while to assess survival of the grafts in the transplant site by magnetic resonance imaging (MRI) scan and the effectiveness of transient immunosuppression are the secondary objectives.

Trials of Other Cells

Schwann cells (SCs) are the main supportive glia in the peripheral nervous system. They were the first cells to be used in SCI animals for the potential of promoting axon regeneration in the CNS[40]. Transplantation of SCs has been extensively investigated as a therapeutic intervention in preclinical SCI studies[41]. In December 2012, the University of Miami announced that a phase I safety trial of autologous human SCs in subacute SCI patients received approval from the FDA. In a completed clinical study of SC transplantation, Saberi *et al.*[42] injected SCs harvested from the sural nerve into multiple locations of the traumatized spinal cord in 33 patients with complete chronic SCI (AIS A-B). During a follow-up of 2 years, considerable improvements were observed in motor function and light touch sensation, especially in the cervical injury group.

Olfactory ensheathing cells (OECs) are specialized glia surrounding olfactory nerve fascicles. OECs can be obtained from either biopsy of the olfactory mucosa or cultured from aborted fetal olfactory bulbs. Mackay-Sim *et al.* treated six chronic SCI patients with cultured autologous OECs obtained by biopsy 4–10 weeks before treatment[43]. Safety was demonstrated, but no significant functional benefit was found after transplantation. Lima *et al.* transplanted small pieces of olfactory mucosa into 20 patients with chronic traumatic SCI (AIS A-B)[44], and found that the lesion site was filled in all patients, with no neoplastic growth or syringomyelia on MRI. Huang *et al.* implanted fetal olfactory bulbs (3–4 months gestation) above and below the injured spinal cord site in 656 patients with chronic SCI[45]. The follow-up MRI did not reveal any new changes in the spinal cord parenchyma.

Macrophages can generate neurotrophic factors and block inhibitors in the peripheral nervous system. Knoller *et al.* initiated a phase I study with eight participants

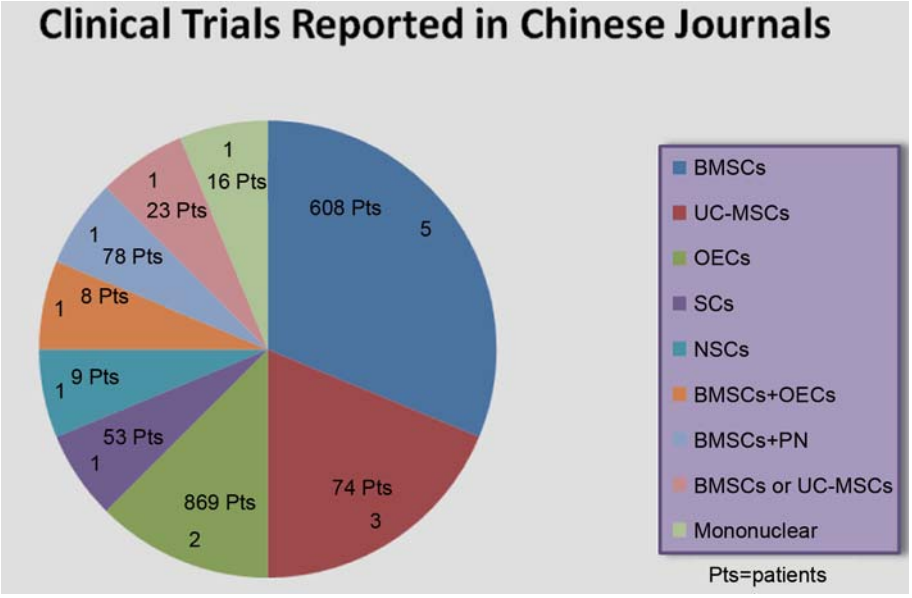

Fig. 3. Clinical trials of various grafts reported in Chinese journals. Number of trials and treated patients.

using direct injection of autologous macrophages into the spinal cord within 14 days after SCI[46]. Mild functional improvements without any critical adverse effects were found in three patients.

Current Status of Clinical Studies in China

Sixteen independent clinical trials have been reported in China, using BMMSCs, UC-MSCs, OECs, bone marrow-derived NSCs, SCs and mononuclear cells[45, 47-61]. More than 1700 SCI patients have received cell-mediated transplantation therapy. However, almost all studies were reported in Chinese journals (Fig. 3).

Wang *et al.* initiated the earliest trial in 420 chronic SCI patients (42 complete and 378 incomplete) in 2003[50], which was also the largest of the eight BMMSC studies[47-54]. The cells were transplanted into all patients through multiple routes including direct parenchymal, intrathecal, and intravenous. The dosage of a single injection was 2×10^2–3×10^2/kg body weight. Incomplete SCI patients exhibited significant functional recovery, but no improvement was observed in the complete group; and no severe adverse effects were reported but several patients developed temporary headache and low fever.

Dai *et al.* treated 23 chronic SCI patients with either 1×10^7 BMMSCs (n = 15) or UC-MSCs (n = 8) *via* lumbar puncture. Incomplete injury patients benefited more from the therapy[49]. The BMMSC group showed more motor function improvement than the UC-MSC group 3 months after transplantation.

Transplantation of autologous BMMSCs combined with peripheral nerve was initiated by Li *et al.* in 2003[52]. Autologous sural nerve was cut into cauda equina-like tissues, which were longitudinally transplanted into the spinal cord or intramedullary cysts. All of the 78 patients were discharged smoothly except for 1 with serious combined injury death with no autopsy. All 77 patients were improved, and no obvious adverse event was found.

Huang *et al.* initiated the first and largest trial of OECs in 2001[45]. They injected 1×10^6 cells directly into the injury site in each of the 656 chronic SCI patients. Two patients died of hypertension and cerebral hemorrhage, and severe pulmonary infection (1.5 and 1 month after operation). There were no postmortem examinations. Cerebrospinal fluid leakage occurred in 38 patients, and 8 suffered varying degrees of functional decline.

Cui *et al.* assessed the short-term curative effect and safety of autologous bone marrow mononuclear cell transplantation in 16 patients with SCI using intravenous or intrathecal delivery[60]. There was no significant functional

improvement. The adverse effects, including headache, abdominal distension, and meningeal irritation, were found in the intrathecal group.

Challenges

Negative Outcomes of Clinical Trials

In the reported completed trials and those in progress, overall complications were rare, with no incidence of death due to transplantation therapy. The reports of specific complication details were variable throughout the published literature, and no adverse events were noted in those studies[14, 25, 62].

The complications seem to be related to the aggressive procedure and application routes of cell-mediated therapy[14, 20]. For example, the most common complications of intrathecal injection, the most frequently used method of transplantation, are headache and neuropathic pain[21, 63].

An additional concern by Kishk *et al.* is the development of neuropathic pain after this therapy, perhaps due to the recovery and formation of neuronal circuitry[21]. Neuroplasticity is the foundation of recovery after SCI, but this contributes to neuropathology at the same time[64]. The negative effects of neuroplasticity vary, depending on conditions. Treatment strategies aiming at increasing neurotrophins in the spinal cord powerfully promote axon growth. However, this effect appears to be most highly related to the negative aspects of neuroplasticity. Treatments or conditions with the objective of mitigating growth inhibition, lead to less incidence of pain due to the moderate side effects. Finally, because of the least pain incidence, neuroprotection focusing on sparing tracts of spinal cord and limiting stimulation by the deafferentation may be the best strategy. Neuroprotection *via* the secretion of various neurotrophic factors is thought to be the major role of MSCs in SCI transplantation therapy. Thus, compared to NSCs and other pluripotent stem cells, MSCs seem to contribute less to complications and adverse effects.

Overall, these cell-mediated therapies are well-tolerated. However, the incidence of adverse events has been shown to correlate with the utilization of independent auditors and predefined definitions of complications[65-68], neither of which was noted in any of the reported series. As a result, it is suspected that the published studies under-report the true incidence of adverse events with these procedures. On the other hand, to mitigate the potential influence of these variables and to understand the incidence with which they occur, further basic investigations and randomized controlled studies are necessary.

Selection of Target Population

To ensure proper conduct of clinical trials in SCI, guidelines from the International Campaign for Cures of SCI Paralysis were published in 2007[69-72]. Nevertheless, the reported clinical trials only partially meet or totally ignore the guidelines even after 2007. The inclusion criteria of staging, severity, and segmenting of SCIs are variable and disputable among the clinical trials reviewed here.

It is unclear whether transplantation should be restricted to a certain stage of injury in future SCI treatment, but there is a suggestion of an optimal temporal window and novel reasonable staging for cell-mediated therapy. Briefly, the acute stage, during which patients are at a high risk of developing complications, would be expected to last until the end-point of spinal shock. The definition of the subacute stage would be the stationary phase of physical status, during which the bodily functions impaired by serious trauma will have been well managed. This period could be prolonged to half a year or even longer. The stability of neurological function should be confirmed by another 6-month observation. It might then be presumed that the SCI patient has entered the chronic stage while there is no confirmed indication of dysfunctional change.

The cell transplantation therapies for SCI patients mainly focus on the cervical, thoracic, and cervicothoracic segments. The neurological recovery potential varies after an acute traumatic SCI; patients with cervical injuries tend to have a greater likelihood of motor improvement than those with thoracic injuries[62, 73]. Currently, there are still no persuasive data to compare the outcomes between cervical and thoracic SCI, but attention should be paid to this lacuna while establishing the inclusion criteria.

Patients from the chronic AIS-A population have limited risk of losing potential neurological function if transplantation therapy has any adverse or unforeseen complications. This is why they are the chief target of reported SCI clinical trials. The stable neurological status allows assessment of the clinical outcomes after transplantation. At the same

time, because of repeated operations and long-term evaluation, the patients without enough will to assume this responsibility should not be recruited into clinical trials[74]. A chronic complete SCI population with stable dysfunction and better tolerance would be suitable for a phase I clinical trial. It is important to ensure that scientific knowledge, not unreasonable expectations of treatment, drives the study[75]. In a phase II clinical trial, the group of subacute incomplete SCI participants is considered to be a better choice. The goal of this phase is to achieve the greatest benefit with the least harm.

Optimization of Transplantation Material

Many sources of cells have already been used in clinical trials of SCI, with an emphasis on stem cells. Selection of the most suitable transplantation material for therapeutic application is a great challenge of clinical design.

Different kinds of cells possess specific properties, so one cell type may be more suitable than others in a specific condition or disease. For example, MSCs seem to be suitable for multiple sclerosis, and large quantities of stem cells are required for the multi-site pathological changes. Application of relatively few cells with restricted differentiation to a specific site is valuable for SCI treatment. The characteristics of NSCs meet the selection criteria. Highly-characterized stem-cell populations, like NSI-566RSC, of which the safety and efficacy have been well defined, would be considered first for SCI therapy.

The majority of transplanted cells differentiate into certain types such as oligodendrocytes or motor neurons, while others have the capacity of unplanned differentiation, even tumorigenesis. Indeed, tumorigenesis is rarely reported in animal studies. However, the length of follow-up in these studies is short, and humans with SCI may survive much longer after cell-mediated transplantation.

The third fundamental issue for the development of cell-mediated therapies is the inherently cumbersome process. These transplantation materials have to be obtained by experienced clinicians, cultured, prepared under Good Manufacturing Practices conditions, and then further prepared immediately before the initiation of therapy. These highly time- and labor-intensive steps lead to risks of failure and expense. This sequence should be modified and the standard of manufacture conditions developed before extensive practice of any transplantation therapy begins.

Practical Issues

Several authors have stated that proof of safety and efficacy through the use of large-animal models is indispensable in the development of stem-cell transplantation therapies, an opinion shared by others engaged in similar research[75-79]. However, the requirement for large-animal models is unsettled, and some authors argue that rodent models provide sufficient preclinical evidence of treatment[80, 81]. The fact that the first pluripotent stem-cell trial approved by the FDA was based on rodent models alone, suggests that this level of preclinical evidence is acceptable. Several articles have highlighted the need for independent replication of promising discoveries before clinical research commences[76, 77]. However, lack of funding is a significant obstacle. It is hoped that collaboration between government and industry will further such projects, in which partners share the risk, burden, and opportunities of transmuting cell-mediated therapy from bench to clinic.

Difficult regulatory procedures are frequently-cited obstacles to the manipulation of stem cells; procedures believed to be needlessly cumbersome inhibit research innovation and product development. In the first pluripotent stem-cell trial for SCI, the company announced its discontinuance partly due to the cumbersome regulatory procedures[82]. Governing bodies should streamline procedures and make necessary adjustments to keep pace with scientific progress.

Perspectives

There are multiple challenges for the efficient and routine practice of transplantation therapy for neurological diseases. Identifying suitable cell populations is the most important step forward; these would be commercially available, well characterized, and ethically free for clinical use. For induced pluripotent stem cells (iPSCs), studies are moving forward very rapidly, and they are thought to be a great optional source for clinical applications in the future. More work should be done to better understand the nature of iPSCs.

In the field of neuroscience, research on cell-mediated regenerative therapy for human diseases is still at the preliminary stage. Although the desire to promote clinical trials with multiple types of stem cells for various

diseases is extremely strong, current knowledge about the mechanisms of cell-mediated regenerative therapy is poor, so the situation once the cells are introduced into the patients remains unclear. Despite all this, it is inspiring that many research groups are pooling their efforts, the consensus of which should open new perspectives for cell-mediated regenerative therapy. Strong support and adequate funding from various organizations worldwide are needed to rapidly develop new clinical trials and make remarkable achievements in the next few years.

