Chapter 6 Looking Beyond Development: Maintaining Nervous System Architecture

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CHAPTER SIX

Looking Beyond Development: Maintaining Nervous System Architecture

Claire Bénard *and* Oliver Hobert

Contents

Abstract

Neuronal circuitries established in development must persist throughout life. This poses a serious challenge to the structural integrity of an embryonically patterned nervous system as an animal dramatically increases its size postnatally, remodels parts of its anatomy, and incorporates new neurons. In addition, body movements, injury, and ageing generate physical stress on the nervous system. Specific molecular pathways maintain intrinsic properties of neurons in the mature nervous system. Other factors ensure that the overall organization of entire neuronal ensembles into ganglia and fascicles is appropriately maintained upon external challenges. Here, we discuss different molecules underlying these neuronal maintenance mechanisms, with a focus on lessons learned from the nematode *Caenorhabditis elegans*.

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1. Introduction

1.1. The maturing brain

A series of well-orchestrated processes build our brain *in utero*. The neural tube forms by 3–4 weeks of gestation, differentiates into regions, and hosts massive neuronal proliferation and migration during weeks 12–20. Synapses then organize, while extensive cell death sculpts the forming brain until the first few weeks of postnatal life. At birth, our brain is made of approximately 100 billion neurons, but a dynamic and lengthy process of maturation of the brain ensues to increase this number further (Fig. 6.1). Profound modifications of the anatomy occur on a large scale, involving growth of the entire brain and some subregions, concomitant with widespread changes at the scale of the neuron, its axon, dendrites, and synapses. The organization of the brain established by the time of birth must be maintained throughout this process of maturation.

In parallel to this expansion of the brain, extensive changes of cortical and subcortical components occur during childhood and adolescence

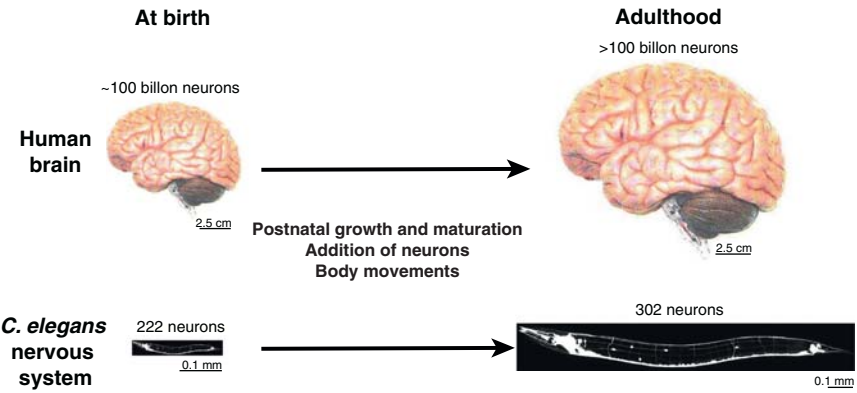

Figure 6.1 After the nervous system is established during embryogenesis, it faces the challenges of growth, maturation, and mechanical strain. At birth, a human brain is only about 1/4–1/3 of our adult brain volume. It grows and develops most dramatically during childhood. The fastest growth occurs in the first 3 years of postnatal life, when the infant's brain reaches 90% of the adult weight. By age 6, the total brain size is 95% of its maximum, reaching the adult volume and weight by about 12 years of age (Lenroot and Giedd, 2006; Toga *et al.*, 2006). Although not shown in the diagram, other regions of the central and peripheral nervous systems also grow expansively during childhood and adolescence. The nervous system of the worm *C. elegans* (bottom) is laid out in embryogenesis. After hatching into a larva (birth), the worm grows 100 times in volume, and the entire nervous system grows considerably as well. The nervous system of both organisms are subjected to mechanical strain originating from growth itself, the addition of neurons and their axons, and the motions of the body.

(Lenroot and Giedd, 2006). In the first few years of life, dendritic branching and synaptic connections increase tremendously, followed by dendritic pruning and synapse elimination, which result in the selection of the most efficient and active connections. In particular, synaptogenesis peaks at 4 months of postnatal life in the visual cortex, and synapse elimination brings about the adult state by 4–5 years of age. In the medial prefrontal cortex, synaptic growth peaks at 3–4 years and the bulk of trimming is completed by mid-to-late adolescence (Toga *et al.*, 2006). Changes in volume and density of gray and white matter continue well into the third decade of life. Vast waves of myelination take place during late adolescence and continue in the adult.

Taken together, brain development and maturation are highly dynamic, continuous processes that involve substantial growth and morphological alteration during embryonic and postembryonic stages, respectively. Moreover, the maturing brain is endowed with great plasticity that is essential for shaping its structure and function. Plasticity is most prevalent in defined time windows or critical periods (Hubel and Wiesel, 1970), but structural changes continue to occur. For example, the adult cortex undergoes structural changes in spines (Trachtenberg *et al.*, 2002), axons (Florence *et al.*, 1998), and dendrites (Tailby *et al.*, 2005). Also, adult neurogenesis generates a substantial number of new neurons that are incorporated into existing circuitries (Li, Mu, and Gage, this volume). In spite of these massive alterations, many of the neuronal features and structures established earlier in development are retained throughout life.

1.2. Challenges faced by nervous systems during maturation

The dramatic changes that occur during brain maturation raise the question of how neuroanatomical structures formed at an earlier time point retain their essential morphology and connectivity amidst the expansive