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·Review·

Axon guidance factor netrin-1 and its receptors regulate angiogenesis after cerebral ischemia

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Neurogenesis and angiogenesis play important roles in functional recovery after ischemic stroke. When cerebral ischemia occurs, axon regeneration can compensate for the loss of apoptotic neurons in the ischemic area. The formation of new blood vessels ameliorates the local decrease in blood supply, enhancing the supply of oxygen and nutrients to newly-formed neurons. New blood vessels also act as a scaffold for the migration of neuroblasts to the infarct area after ischemic stroke. In light of this, researchers have been actively searching for methods to treat cerebral infarction. Netrins were first identified as a family of proteins that mediate axon guidance and direct axon migration during embryogenesis. Later studies have revealed other functions of this protein family. In this review, we focus on netrin-1, which has been shown to be involved in axon migration and angiogenesis, which are required for recovery after cerebral ischemia. Thus, therapies targeting netrin-1 may be useful for the treatment of ischemic stroke.

Keywords: netrin-1; angiogenesis; cerebral ischemia; neuronal recovery

Introduction

Stroke, especially acute ischemic stroke (which accounts for 87% of stroke cases), is a major cause of mortality and disability worldwide[1]. Ischemic stroke is primarily caused by blockage of blood vessels in the brain due to thrombi or cardiogenic emboli. The affected tissue loses its supply of oxygen and glucose, with immediate disturbance of function. Research regarding therapies for stroke has long focused on neuroprotective agents. However, clinical use of the only approved drug, tissue plasminogen activator, is limited due to the short time-window of administration and the potential for life-threatening hemorrhage[2]. Safer and more effective treatment strategies for stroke are urgently needed. The vasculature in the adult brain is stable under normal conditions; however, it responds to ischemia through angiogenesis, which drives the formation of new blood vessels. Angiogenesis has been identified as a potential pathway to promote the recovery of neuronal function.

Members of the netrin protein family act as bifunctional axon-guidance cues that regulate migration during neuronal development. Netrins either attract or repel pathfinding axons, depending on the identity of the receptors. Netrin-1 was the first to be purified and is the best-characterized member. In addition to its involvement in guiding axon migration during embryonic development, netrin-1 functions in organ formation[3-6], tumorigenesis[7], inflammation[8, 9], and anti-apoptotis[10], as well as being a potential biomarker for renal injury and certain cancers[11, 12]. It also promotes the recovery of neuronal function after cerebral ischemia in animal models[13-16]. Importantly, a growing number of studies have focused on elucidating the effects of netrin-1 on angiogenesis. In this review, we discuss the implications of the actions of netrin-1 in angiogenesis after cerebral ischemia.

Structures and Functions of Netrin-1 and Its Receptors

Netrins are laminin-related proteins with highly-conserved structures. The N-terminus is composed of two domains that are similar to laminin domains V and VI. The domain V-like region contains three epidermal growth factor (EGF)-like repeats. The C-terminal domain contains binding sites for membrane glycolipids and extracellular matrix components, such as heparin sulfate proteoglycans, integrin α3β1, and integrin α6β4[17, 18]. Three secreted netrins (netrin-1, -3, and -4) and two glycosylphosphatidylinositol (GPI)-anchored membrane-bound netrins (netrin-G1 and -G2) have been identified in mammals[19, 20]. In netrin-1, -3, -G1, and -G2, the N-terminus is homologous to the laminin γ chain; in netrin-4 (also called β-netrin), the N-terminus is more similar to the laminin β chain[17] (Fig. 1A and B).

Netrin receptors mainly include members of the DCC (deleted in colorectal cancer) family (including neogenin), the UNC5 (uncoordinated 5) protein family, and DSCAM (Down syndrome cell-adhesion molecule). All of these transmembrane receptors belong to the immunoglobin (Ig) superfamily (Fig. 1C)[19]. DCC mediates axon attraction, whereas UNC5 homodimers and UNC5-DCC heterodimers mediate axon repulsion[17]. The extracellular portion of DCC family members contains four Ig and six fibronectin type 3 (FNIII) domains, and the intracellular region consists of three highly-conserved domains (P1, P2, and P3) that play important roles in intracellular signal transduction. The extracellular region of UNC5 contains two Ig domains, followed by two thrombospondin (TSP) type-I modules. Its intracellular region contains a ZU5 domain of undetermined function, a DCC-binding site, and a death domain that is associated with apoptotic signaling. In vertebrates, the UNC5 family is composed of four members, UNC5A, B, C, and D[20]. Of these, UNC5B, expressed during early blood-vessel formation, is the most important and is implicated in netrin-1-regulated angiogenesis[21]. It is expressed in the semicircular canals and retina, as well as in the epiphysis, thalamus, and placenta[22-24]. UNC5B has been reported to act as a pro- or anti-angiogenic receptor in different studies[25-28]. DSCAM is a type I transmembrane protein that contains 10 Ig domains and six FNIII repeats in its extracellular domain[19, 20]. In addition, the membrane-associated, G-protein-coupled adenosine A2b receptor functions as a netrin-1 receptor. However, it is unclear whether the A2b receptor is involved in netrin-1-mediated axon-guidance signaling[29-31]. In epithelial cells, binding of the netrin-1 C-terminus with integrins α3β1 and α6β4 provides cues for axon adhesion and migration[32].

When combined with different receptors, netrins provide chemotropic guidance cues. Receptors for secreted netrins include DCC, neogenin, UNC5, and DSCAM. GPI-anchored membrane proteins bind the netrin G ligands NGL-1 and NGL-2[20].

Netrin-1 is currently the best-studied of the netrins. Initially, the UNC-6 protein was identified in *Caenorhabditis elegans* as a guide for cell and axon migration[33]. Thereafter, a homolog of UNC-6, netrin-1, was purified from embryonic chick brain. The root 'netr' originates from a Sanskrit word meaning 'one who guides'[34]. Netrin-1 is expressed in the developing and mature nervous systems, including the spinal cord, cerebellum, visual system, olfactory system, substantia nigra, corpus striatum, ganglionic eminence, and internal capsule[35-37]. It is also expressed in the lung, pancreas, placenta, and mammary gland[24, 38-40]. Netrin-1 acts as a chemoattractant or chemorepellent for migrating cells and axons in the developing central nervous system. It also plays a crucial role in the survival of neurons expressing UNC5 and DCC[41]. Netrin-1 is important in oligodendrocyte development[42]. Netrin-1 silencing in mice is associated with developmental disorders of the spinal cord, corpus callosum, and hippocampus, leading to lethal developmental defects of the nervous system[43].

Netrin-1 and Angiogenesis

Axon guidance factors primarily belong to one of the four receptor/ligand families[44-46]: the roundabout (Robo) receptors bind Slit ligands; neuropilins bind semaphorins or vascular endothelial growth factor (VEGF); Ephs bind ephrins; and UNC5 and DCC bind netrins. These receptors are expressed on neurons and endothelial cells (ECs). They regulate neuronal and vascular development, as well as tumor angiogenesis, by binding to their corresponding ligands[47].

The formation of new blood vessels occurs by vasculogenesis, angiogenesis, and arteriogenesis[48]. The

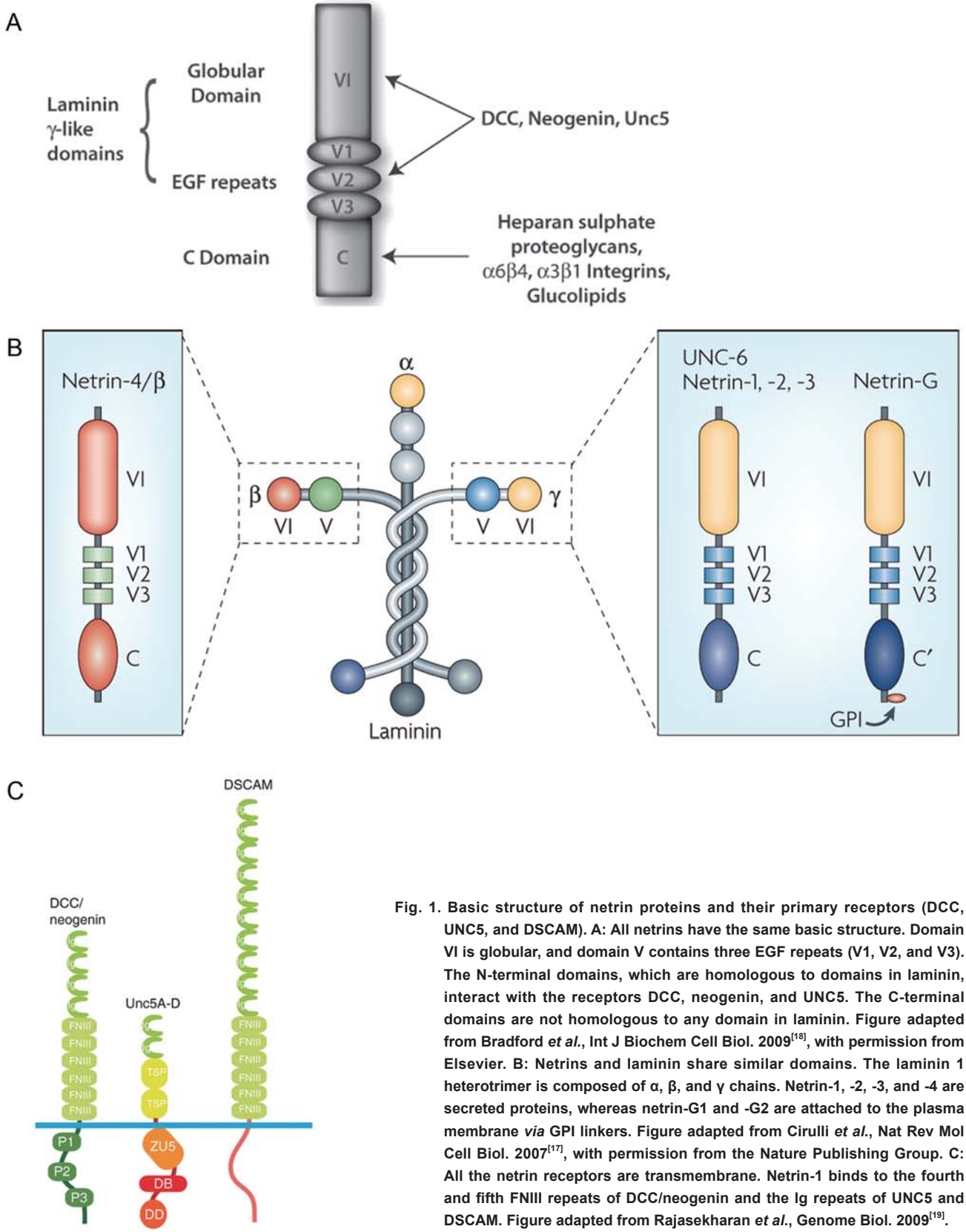

Fig. 1. Basic structure of netrin proteins and their primary receptors (DCC, UNC5, and DSCAM). A: All netrins have the same basic structure. Domain VI is globular, and domain V contains three EGF repeats (V1, V2, and V3). The N-terminal domains, which are homologous to domains in laminin, interact with the receptors DCC, neogenin, and UNC5. The C-terminal domains are not homologous to any domain in laminin. Figure adapted from Bradford *et al.*, Int J Biochem Cell Biol. 2009[18], with permission from Elsevier. B: Netrins and laminin share similar domains. The laminin 1 heterotrimer is composed of α, β, and γ chains. Netrin-1, -2, -3, and -4 are secreted proteins, whereas netrin-G1 and -G2 are attached to the plasma membrane *via* GPI linkers. Figure adapted from Cirulli *et al.*, Nat Rev Mol Cell Biol. 2007[17], with permission from the Nature Publishing Group. C: All the netrin receptors are transmembrane. Netrin-1 binds to the fourth and fifth FNIII repeats of DCC/neogenin and the Ig repeats of UNC5 and DSCAM. Figure adapted from Rajasekharan *et al.*, Genome Biol. 2009[19].

timing of vasculogenesis is during embryonic development, when the endothelial precursors, or angioblasts, assemble and differentiate into ECs to form the vascular plexus. Angiogenesis is the subsequent sprouting of new capillaries from pre-existing blood vessels. Arteriogenesis is the process of stabilizing the new vessels, and involves surrounding the vessels with pericytes and vascular smooth muscle cells, as well as remodeling *via* increased blood flow[48, 49]. Blood vessels in adults are generally in a quiescent state. New vessel formation primarily occurs under pathological conditions through angiogenesis. Research may provide new targets for the treatment of diseases associated with excessive angiogenesis, such as cancer and retinopathy, as well as diseases associated with insufficient angiogenesis, such as coronary heart disease and ischemic stroke. Angiogenesis involves degradation of the extracellular matrix by proteolytic enzymes, and the proliferation, differentiation, and migration of ECs[50, 51]. ECs maintain high plasticity and extend filopodia after stimulation by angiogenic signals[49]. Apoptosis of ECs impedes vascular regeneration; thus, the inhibition of apoptosis is an important mechanism for EC survival and a key step in angiogenesis[50]. According to Castets *et al*.[25], netrin-1 is a survival factor for ECs. When it binds to UNC5B, the action of the downstream apoptotic signal serine/threonine kinase DAPK (death-associated protein kinase) is inhibited, and then the UNC5B-dependent EC apoptosis is blocked. In contrast, EC apoptosis is induced when netrin-1 is not bound to UNC5B. In zebrafish, vascular sprouting defects induced by netrin-1 silencing are reversed by inhibition of caspase activity, UNC5B silencing, or DAPK silencing[25]. Therefore, it can be inferred that netrin-1 promotes angiogenesis by inhibiting EC apoptosis.

Park *et al*.[52] demonstrated that netrin-1 is involved in the development of the nervous and vascular systems during mouse embryonic development. Mice lacking netrin-1 exhibit blood-vessel defects, suggesting that netrin-1 facilitates vascular development. Another study showed that UNC5B deficiency results in placental arteriole dysplasia, leading to embryonic death[26]. Therefore, the receptor for netrin-1, UNC5B, is essential for the process of placental vascular formation.

Importantly, netrin-1 mediates angiogenesis by promoting adhesion between ECs and vascular smooth muscle cells. Netrin-1 enhances the reaction of vessels with VEGF, thereby stimulating angiogenesis. Therefore, some researchers have proposed that netrin-1 is also a pro-angiogenic factor[52]. Highly-purified netrin-1 stimulates EC proliferation, migration, and tube-formation *in vitro*, with non-significant expression levels of UNC5B, DCC, and neogenin, suggesting that the process does not depend on netrin-1 receptors[53]. These findings indicate that netrin-1 is a pro-angiogenic factor that can act independently of its receptors.

Consistently, Fan *et al*.[54] showed that netrin-1 stimulates the proliferation and migration of human cerebral ECs. In the adult brain, netrin-1 hyperstimulation facilitates focal angiogenesis. Newly-formed vessels induced by netrin-1 contain an intact EC monolayer surrounded by multiple cell layers. Nguyen *et al*.[55] demonstrated that netrin-1 induces angiogenesis by increasing endothelial nitric oxide production *via* a DCC-dependent ERK1/2-eNOS feed-forward mechanism in aortic ECs. All these studies suggest that netrin-1 is a pro-angiogenic factor, albeit with some discrepancies (Table 1).

However, in some cases, netrin-1 has also been found to block angiogenesis. For example, Lu *et al*.[27] reported that netrin-1 reduces endothelial migration and filopodial extension, and this is mediated by signaling through UNC5B. UNC5B is a repulsive netrin-1 receptor expressed by endothelial tip cells of the vascular system during mouse embryonic development. Disruption of the *Unc5b* gene in mice leads to excessive vessel branching, confirming that netrin-1 inhibits angiogenesis *via* the UNC5B receptor. Likewise, Larrivee *et al*.[28] demonstrated that deletion of the *Unc5b* gene ameliorates the netrin-1-mediated inhibition of angiogenesis. Netrin-1 repels ECs when UNC5B is expressed, with angiogenesis being suppressed throughout this process. Both studies imply that netrin-1 functions as an anti-angiogenic factor when acting in concert with UNC5B (Table 1).

In conclusion, it remains controversial whether netrin-1 promotes or inhibits angiogenesis. The precise role may depend on experimental conditions, animal models, and types of ECs or vessels examined. Besides, it is unclear which netrin-1 receptors mediate specific functions. According to Yang *et al*.[56], the concentration of netrin-1 affects its impact on vessels: low doses appear to promote

Table 1. Effects of netrin-1 and its receptors on angiogenesis

Effects	Receptors involved
Pro-angiogenic effect	
Inhibit EC apoptosis[25]	UNC5B
Stimulate EC Proliferation, migration, and TF[53]	Independent of UNC5B, DCC, neogenin/--
Increase endothelial NO production[55]	DCC
Promote vascular development[52]	UNC5B/-----
Facilitate focal angiogenesis in adult[54]	------
Anti-angiogenic effect	
Block EC migration[27]	UNC5B
Repel EC[28]	UNC5B
Dual function	------

EC, endothelial cell; NO, nitric oxide; TF, tube formation; -----, unknown.

angiogenesis, whereas higher doses inhibit it *in vitro*. Taken together, the purity and concentration of netrin-1, and the specific type of receptor expressed in a given cell type, may be important for defining the role of this protein in angiogenesis.

Angiogenesis after Cerebral Ischemia

The process of angiogenesis—the sprouting of new capillaries from pre-existing blood vessels—participates in neuronal recovery after ischemic stroke. In the early 1990s, Krupinski *et al.*[57] analyzed brain tissues from 10 patients, and found that the number of microvessels, particularly in the penumbra, is significantly increased after cerebral ischemic stroke. The microvessel density correlates with the long-term survival of patients. Sbarbati *et al.*[58] found that microvessels form 2 weeks after middle cerebral artery occlusion (MCAO) in rats, implying that microvessel formation mediates the recovery of blood flow and helps to compensate for the collateral circulation after permanent MCAO.

Interruption of cerebral blood flow rapidly triggers the transcription of angiogenesis-related genes. Using cDNA analysis, Hayashi *et al.*[59] examined the expression levels of 96 angiogenesis-related genes after transient MCAO (tMCAO) in mice, and found that 42, 29, and 13 genes are upregulated at 1 h, 1 day, and 21 days, respectively, after transient occlusion. Beck *et al.*[60] found that the mRNA expression of angiogenin-2, which promotes angiogenesis, is upregulated after 6 h of occlusion in MCAO rats. These changes lead to the synthesis of pro-angiogenic proteins such as VEGF. The expression levels of both VEGF and endostatin increase after cerebral ischemia, although they play opposite roles in angiogenesis[61]. These studies suggest that pro- and anti-angiogenic factors work together to regulate angiogenesis.

Pro-angiogenic factors promote EC proliferation. Initially, microvessels form in the boundary zone of the ischemic region, increasing the supply of oxygen and nutrients. Gradually, the newly-formed vessels grow and increase the cerebral blood volume. However, whether angiogenesis leads to the formation of an intact and functional vessel network in the ischemic zone after stroke needs to be determined[62].

With regard to the role of angiogenesis after cerebral ischemia, Yu *et al.*[63] proposed that the major function of ischemia-stimulated vessel formation is to eliminate necrotic tissue and debris *via* macrophages. Wei *et al.*[64] demonstrated that angiogenesis helps to restore blood flow in the ischemic zone by interacting with the arteriole collateral circulation established after cerebral ischemia, thereby improving the long-term recovery of neurological function in rats. Angiogenesis after ischemic stroke protects damaged tissue by supplying nutrition for neuronal remodeling and improving the metabolism of surviving neurons.

In addition, mounting evidence indicates that the vasculature acts as a scaffold for neuroblasts in the subventricular zone (SVZ) to migrate to the infarct area after ischemic stroke[65-67]. SVZ-derived neuroblasts assemble around blood vessels adjacent to the infarct area, migrate along the vessels, and finally reach the infarct zone. The ECs secrete stromal-derived factor-1α (SDF-1α) to attract neuroblasts expressing CXCR4, the receptor for SDF-1α. These neuroblasts gradually differentiate into mature neurons, and new blood vessels supply nutrition to the newly-developed neurons[68]. Migrating neuroblasts pass through areas of vascular remodeling and regeneration, suggesting that angiogenesis assists neurogenesis, and that both processes promote functional recovery after ischemic stroke. Taken together, angiogenesis helps to restore neuronal function after ischemic stroke, which may offer useful insights into new treatment approaches for the condition[62, 68, 69].

Netrin-1 Promotes Angiogenesis after Cerebral Ischemia

The expression levels of netrin-1 and its receptors change after injury to the nervous system. In rats subjected to MCAO, netrin-1 and UNC5B are expressed in neuronal perikarya in the peri-infarct area, and DCC is expressed in perivascular astrocytes[70]. In a similar study, netrin-1 was reported to be expressed in neurons in the peri-infarct region, DCC in neurons and astrocytes, and neogenin in ECs in the infarct area[71]. The expression levels of netrin-1 and its receptors peak at 14 days after MCAO. Because netrin-1 promotes axon migration, the spatial and temporal similarities between the netrin-1 and DCC expression indicate that they are simultaneously involved in axon migration.

Neogenin may also play a role in angiogenesis. The temporal overlap of netrin-1 and neogenin expression implies that axon regeneration and angiogenesis occur concomitantly. Neurons and vessels share similar signaling pathways that mediate differentiation, maturity, and migration. They also have similar parallel branching structures[72]. Therefore, neurogenesis and angiogenesis may influence each other after cerebral ischemia.

Netrin-1 is involved in the functional recovery of neurons after cerebral ischemia. Bayat *et al.*[13] found that hippocampal administration of exogenous netrin-1 significantly improves spatial memory and enhances synaptic plasticity in a dose-dependent manner 24 h after global cerebral ischemia secondary to cardiac arrest in rats. Wu *et al.*[14] reported that the presence of netrin-1 decreases the infarct size and the number of apoptotic neurons in MCAO mice, suggesting that it has protective effects after cerebral ischemia. Liu *et al.*[70] showed that netrin-1 and its receptors may be involved in remodeling the peri-infarct neuronal circuitry after treadmill exercise, suggesting that exercise encourages neuronal survival in the infarct region by regulating the netrin-1/UNC5B signaling pathways.

Lu *et al.*[15] constructed an adeno-associated viral netrin-1 vector (AAV-NT-1), and delivered it into mouse brain after tMCAO. Netrin-1 expression increased and neurobehavioral outcomes significantly improved at 7 days after tMCAO in the mice with AAV-NT-1 transduction compared to controls. They therefore proposed that netrin-1 promotes functional recovery after cerebral ischemia, and that netrin-1 gene transfer could be used to treat cerebral ischemic diseases.

Our research group has been actively investigating the role of netrin-1 after cerebral ischemia. Recently, we demonstrated that the administration of exogenous netrin-1 protects neurons by attenuating secondary apoptosis in the ventroposterior thalamic nucleus (VPN) ipsilateral to a focal cerebral infarction in rats. This process may depend on the UNC5H2 receptor. Insufficient expression of endogenous netrin-1 may cause secondary damage in the VPN after ischemic stroke[16].

Other recent studies have explored the effects of netrin-1 overexpression using the AAV-NT-1 vector. Sun *et al.*[73] reported that netrin-1 overexpression notably increases the peri-infarct vessel density and promotes the migration of immature neurons to the infarct territory. Netrin-1 overexpression also assists in the recovery of motor function after cerebral infarction in rats. Lu *et al.*[74] found that netrin-1 overexpression contributes to functional recovery by diminishing the infarct size and promoting angiogenesis after mouse tMCAO.

Therefore, netrin-1 appears to play an important role in functional recovery. Although the underlying mechanism

remains unclear, the current evidence suggests that netrin-1-mediated angiogenesis is one such mechanism. Future studies are needed to clarify its role in promoting angiogenesis after cerebral ischemia.

Prospects

Ischemic stroke has high mortality and disability, and numerous studies have attempted to find effective therapies for this condition. Netrin-1 promotes axon migration and regeneration, inhibits neuronal apoptosis, and facilitates angiogenesis in the infarct area, increasing the blood supply to ischemic tissues and improving the prognosis. Current studies of netrin-1 have focused on the cellular and animal levels, and its role in the promotion or inhibition of angiogenesis remains controversial. Thus, future studies addressing the seemingly contradictory pro-angiogenic and anti-angiogenic effects of netrin-1 are sorely needed. Nevertheless, netrin-1 may be a potential therapeutic target for the promotion of neuronal recovery following ischemic stroke. To date, the functions of other members of the netrin protein family have not been fully elucidated, and therefore are critically in need of investigation.

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·Review·

Carboxypeptidase E (NF-α1): a new trophic factor in neuroprotection

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Carboxypeptidase E (CPE) is a prohormone-processing enzyme and sorting receptor that functions intracellularly. However, recent studies have demonstrated that CPE acts as a trophic factor extracellularly to up-regulate the expression of a pro-survival gene. This mini-review summarizes the roles of CPE in neuroprotection and the implications for neurodegenerative diseases.

Keywords: carboxypeptidase E; NF-α1; neuroprotection; stress; neurodegenerative disease

Introduction

Carboxypeptidase E (CPE) was first identified as a prohormone-processing enzyme[1, 2] that cleaves the C-terminally-extended basic residues (arginine and/or lysine) from peptide intermediates to produce bioactive neuropeptides and peptide hormones[3]. Since then, CPE has been found to possess various non-enzymatic activities. It is a regulated secretory pathway (RSP) sorting receptor[4] and targets pro-BDNF (brain-derived neurotrophic factor) to the regulated secretory pathway[5], but not nerve growth factor (NGF)[6]. Moreover, the cytoplasmic tail of CPE drives bi-directional transport of BDNF vesicles and its secretion in hippocampal neurons[7] and mediates the localization of synaptic vesicles to the pre-active zone in hypothalamic neurons[8]. Recent studies suggest that CPE is a new trophic factor that functions independently of its enzymatic activity. Here, we review the role of CPE in neuroprotection and the implications for neurodegenerative diseases.

CPE Expression in Brain Is Modulated by Stress

The first evidence that CPE functions as a neuroprotective protein came from correlative studies showing that it is up-regulated after stress. In the hippocampal CA1 and CA3 regions and in the cortex, increased levels of CPE mRNA and protein occur after 15 min of transient global ischemia followed by 8 h of reperfusion[9]. Moreover, Zhou *et al.* showed that in mice lacking an active CPE protease, a sublethal episode of focal cerebral ischemia results in abundant TUNEL-positive cells in the ischemic cortex, in contrast to only a few in the ischemic cortex of wild-type mice, suggesting that neurons are more susceptible to cell death in the absence of CPE[10]. CPE gene expression is up-regulated in the amygdala of rats exposed to cat odor, a stressor that induces anxiety-like behavior[11]. Also, CPE protein and mRNA are significantly elevated in the mouse CA3 region after mild chronic restraint stress[12]. This form of stress in mice also results in elevation of the pro-survival Bcl-2 protein/mRNA and p-AKT levels in the hippocampus, while CPE knockout (KO) mice[13] show a decrease[12]. Thus the up-regulation of CPE during stress contributes to neuronal survival. In contrast to the increased CPE, the offspring of pregnant ewes subjected to aversive interactions with human handlers show a decrease in CPE concomitant with abnormal dendritic spine density and morphogenesis in the prefrontal cortex and hippocampus[14]. This is consistent with a report showing that CPE KO mice exhibit abnormal dendritic arborization and spine morphology in these areas[15], demonstrating that

CPE plays a role in normal cytoarchitecture and neuronal function in these brain regions. Supporting evidence came from the finding that CPE is a binding partner for nitric oxide synthase 1 adaptor protein, a protein involved in the regulation of dendritic patterning in hippocampal neurons[16].

CPE Knock-out Mice Have Neurological Deficits

Since CPE has been implicated in neuroprotection, one would expect that a lack of CPE in the brain would result in neurodegeneration and behavioral abnormalities. Indeed, CPE KO mice display memory deficits as revealed by the Morris water maze, object preference, and social transmission of food preference[17], and show no evoked long-term potentiation (which is required for memory and learning) in hippocampal slices. Neonatal CPE KO mice also exhibit a significant delay in eye opening, which reflects a developmental delay in the central nervous system (Cawley *et al*., unpublished data). In addition, *Cpe*$^{fat/fat}$ mutant mice lacking CPE exhibit anxiety- and/or depression-like behaviors[18]. CPE KO mice at 4 weeks of age or older, but not at 3 weeks, exhibit marked degeneration of the CA3 region which normally expresses high levels of CPE[17]. The neurodegeneration in CPE KO mice was initially thought to be a developmental defect. However, a recent study[19] suggests that this is due to weaning, because the hippocampus is intact in 4-week-old CPE KO mice that have not yet weaned, but weaning of CPE KO mice at 2 or 3 weeks of age, which involves maternal separation (emotional stress) and ear-tagging and tail-snipping for genotyping (physical stress), each results in degeneration of the CA3 neurons by 3 and 4 weeks. Interestingly, daily treatment with carbamazepine, an antiepileptic agent, in 2-week-old CPE KO mice for 2 weeks prevents the neurodegeneration, despite the weaning process at 3 weeks[19]. Therefore, emotional and physical stress in early life lowers the seizure threshold and exacerbates the degeneration of susceptible neurons in the CA3 region in the absence of the neuroprotective protein, CPE.

CPE Acts as a Trophic Factor to Promote Neuronal Survival

The animal model studies discussed above along with *ex vivo* studies suggest that CPE is a neuroprotective protein. For example, primary cultured hippocampal neurons from CPE KO mice are more prone to die in culture than those from wild-type littermates[20]. Also, low-potassium-induced apoptosis is significantly increased in CPE$^{+/-}$ cerebellar granule neurons (CGNs) in comparison to CPE$^{+/+}$ CGNs, indicating that CPE plays a neuroprotective role in this type of neuron as well as hippocampal neurons[21]. More direct evidence came from a study showing that transduction of an adenovirus carrying CPE into primary cultured hippocampal neurons, causing over-expression of CPE, protects against hydrogen peroxide-induced neurotoxicity[17]. Although the mechanism was unknown, CPE was assumed to function intracellularly to process some precursor protein that had neuroprotective activity. However, a recent study suggests that CPE acts extracellularly as a neuroprotective trophic factor, independent of its enzymatic activity. Extracellular CPE functions by signaling through the ERK and Akt pathways to up-regulate the expression of the pro-survival protein Bcl-2 and inhibit caspase-3 activation, indicating that it confers neuroprotection against cell death by modulating mitochondrial energetics[20]. CPE also protects hippocampal neurons against cell death induced by oxidative stress and glutamate neurotoxicity. In addition, it promotes the long-term survival of embryonic hippocampal neurons from CPE KO mice in culture[20]. Since the pattern of the CPE-induced activation of the ERK and Akt signaling pathways is similar to classic trophic factors such as BDNF and NGF, an alternative name was given to CPE, “Neurotrophic Factor-α1” (NF-α1), indicating its functions as a trophic factor. In addition to primary cultured neurons, a recent study further found that secreted NF-α1 protects PC12 cells, a pheochromocytoma cell line, against starvation- and hypoxia-induced cell death[22].

CPE in Neurodegenerative Diseases

Since CPE can function as a trophic factor, it is not surprising to find that aberrations of its expression and/or distribution occur in neurodegenerative diseases. In contrast to normal human cortex where CPE is preferentially localized in dendrites and perikarya, cortices from patients with Alzheimer’s disease (AD) show a high accumulation of CPE in dystrophic neurites surrounding amyloid beta. Of note, a similar accumulation of CPE occurs in a mouse model of AD[23]. This pattern of accumulation is similar to

trophic factors and neuropeptides such as galanin. The overexpression of galanin in AD brains has been shown to promote neuronal survival[24] and exogenous galanin has neuroprotective effects in a rodent model of AD[25]. Thus we hypothesize that the accumulation of CPE in dystrophic neurites in AD is a self-defense mechanism to delay the onset and progression of AD. Interestingly, a CPE mutant named "QQ CPE", has been found in the cortex of a patient with AD[3]. Cell biological studies demonstrated that QQ CPE is synthesized but fails to be secreted when transfected into neuro2a cells, a neuroendocrine cell line. In addition, co-expression of wild-type and QQ CPE results in the degradation of both forms and a reduction in the secretion of wild-type CPE, indicating that the mutant acts in a dominant-negative manner[3]. Overexpression of QQ CPE by adenovirus transduction in rat hippocampal and cortical neurons results in increased levels of CHOP (C/EBP homologous protein), a transcription factor induced by endoplasmic reticulum stress-induced apoptosis, decreased levels of Bcl-2, and increased cytotoxicity and neuronal death[3]. Hence, neurons expressing QQ CPE may lack the neuroprotective functions of CPE and this may lead to neurodegenerative diseases, including AD, with aging. Indeed, analysis of CPE proteolytic activity in Brodmann's area 21 of normal and AD patients postmortem shows changes in the activity of both soluble and membrane forms of CPE, suggesting changes in the levels of CPE protein in the AD patients[26]. In addition, in cathepsin B and L double-knockout mice, a model of neuronal ceroid lipofuscinoses with early-onset neurodegeneration, CPE is increased >10 folds[27], presumably to compensate for the lack of the two enzymes. However, given the new finding that CPE has trophic properties, this 10-fold increase is likely to protect neurons from further degeneration. In addition, in experimental autoimmune encephalomyelitis (EAE)[28], a mouse model of multiple sclerosis, a trait locus for EAE has been mapped to the *Cpe* gene on chromosome 8, while microarray data from the inflamed spinal cord of EAE mice shows a decrease in CPE concomitant with an increase in the severity of the disease[29].

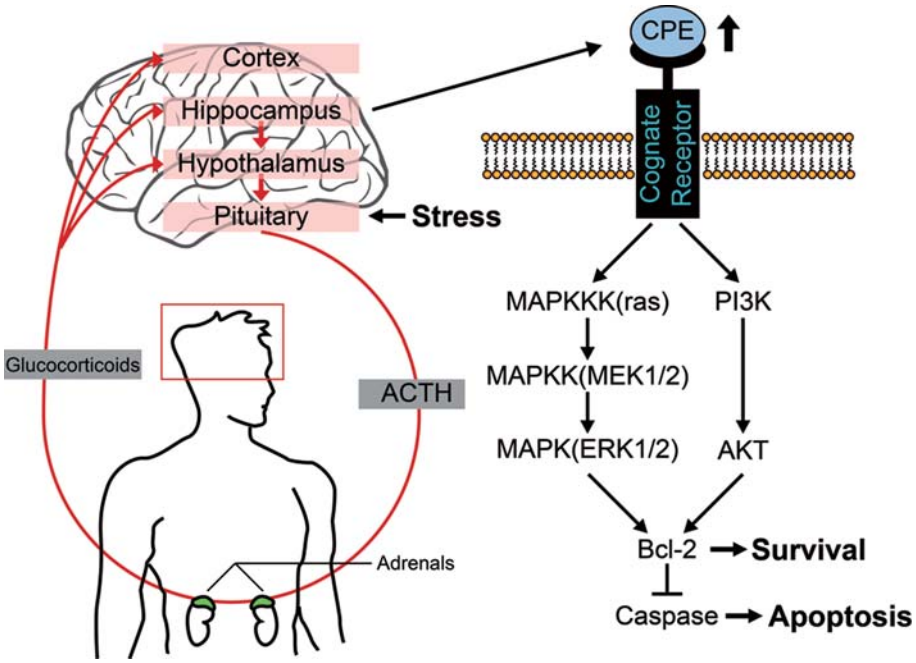

Fig. 1. Pathways for CPE-mediated neuronal survival during stress. During stress, ACTH is released into the circulation from the pituitary, which then stimulates glucocorticoid release from the adrenals. Glucocorticoids are then transported to the hippocampus which enhances the expression and secretion of CPE. CPE binds to a cognate receptor in hippocampal neurons to activate ERK and AKT signaling pathways which then mediate the upregulation of expression of Bcl-2, an anti-apoptotic protein. Bcl-2 inhibits the activation of caspases to prevent cell death and promote cell survival.

Conclusions

Here we have reviewed recent evidence that CPE is a new trophic factor and is involved in neurodegenerative diseases. CPE joins the ranks of other important neurotrophins such as BDNF and NGF, and plays pivotal roles in neuroprotection and neuronal survival during stress-induced apoptosis (Fig. 1) and neural development, given a recent study showing that CPE is highly expressed in neural stem cells[30]. Indeed, CPE is a negative regulator of proliferation in adult neural stem cells[30]. In addition, the mechanisms underlying the neuroprotection by CPE deserve further investigation, such as identifying the receptor to which it binds to activate the ERK and Akt signaling pathways (see Fig. 1). CPE could directly bind to a cognate receptor to function as a trophic factor, or act as a binding partner to activate the downstream signaling pathways. One example of the latter is that CPE forms a complex with the Wnt3a ligand and the Frizzled receptor to inhibit the wnt signaling pathway[31]. In addition, the molecular domain of CPE, which is responsible for the neuroprotective effects, needs to be explored for therapeutic use in drug design. Although many efforts have been made to understand and cure neurodegenerative diseases, successful treatment is still lacking, and the available therapies provide only symptomatic improvement. CPE is an emerging and a promising therapeutic target for neurodegenerative diseases. Encouragingly, clinical trials have shown that neurotrophic factors are potentially effective in treating AD[32]. Thus, continued investigations into the function of CPE/NF-α1 as a new trophic factor are warranted.

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·Original Article·

Voltage-dependent blockade by bupivacaine of cardiac sodium channels expressed in *Xenopus* oocytes

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ABSTRACT

Bupivacaine ranks as the most potent and efficient drug among class I local anesthetics, but its high potential for toxic reactions severely limits its clinical use. Although bupivacaine-induced toxicity is mainly caused by substantial blockade of voltage-gated sodium channels (VGSCs), how these hydrophobic molecules interact with the receptor sites to which they bind remains unclear. $Na_v1.5$ is the dominant isoform of VGSCs expressed in cardiac myocytes, and its dysfunction may be the cause of bupivacaine-triggered arrhythmia. Here, we investigated the effect of bupivacaine on $Na_v1.5$ within the clinical concentration range. The electrophysiological measurements on $Na_v1.5$ expressed in *Xenopus* oocytes showed that bupivacaine induced a voltage- and concentration-dependent blockade on the peak of I_{Na} and the half-maximal inhibitory dose was 4.51 μmol/L. Consistent with other local anesthetics, bupivacaine also induced a use-dependent blockade on $Na_v1.5$ currents. The underlying mechanisms of this blockade may contribute to the fact that bupivacaine not only dose-dependently affected the gating kinetics of $Na_v1.5$ but also accelerated the development of its open-state slow inactivation. These results extend our knowledge of the action of bupivacaine on cardiac sodium channels, and therefore contribute to the safer and more efficient clinical use of bupivacaine.

Keywords: bupivacaine; $Na_v1.5$; voltage-dependent blockade; inactivated state

INTRODUCTION

Bupivacaine is one of the aminoamide drugs belonging to the class I local anesthetics (LAs) which include lidocaine, ropivacaine, and mepivacaine. It is generally used for infiltration and nerve block, as well as epidural and intrathecal anesthesia in clinical management. Despite its long-lasting effect when provided systemically for pain relief, patients still risk adverse drug reactions with accidental intravascular injection, inadvertent intrathecal injection, or an excessive systemic dose[1]. Systemic exposure to excessive bupivacaine mainly results in epilepsy-like syndromes due to central nervous system (CNS) excitation, and arrhythmias or cardiac arrest caused by cardiovascular toxicity[2]. Although efforts have been made to develop a controllable and safer delivery/release system for bupivacaine, a better strategy to avoid the overall toxicity is needed.

It is well-established that the main action of bupivacaine involves the use-dependent blockade of voltage-gated sodium channels (VGSCs) that are responsible for action potential initiation and axonal conduction[3]. Meanwhile, it also has a significant inhibitory effect on K^+ and Ca^{2+} channels that contribute to the repolarization and modulatory shaping of action potentials[4, 5]. Therefore, it is reasonable to infer that membrane depolarization and increased neuronal

excitability associated with these ion channels may account for the systemic toxicity of bupivacaine. However, knowledge about the links between pharmacological interactions and behavioral consequences have still to be worked out.

VGSCs are transmembrane proteins consisting of an ion-conducting α-subunit and one or more auxiliary subunits[6]. Generally, the α-subunit comprises four repeated domains (DI–DIV), each containing six transmembrane α-helixes (S1–S6) and a hairpin-like pore loop lining between S5 and S6[7]. Despite the high structural similarity among VGSC isoforms, they have distinct distributions, gating properties, and functional activities[8]. To date, the bupivacaine-binding site on sodium channels has been located at the intracellular portion[9, 10]. Thereby, bupivacaine blocks Na^+ influx into neurons and prevents depolarization. However, stereoselectivity has been reported in the bupivacaine-induced blockade of the inactivated state of Na^+ channels, but not on the blockade of activated (open-state) Na^+ channels[11]. Point-directed mutagenesis of the rat skeletal muscle $Na_v1.4$ channel revealed that the binding sites of bupivacaine are located in the pore-lining transmembrane segment 6 (S6) of domains 1, 3, and 4 (D1-S6, D3-S6, D4-S6), in which residues L1280 in D3-S6 and N434 in D1-S6 interact directly with bupivacaine and face each other in the ion-conducting pore[9, 10]. Even so, given that a variety of VGSC isoforms are distributed throughout human tissues, thorough investigation of how bupivacaine interacts with other Na^+ channels is still needed.

$Na_v1.5$ is responsible for the upstroke (phase 0) of the action potential in cardiac cells. Opening of the channel leads to a rapid influx of Na^+ (I_{Na}), which depolarizes the membrane potential within tenths of a millisecond[12]. Dysfunction of $Na_v1.5$ channels leads to various arrhythmias, such as long QT syndrome, Brugada syndrome, and cardiac conduction disease (also known as Lev-Lenegre syndrome)[13-15]. In light of this, there may be a relationship between the cardiac toxicity of bupivacaine and its use-dependent blockade of Na^+ channels. But it remains uncertain whether inhibition of VGSCs contributes to the systemic toxic effects of LAs, including the initial CNS excitation and pro-convulsive action[16, 17]. In this study, we investigated the pharmacological kinetics of bupivacaine on $Na_v1.5$ expressed in *Xenopus* oocytes.

MATERIALS AND METHODS

Chemicals and Solutions

Bupivacaine (Sigma-Aldrich, St. Louis, MO) was dissolved at room temperature in the bath solution ND96 (in mmol/L: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2, and HEPES 5, pH 7.4) at 100 mmol/L as stock solution and stored at –20°C. Different doses of bupivacaine were prepared before use and applied to oocytes by continuous perfusion *via* a fast gravity-driven perfusion system. After 10 min of perfusion, step pulses were used to investigate the effect of bupivacaine on $Na_v1.5$ channels. The rate of perfusion with blank or test solution was adjusted to 0.1 drop per second to minimize changes in the flow rate.

Plasmid

The gene encoding the rat Na^+ channel α-subunit of $Na_v1.5$ in pcDNA 3.1 vector was a generous gift from Dr. Kaoru Yamaoka (Hiroshima International University, Higashi-Hiroshima, Hiroshima, Japan) and was sub-cloned into pSP64 Poly(A) vector (Promega, Madison, WI) with SP6 promoter to ensure robust expression in *Xenopus* oocytes.

RNA Transcription and Expression in *Xenopus* Oocytes

The $Na_v1.5$ cRNA was synthesized from an EcoR I linearized DNA template and was transcribed *in vitro* using SP6 RNA-polymerase and the mMESSAGE mMACHINE™ system (Ambion, Austin, TX). The quality of mRNA produced was checked by running on a 1% agarose gel and Nanodrop 2000 (Thermo Scientific, Waltham, MA).

Xenopus laevis oocytes were injected with 10–20 ng of $Na_v1.5$ cRNA. Oocytes were incubated at 20°C for 2–4 days in ND96 solution supplemented with 5 mmol/L pyruvate and 0.1 mg/mL gentamycin.

Xenopus oocytes that expressed $Na_v1.5$ were clamped at −100 mV before electrophysiological recordings. Robust Na^+ currents were induced in oocytes when depolarized by a series of step stimuli ranging from −100 mV to +70 mV. To minimize individual difference between samples, only oocytes with peak I_{Na} currents elicited at −20 mV or −30 mV were chosen for subsequent tests[18, 19].

Electrophysiological Recording

Two-electrode voltage-clamp recordings were performed using an Axon 900A amplifier (Molecular Devices,

Sunnyvale, CA) and pClamp 10.0 software (MDC). Data were acquired using Clampfit 10.3 (MDC) and analyzed with Origin 7.5 (Northampton, MA). The voltage and the current electrodes were filled with 3 mol/L KCl. Currents were filtered at 1.3 kHz and sampled at 10 kHz with a four-pole Bessel filter. The bath solution contained (in mmol/L): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2, and HEPES 5 (pH 7.4).

Data Analysis

Mean conductance (G) was calculated using the equation $G = I/(V-V_r)$, where I is the peak current elicited upon depolarization, V is the membrane potential, and V_r is the reversal potential. The voltage-dependence for the activation was fit with the Boltzmann relation, $G/G_{max} = 1/[1+\exp(V-V_m)/k_m]$, where V_m is the voltage for half-maximum activation and k_m is the slope factor. Current decays were fit with a double exponential equation: $I = A_{fast}*\exp[-(t-K)/\tau_{fast}]+A_{slow}*\exp[-(t-K)/\tau_{slow}]+I_{SS}$, where I is the current, A_{fast} and A_{slow} represent the percentage of channels inactivating with time constants τ_{fast} and τ_{slow}, K is the time shift, and I_{SS} is the steady-state asymptote.

The Hill formula was used to fit the dose-response relationship of bupivacaine: $I_{drug}/I_{control} = A*[\text{Bupivacaine}]\text{^}n/(EC_{50}\text{^}n+[\text{Bupivacaine}]\text{^}n)$, where [Bupivacaine] is the concentration of bupivacaine, and n is the Hill coefficient. A is a constant representing the maximum reduction rate of Na^+ currents by bupivacaine. The value of $I_{drug}/I_{control}$ provides the maximal value of normalized inhibition of Na^+ currents at each concentration of bupivacaine. EC_{50} is the concentration of half-maximal inhibition of Na^+ currents by bupivacaine.

The voltage-dependence of fast inactivation and slow inactivation was described by the two-state Boltzmann equation: $I/I_{max} = A+(1-A)/\{1+\exp[(V-V_{1/2})/k]\}$, where A reflects the fraction of channels that are resistant to slow inactivation, V is the membrane potential of the conditioning step, $V_{1/2}$ is the membrane potential at which half-maximal inactivation is achieved, and k is the slope factor. The parameters for fast inactivation were characterized by the half-maximal voltage V_f and the slope factor k_f; and for slow inactivation were V_s and k_s.

Current decays were fitted with a double-exponential equation: $I = A_{fast}*\exp[-(t-K)/\tau_{fast}]+A_{slow}*\exp[-(t-K)/\tau_{slow}]+C$, where I is the current, A_{fast} and A_{slow} represent the percentage of channels inactivating with time constants τ_{fast} and τ_{slow}, t is the conditioning pulse duration, and K is the time shift.

The time constants for the development of slow inactivation were determined by fitting the data with a double-exponential equation: $I/I_{max} = A_{fast}*\exp(-t/\tau_{fast})+A_{slow}*\exp(-t/\tau_{slow})+C$. The normalized currents I/I_{max} provide information about how many channels entered slow inactivation during the conditioning pulse.

In each testing sample, control and bupivacaine data were acquired from the same oocyte. Only recordings with leakage <0.08 μA and fluctuation within 0.05 μA were selected for statistical analysis. Data are presented as mean ± SEM. Statistically significant differences between parameters of currents measured in control and drug-containing solutions were assessed with one-way ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) or Student's *t*-test in Origin 7.5.

RESULTS

Voltage- and Use-dependent Blockade by Bupivacaine

Although it has been reported that blockade of the Na^+ channel by LAs is differentially modulated by β1 and β3 subunits[20], this was not of concern in the present study in that we intended to provide a direct assessment of the pharmacological effects of bupivacaine on the $Na_v1.5$ channel. Hence, *Xenopus* oocytes were chosen as an expression system of $Na_v1.5$ alone.

Robust Na^+ currents of $Na_v1.5$ were elicited by step stimuli from a holding potential of −100 mV to +70 mV with 100 ms duration (Fig. 1A). To reach equilibrium for each recording, oocytes were perfused with the external solution of ND96 for 10 min prior to the subsequent protocols. After application of 50 μmol/L bupivacaine for 10 min, the peak $Na_v1.5$ current was inhibited by ~70%. The I–V curves showed that the blockade of I_{Na} occurred at quite depolarized potentials (about −40 mV for 10–100 μmol/L) (Fig. 3C). To quantify the dose-dependent blockade potency, clinical-range concentrations were selected to determine the IC_{50}. The results showed that bupivacaine blocked I_{Na} in a dose-dependent and "slow-out" (in that the effect was hard to be abolished during washing step) manner (Fig. 1B). The highest concentration (200 μmol/L) almost completely blocked I_{Na}, and the remaining currents were

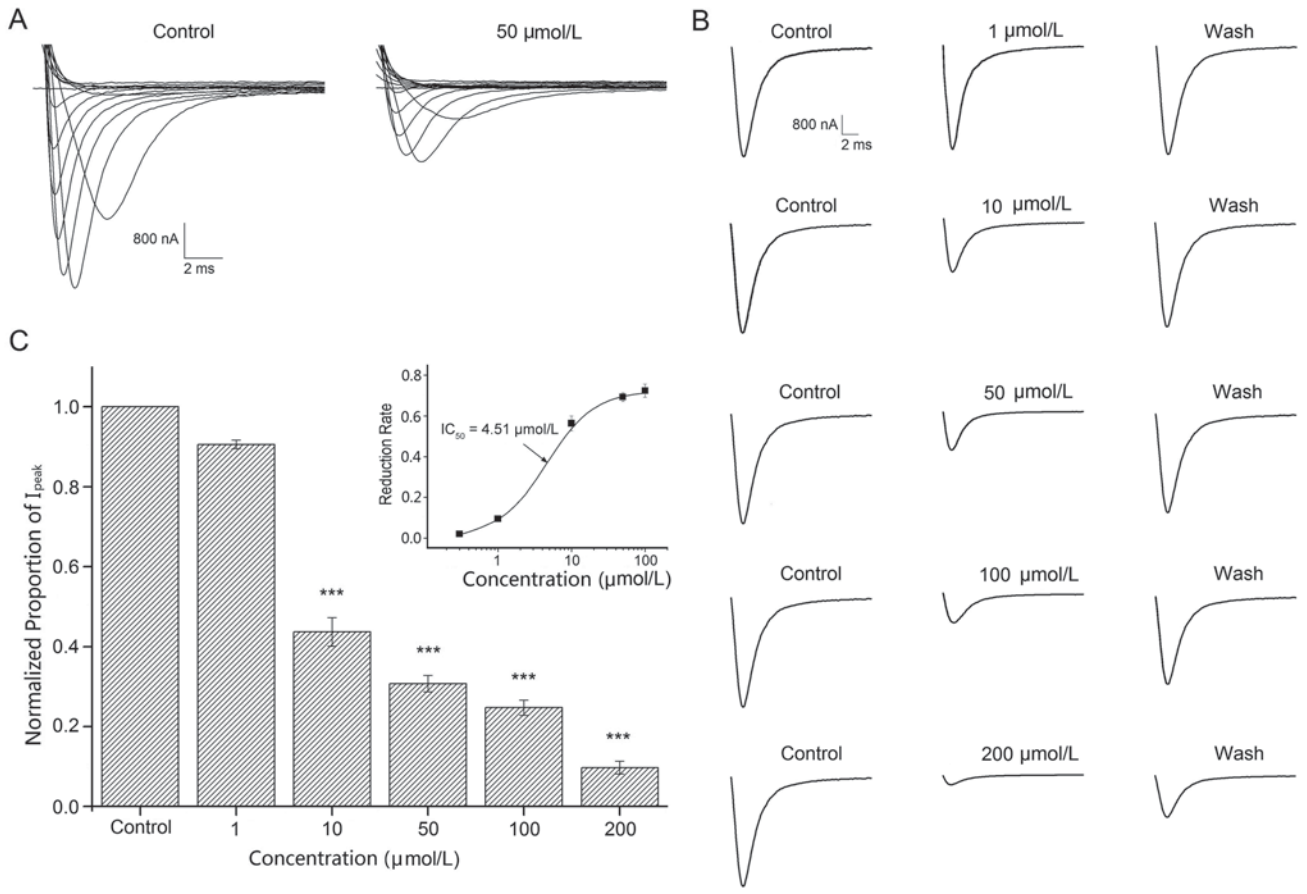

Fig. 1. Voltage and dose-dependent blockade of $Na_v1.5$ channels by bupivacaine. A: Representative traces of I_{Na} in blank (Control, left) and 50 μmol/L bupivacaine (right). B: Representative traces of I_{Na} illustrating the blocking potency of bupivacaine on $Na_v1.5$ at different concentrations at −20 mV; C: Dose-dependent blockade of $Na_v1.5$ by bupivacaine. Mean ± SEM. ***P <0.001 *vs* control; one-way ANOVA, n = 6 for each. Inset: dose-response curve for I_{Na} reduction and bupivacaine concentration.

only 9.75 ± 1.61% (P <0.001, n = 6) of control, while 1 μmol/L had little effect on I_{Na}, the remaining currents being 90.58 ± 1.06% of control (P >0.05, n = 6). The I_{Na} reduction rates induced by bupivacaine were 56.31 ± 3.60% at 10 μmol/L (P <0.001, n = 6); 69.24 ± 2.08% at 50 μmol/L (P <0.001, n = 5); and 72.37 ± 3.24% at 100 μmol/L (P <0.001, n = 5) (Fig. 1C). Accordingly, the dose-response relationship fit the Hill equation well, giving an IC_{50} of 4.51 μmol/L with a Hill coefficient of 1.33 (Fig. 1C, inset).

Since the hallmark of most class I LAs is the induction of a use-dependent blockade of Na^+ channels, the kinetic properties of bupivacaine blockade was characterized in $Na_v1.5$ with steps of depolarizing stimuli from −100 mV to +10 mV at 0.5, 1, and 2 Hz for 60 pulses each. Each peak Na^+ current was normalized to the peak current during the first pulse. Under control conditions, there was hardly any reduction in peak I_{Na} (Fig. 2). After treatment with bupivacaine at different concentrations, the potency of blockade was progressively enhanced. Notably, the blockade by bupivacaine at 1 μmol/L tested at 2 Hz was more efficient than that tested at 1 Hz, while this relationship was reversed at higher concentrations (50 and 100 μmol/L) (P <0.001, n = 5–6, Fig. 2C). The development of blockade was accelerated with increasing bupivacaine concentration at all frequencies tested (Fig. 2B, D). Almost all the use-dependent blockade at different concentrations was achieved within the first 15 pulses, indicating a “fast-in” (in that the inhibition rate was fast) manner. When fitted to the first order exponential equation, the resultant time constants for entry into the steady-state blockade

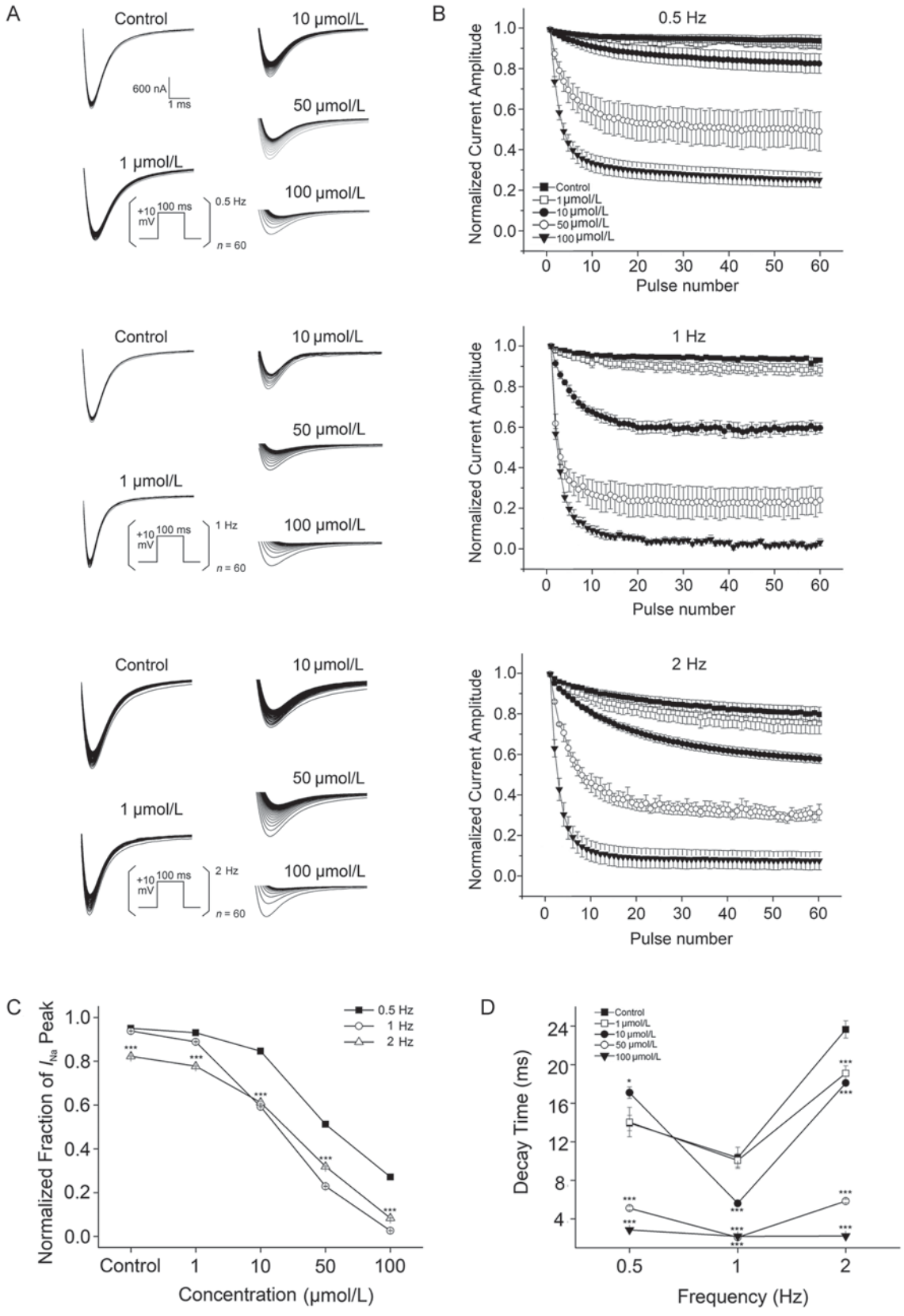
A
Control
10 μmol/L
600 nA
1 ms
50 μmol/L
1 μmol/L
100 μmol/L
+10 mV
100 ms
0.5 Hz
n = 60
1 Hz
2 Hz
B
0.5 Hz
1 Hz
2 Hz
Normalized Current Amplitude
Pulse number
Control
1 μmol/L
10 μmol/L
50 μmol/L
100 μmol/L
C
Normalized Fraction of I_{Na} Peak
Concentration (μmol/L)
D
Decay Time (ms)
Frequency (Hz)

Fig. 2. Use-dependent blockade of $Na_v1.5$ by bupivacaine at different concentrations. Oocytes were held at –100 mV and a train of sixty 100-ms pulses was applied to +10 mV at three frequencies (0.5, 1, and 2 Hz, inset). The peak currents elicited by each pulse (A) were normalized to the current of the first pulse (P_n-P_1, where n = 1–60) and were then plotted *versus* pulse number (B). Values represent mean ± SEM. Control (n = 6); 1 μmol/L bupivacaine (n = 6); 10 μmol/L bupivacaine (n = 5); 50 μmol/L bupivacaine (n = 3); 100 μmol/L bupivacaine (n = 4). C: Plot of normalized fraction of peak I_{Na} against different concentrations of bupivacaine tested at 0.5, 1, and 2 Hz. ***P <0.001 for normalized I_{Na} tested at 1 Hz (open circles) compared with that at 2 Hz (open triangles); Student's t-test (n = 5–6). D: Time constants for the entry into the steady-state blockade of I_{Na} by bupivacaine tested at 0.5, 1, and 2 Hz. *P <0.05, ***P <0.001 *vs* control; one-way ANOVA (n = 5–6).

decreased in a nonlinear frequency-dependent manner, prominently for 0.5 and 2 Hz but less efficiently for 1 Hz (Fig. 2D).

Bupivacaine Shifts the Voltage-dependent Relationship of Activation and Inactivation

To give a full assessment of the pharmacological profile of bupivacaine, the voltage-dependent activation and steady-state inactivation of expressed $Na_v1.5$ channels were tested. Since 200 μmol/L bupivacaine significantly depressed the channel activity, amplitudes recorded at this concentration did not reflect its real pharmacological effect. Among the four concentrations tested (except 1 μmol/L), the voltage-dependent activation curves were substantially shifted to more depolarized potentials in a dose-dependent manner (7.71 mV for 10 μmol/L, 9.21 mV for 50 μmol/L, and 12.01 mV for 100 μmol/L) (Fig. 3B, Table 1).

Compared with the voltage-dependent activation, the steady-state inactivation was less sensitive to bupivacaine. All the inactivation curves were shifted to more hyperpolarized potentials, except for the 10 μmol/L bupivacaine treatment (Fig. 3B). Bupivacaine at 50 μmol/L hyperpolarized the steady-state inactivation curve by 8.25 mV (P <0.001, n = 6), about double that of 1 μmol/L (3.47 mV; P <0.001, n = 6) and 100 μmol/L (4.31 mV; P <0.001, n = 6). In contrast, 10 μmol/L bupivacaine slightly depolarized the steady-state inactivation by 1.26 mV (P <0.01, n = 6) (Table 1). Bupivacaine also significantly altered the slope factor of the inactivation curves at all concentrations except 1 μmol/L (Table 1).

Bupivacaine Modifies the Gating Kinetics of Inactivation

Since LAs have a higher affinity for channels in the inactivated state, we considered that the blockade of I_{Na} by bupivacaine may be due to changes in the fast and slow inactivation components of $Na_v1.5$. To test this hypothesis, the voltage-dependent relationships of these two components were explored.

The voltage-dependence of fast and slow inactivation was investigated using protocols with prepulses from −100 mV to +60 mV for different durations (10 ms for fast and 2 000 ms for slow inactivation) (Fig. S1A, B, inset).

Overall, the slow inactivation was more vulnerable to modulation by bupivacaine, with $V_{1/2}$ shifted to a more hyperpolarized potential than that of fast inactivation at all tested concentrations (1, 10, 50, and 100 μmol/L). In slow inactivation, 50 μmol/L bupivacaine induced the most pronounced hyperpolarization shift (20.22 mV) (P <0.001, n = 6) and modest shifts at 10 and 20 μmol/L (3.14 and 11.98 mV respectively; both P <0.001, n = 6), Bupivacaine at 1 μmol/L barely caused any shift in the slow inactivation curve (P >0.05, n = 6). Finally, the fraction of channels resistant to slow inactivation was decreased by bupivacaine in a dose-dependent manner (Fig. S2).

The effect of bupivacaine on the fast inactivation of $Na_v1.5$ was not as prominent as that on slow inactivation. At 100 μmol/L, bupivacaine even markedly depolarized the voltage-dependency by 5.46 mV (P <0.01, n = 6), accompanied by a decrease in the steepness of the inactivation curve (Δk = 5.08 mV), in contrast to the other groups (Table 2, Fig. S1).

The time-constants of decay for the fast and slow components were obtained by fitting the current decay of the activation traces to the double exponential equation. The time constant was several milliseconds in the fast component but dozens of milliseconds in the slow component (Fig. 4A). Bupivacaine preferentially acted on the slow component, in that 50 μmol/L bupivacaine increased the time constants at most of the potentials tested (−20 mV, τ_{slow} = 11.01 ± 1.08 ms, n = 5; –10 mV, τ_{slow} = 21.05 ± 4.61 ms, n = 5; 0 mV, τ_{slow} = 44.26 ± 2.71 ms, P <0.001, n = 5; +10 mV, τ_{slow} = 87.65 ± 8.32 ms, P <0.001,

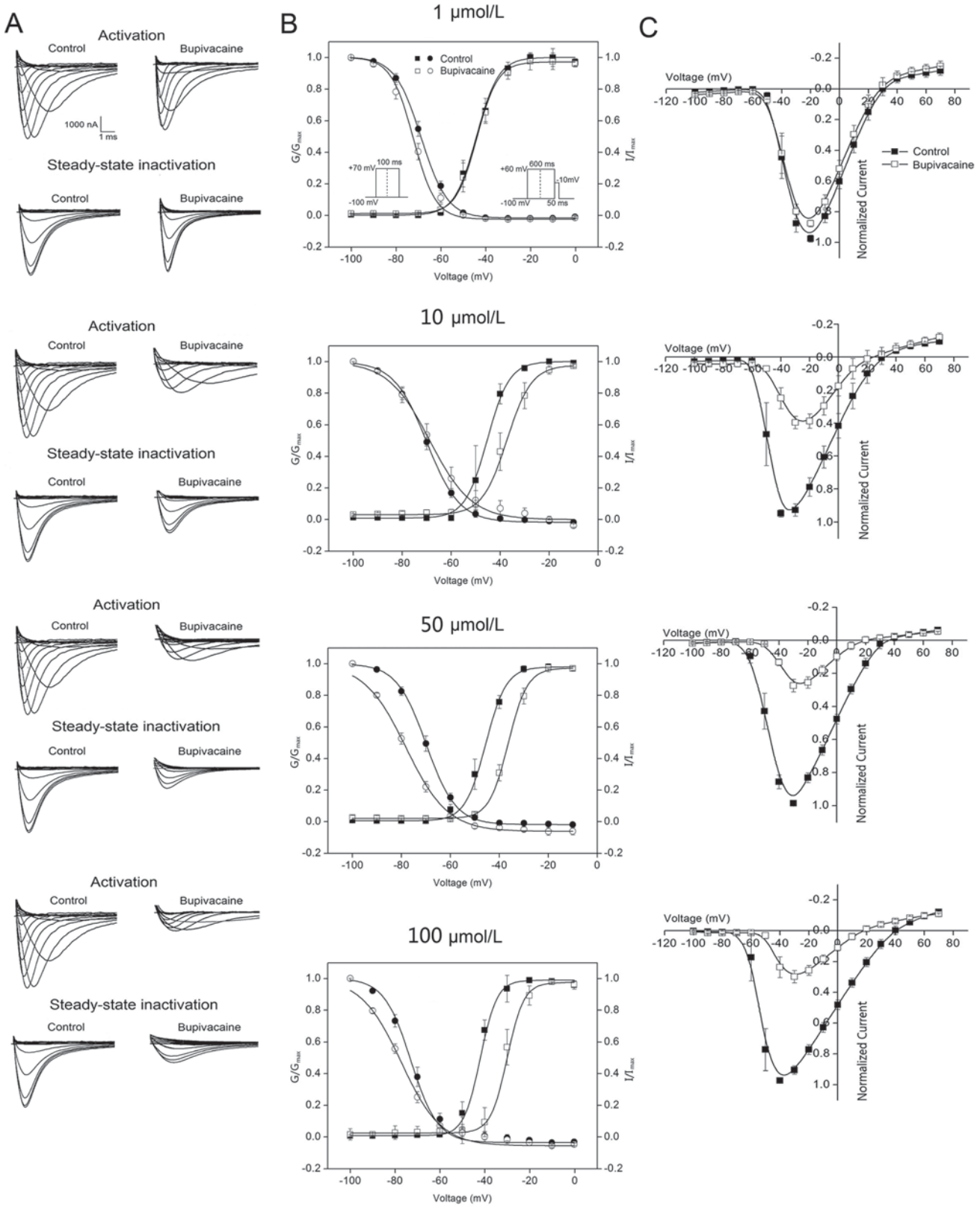
A
Activation
Control
Bupivacaine
1000 nA
1 ms
Steady-state inactivation
Control
Bupivacaine
B
1 μmol/L
Control
Bupivacaine
+70 mV
100 ms
-100 mV
+60 mV
600 ms
10mV
-100 mV
50 ms
G/Gmax
I/Imax
Voltage (mV)
10 μmol/L
50 μmol/L
100 μmol/L
C
Voltage (mV)
Control
Bupivacaine
Normalized Current

Fig. 3. Voltage-dependent activation and steady-state inactivation of $Na_v1.5$ before and after application of 1, 10, 50, and 100 μmol/L bupivacaine. A: Na^+ currents were elicited by depolarizing pulses from a holding potential of −100 mV to +70 mV in 10-mV increments. The voltage-dependence of steady-state inactivation was determined using a two-step protocol in which a conditioning pulse to potentials ranging from –100 mV to +60 mV was followed by a test pulse to –10 mV to measure the peak current amplitude (protocols in B, insets). B: Conductance values were calculated by dividing the peak current amplitude by the driving force at each potential and normalizing to the maximum conductance. For steady-state inactivation, the peak current amplitude during the test pulse was normalized to the maximum current amplitude and plotted as a function of the conditioning pulse potential. Values are averages, and error bars indicate SEMs. The data were fitted to a two-state Boltzmann equation, and the parameters of the fits are shown in Table 1. Sample sizes of each group are shown in Table 1. C: Normalized current-voltage (*I-V*) relationship of $Na_v1.5$ before and after bupivacaine adiministration. Mean ± SEMs.

Table 1. Parameters for activation and steady-state inactivation of $Na_v1.5$ in blank and bupivacaine treatment conditions.

Concentration	Treatment	Activation			Steady-state inactivation		
		n	$V_{1/2}$ (mV)	*k*	*n*	$V_{1/2}$ (mV)	*k*
1 μmol/L	Control	6	–44.03±0.51	4.89±0.29	6	–68.74±0.34	5.84±0.20
	Bupivacaine	6	–43.84±0.49	4.62±0.91	6	–72.21±0.36***	5.91±0.29
10 μmol/L	Control	6	–44.96±0.61	4.49±0.09	6	–70.02±0.34	6.70±0.36
	Bupivacaine	6	–37.25±0.60*	5.03±0.28	6	–68.76±0.29**	8.51±0.30***
50 μmol/L	Control	5	–45.59±0.82	4.21±0.46	6	–69.90±0.35	6.01±0.32
	Bupivacaine	5	–36.38±0.41*	3.91±0.23	6	–78.15±0.34***	8.52±0.35***
100 μmol/L	Control	6	–41.93±1.98	3.69±1.21	6	–72.94±0.37	6.00±0.40
	Bupivacaine	6	–29.92±1.08***	3.71±0.93	6	–77.25±0.29***	8.91±0.39***

*P <0.05, **P <0.01 and ***P <0.001, one way ANOVA; *n* indicates the number of samples tested and *k* is the slope factor. Values are mean ± SEM.

Table 2. Parameters for fast and slow inactivation of $Na_v1.5$ in blank and bupivacaine treatment conditions

Treatment	Concentration	Fast inactivation			Concentration	Slow inactivation		
		n	$V_{1/2}$ (mV)	*k*		*n*	$V_{1/2}$ (mV)	*k*
Control	1 μmol/L	6	–46.68±0.55	6.83±0.47	1 μmol/L	6	–58.46±0.53	14.97±0.31
Drug		6	–48.23±0.79	7.12±0.43		6	–58.88±0.78	14.07±0.57
Control	10 μmol/L	6	–45.36±0.53	6.86±0.21	10 μmol/L	6	–61.77±0.65	13.19±0.61
Drug		6	–47.14±0.77	8.35±0.48*		6	–64.91±0.21***	9.89±0.32***
Control	50 μmol/L	6	–49.89±0.73	6.20±0.33	20 μmol/L	6	–61.64±0.67	15.64±0.42
Drug		6	–51.19±1.21*	7.93±0.79		6	–73.62±0.43***	12.14±0.68***
Control	100 μmol/L	6	–51.75±0.64	6.63±0.27	50 μmol/L	6	–61.65±0.73	14.65±0.31
Drug		6	–46.29±0.74**	11.71±0.57***		6	–81.87±0.49***	11.02±0.46***

*P <0.05, **P <0.01, ***P <0.001; *n* indicates the number of samples tested. Values are mean ± SEM.

n = 3), while the time constants of the fast component were resistant to bupivacaine at all concentrations. These findings were consistent with the voltage-dependent relationship of inactivation described above. Accordingly, with the delayed time constants of the slow component induced by bupivacaine, the fractions of the fast component were slightly increased. Among all the potentials considered, the increased proportion of fast component

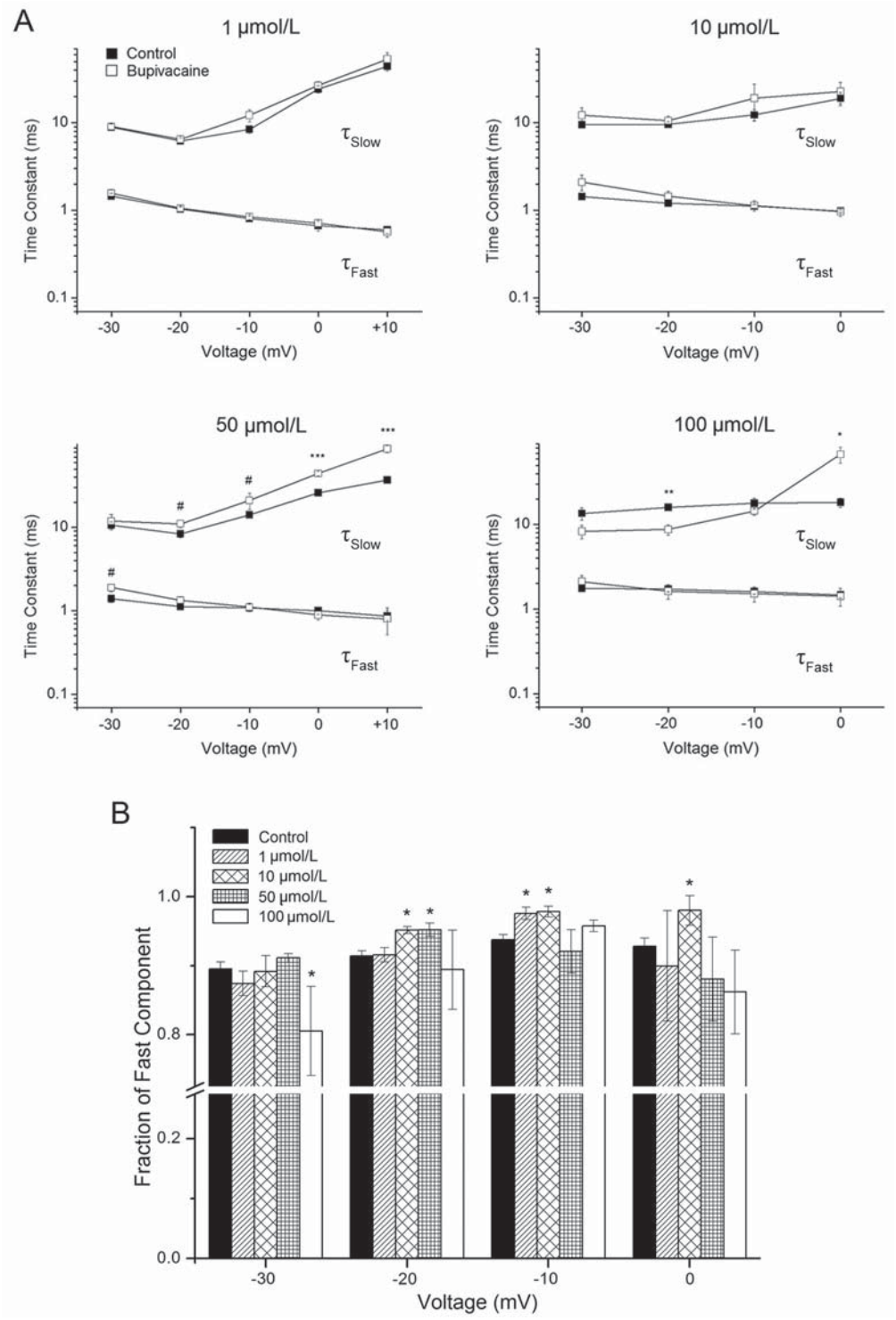

Fig. 4. Modulation of the inactivation kinetics of $Na_v1.5$ before and after 1, 10, 50, and 100 μmol/L bupivacaine. *n* = 5–6. A: Time-courses of decay of Na^+ currents at −30, −20, −10, and +10 mV were fitted to a double-exponential equation. The currents were separated into two inactivation components (τ_{fast} and τ_{slow}) based on the inactivation rate. The effect of bupivacaine was calculated at 1, 10, 50, and 100 μmol/L (open boxes). B: Modulation of the fast component of inactivation of $Na_v1.5$ by bupivacaine. **P* <0.05, ***P* <0.01, ****P* <0.001 *vs* control, Student's *t*-test.

induced by 10 μmol/L bupivacaine was evident, which was increased by 3.8% at −20 mV (P <0.05, n = 5), 4.1% at −10 mV (P <0.05, n = 4), and 5.2% at 0 mV (P <0.05, n = 3) (Fig. 4B). In addition, this increase was also found at −20 mV (3.9%, P <0.05, n = 5) for 50 μmol/L, and at −10 mV (3.8%, P <0.05, n = 6) for 1 μmol/L (Fig. 4B). Notably, a small but notable decrease for 100 μmol/L at −30 mV (9.0%, P <0.05, n = 4) was seen, which may have been caused by the disrupted gating property of channels at high concentrations of bupivacaine.

Bupivacaine Modulates the Development of Slow Inactivation and the Recovery from Inactivation

Since slow inactivation is an important factor governing

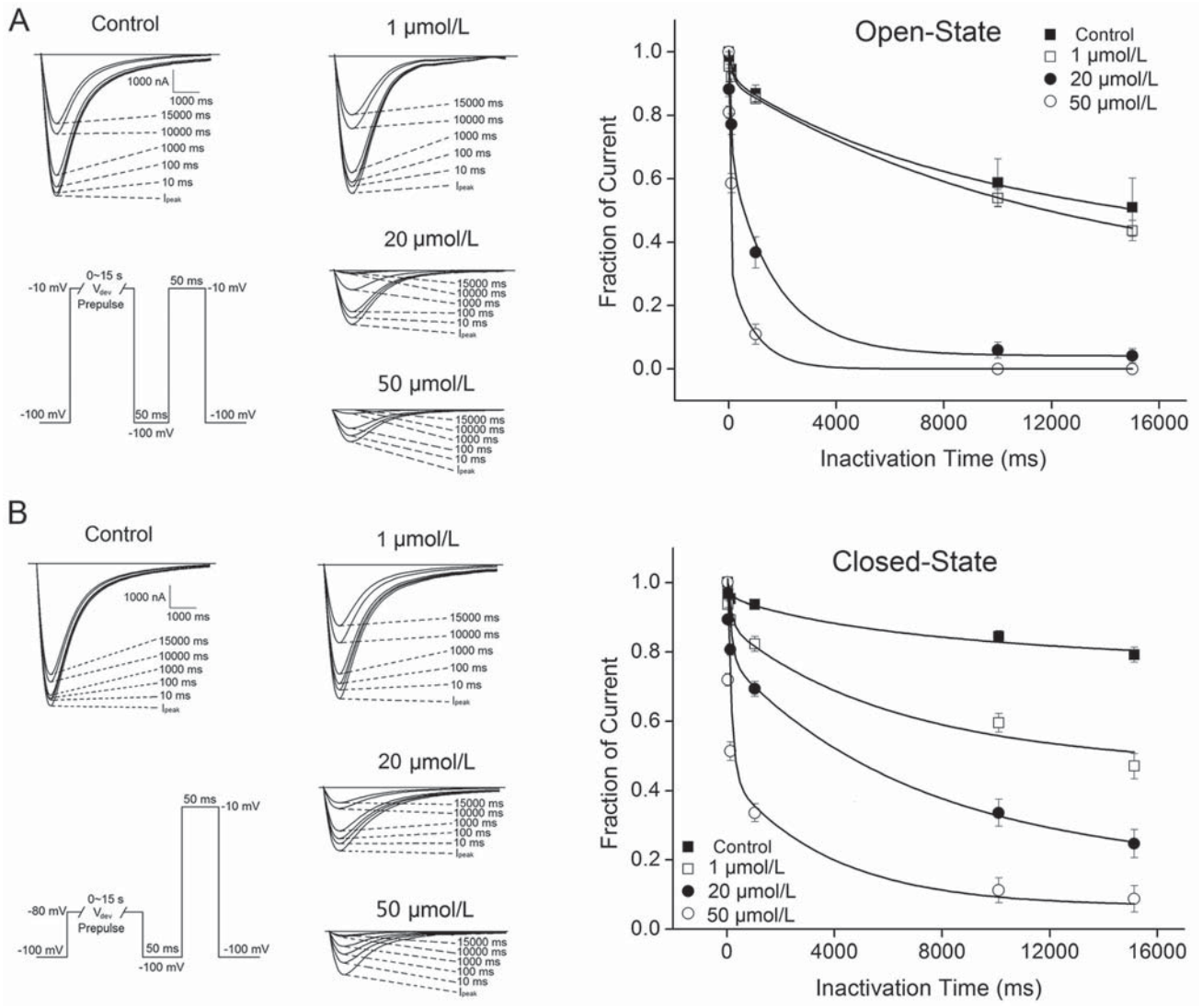

Fig. 5. Development of slow inactivation of $Na_v1.5$ is accelerated by different concentrations of bupivacaine. A: Superimposed current traces of $Na_v1.5$ in control or with different concentrations of bupivacaine showing the rate of development of open-state inactivation. B: Superimposed current traces of $Na_v1.5$ showing the rate of development of closed-state inactivation. Right panels in A and B: time courses of development of inactivation for the peak $Na_v1.5$ currents. Insets: oocytes were prepulsed to V_{dev} for increasing durations, then stepped to −10 mV to determine the fraction of current inactivated during the prepulse. The duration of the inactivation prepulse for each trace is indicated. Averaged data are presented at a V_{dev} of −10 mV (A, n = 6) or −80 mV (B, n = 6) to compare the extent of inactivation. Normalized currents are plotted as a function of V_{dev} duration.

$Na_v1.5$ availability in the activated state, we then determined whether bupivacaine affects the entry into slow inactivation (Fig. 5). Prepulses to −10 mV and −80 mV of variable durations were used to monitor the inactivation onset of $Na_v1.5$ in the open (Fig. 5A) and closed states (Fig. 5B). By fitting the decay currents to the double-exponential equitation, we compared the time constants for the development of slow inactivation at different concentrations of bupivacaine (Fig. 5, right panels). At −10 mV, bupivacaine at all concentrations accelerated both phase I (t_1) and phase II (t_2) of the decay in a dose-dependent manner (Table 3). But at −80 mV, bupivacaine at all concentrations delayed phase I, and the time constants of phase II were delayed at 20 and 50 μmol/L (Table 3). These results suggest that bupivacaine is capable of accelerating the development of open-state slow inactivation of $Na_v1.5$ but not that of closed-state slow inactivation.

The acceleration of entry into slow inactivation may be one of the reasons for the voltage-dependent block of $Na_v1.5$ currents by bupivacaine. However, the high-affinity binding of bupivacaine to the inactivated Na^+ channel might also affect the recovery time from inactivation, which would reduce the number of channels available to reopen, as seen in the actions of lidocaine[21]. Therefore, the recovery time and rate of kinetics were examined for $Na_v1.5$ at different concentrations of bupivacaine.

Recovery from inactivation was determined using a two-pulse protocol consisting of a 50-ms conditioning

Table 3. Time constants for the development of slow inactivation modulated by different concentrations of bupivacaine

Concentration(μmol/L)	Open-State			Closed-State		
	n	t_1 (ms)	t_2 (ms)	*n*	t_1 (ms)	t_2 (ms)
Control	6	151.57±0.49	17889.56±0.59	6	130.48±0.31	13469.90±0.52
1	6	132.93±0.33	12011.27±0.64	6	155.37±0.23	12945.21±0.91
20	6	83.75±0.41	2158.37±0.83	6	141.25±0.44	13511.22±1.03
50	6	35.66±0.21	958.46±0.77	6	147.11±0.56	14034.52±1.67

n indicates the number of samples tested. Values represent means ±S.E.M.

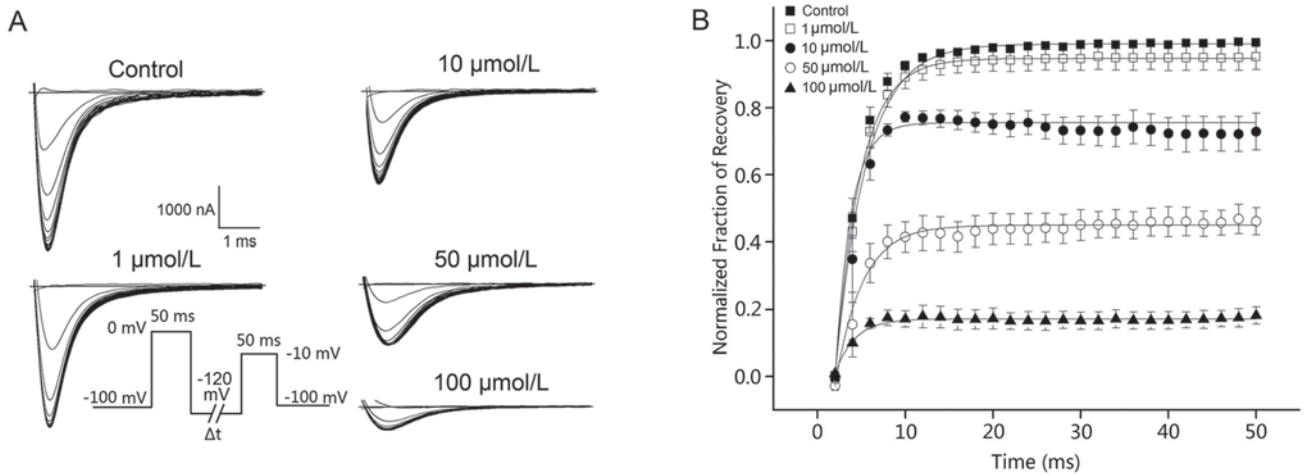

Fig. 6. Bupivacaine attenuates the recovery kinetics from inactivation. A: Time course of recovery at −120 mV as determined by a two-pulse protocol (below). Currents were recorded at a test pulse to −10 mV for 50 ms after a variable (0–100 ms) recovery time at −120 mV from a 50-ms conditioning prepulse at 0 mV. B: The recovery time course was then fitted to a single-exponential function to obtain the time constant of recovery. *n* = 3–6; mean ± SEM.

prepulse at 0 mV followed by a varied recovery time (0–100 ms) at −120 mV, after which a test pulse to −10 mV for 50 ms was applied (Fig. 6A, inset). The recovery kinetics of currents was measured at –10 mV and single exponential fits were used to estimate the recovery time constants and the proportion of recovered Na^+ channels. Under control conditions, 99.02 ± 0.10% of $Na_v1.5$ readily recovered after 50-ms depolarization at 0 mV, with a recovery time constant of 1.76 ± 0.09 ms. Bupivacaine reduced not only the number of channels recovered but also the rates of recovery from inactivation. The proportions of recovered Na^+ channels and time constants for the recovery (τ_{rec}) after treatment with bupivacaine were 94.70 ± 0.17% and 1.44 ± 0.03 ms for 1 μmol/L; 75.57 ± 0.53% and 0.89 ± 0.10 ms for 10 μmol/L; 44.92 ± 0.29% and 1.51 ± 0.10 ms for 50 μmol/L; and 17.17 ± 0.19% and 1.10 ± 0.06 ms for 100 μmol/L. These results suggest that bupivacaine is capable of attenuating the recovery potency of $Na_v1.5$ and slightly accelerating the time constant for partial recovery from the inactivated state (Fig. 6B).

DISCUSSION

Regarded as the most long-acting and efficient LA widely used in clinical management, bupivacaine is still associated with severe cardiac and CNS toxicity, which restricts its use as a safe and controllable LA[22]. The major mechanism for bupivacaine depression of cardiac conduction is considered to be the fast block of Na^+ channels during action potential transmission, which results in slow recovery from block during diastole[23]. Recently, levobupivacaine, a single enantiomer of bupivacaine, has been introduced as a new long-acting LA with potentially reduced toxicity compared with bupivacaine[24]. Even so, bupivacaine has not been replaced in the market, probably due to the lack of perceived safety benefits and/or the consideration of additional costs for the switch to levobupivacaine, which is ~57% more expensive than bupivacaine[22]. Therefore, a safer strategy for the current use of bupivacaine is urgently needed. To achieve this, an in-depth investigation of how bupivacaine interacts with Na^+ channels and the underlying mechanisms need to be illuminated.

In the present study, we examined the pharmacological profile of bupivacaine on $Na_v1.5$, with particular interest in how it interacts with channels expressed in background-free *Xenopus* oocytes.

It is well-established that bupivacaine has a higher affinity for inactivated Na^+ channels[21]. However, apart from the consistency of hyperpolarized inactivation found in this research, bupivacaine also shifted the voltage-dependent activation of $Na_v1.5$ to more depolarized values. The enhanced inactivation and impaired activation of $Na_v1.5$ caused by bupivacaine would reduce cell excitability since larger depolarizing stimuli would be required to activate the $Na_v1.5$ channel.

To date, the mechanisms underlying the blockade of VGSCs by class I LAs have been investigated to follow two independent stages. One is related to the voltage-dependent block, which involves voltage sensor inhibition in the open state. The other is defined as a lipophilic block resulting from interaction with the drug in the closed state[25]. The latter type of inactivation only occurs at very high concentrations and is therefore considered to be a low-affinity block, so this was not a concern in the current study. Consistent with the previous findings, bupivacaine greatly affected the inactivation of $Na_v1.5$ and decreased the number of Na^+ channels that recovered. Moreover, the development of slow inactivation and the voltage-dependent delay in slow inactivation time constants means that bupivacaine has an apparent bias for the open-state Na^+ channel (Fig. 5A). All the results support the idea that the blockade of $Na_v1.5$ by bupivacaine is due to a voltage-dependent block in the open state.

The binding sites of class I LAs on Na^+ channels are localized in DIII-S6 and DIV-S6. However, the key residues involved in bupivacaine binding have been less investigated, unlike that of lidocaine. Currently, it is generally considered that the voltage-dependent blockade may be attributed to the hydrophobic and aromatic residues within S6, such as L1280 and P1759[10]. Here, we found that between the fast and slow inactivation, bupivacaine preferably acted on the latter, for which the voltage-dependency and time constants were substantially changed. This finding is in agreement with the fact that the slow inactivation is thought to be accompanied by rearrangement of the channel pore in DIV[26]. On the other hand, the significant changes in steepness of voltage-dependency of fast inactivation induced by bupivacaine indicated an interaction between bupivacaine and the fast-inactivation lid associated with DIII-S6. Together, we

postulate that the binding of bupivacaine to the inactivated $Na_v1.5$ channel involves DIII-S6 and DIV-S4.

In addition, at extremely high concentrations, bupivacaine dramatically inhibited $Na_v1.5$ activity, leading to a permanent block that barely recovered. In contrast, at lower concentrations, bupivacaine induced a relatively small, reversible inhibition of $Na_v1.5$ currents. This phenomenon indicates that the CNS toxicity induced by bupivacaine may follow a two-stage process: at lower concentrations, inhibitory neurons are blocked first resulting in generalized convulsions, and at higher concentrations a global CNS depression occurs[2]. Hence, although treatment with bupivacaine may have the risk of clinical CNS syndromes, permanent damage can be avoided at appropriate dosages.

Of note, the bupivacaine-induced block of the inactivated state of the Na^+ channel displays stereoselectivity. Both enantiomers of bupivacaine bind with high affinity to the activated or open-state cardiac Na^+ channel, with binding kinetics faster for S(-)- than for R(+)-bupivacaine. The higher potency of R(+)-bupivacaine in blocking the inactivated state of the cardiac Na^+ channel may explain its higher toxicity because of the large contribution of the inactivated-state blockade during the plateau phase of the cardiac action potential. These results support the use of the S(-)-enantiomer to reduce cardiac toxicity[11]. However, as pure S(-)-enantiomer is far more expensive and bupivacaine is clinically used as a racemic mixture, this study provides clues, at least in part, for a safer strategy of the current use of bupivacaine.

In summary, our results revealed that the voltage-dependent block of $Na_v1.5$ by bupivacaine arises not only from a depolarized shift in voltage-dependent activation but also from hyperpolarized inactivation. In particular, bupivacaine has a preference for the open-state inactivated channels, the binding sites of which may rely on the hydrophobic residues within DIII-S6 and DIV-S6. In addition, overdose of bupivacaine could cause a drastic decrease in channel activity that may partially contribute to the clinical cardiac or CNS toxicity.

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SUPPLEMENTAL DATA

Supplemental data include two figures and can be found online at http://www.neurosci.cn/epData.asp?id=192.

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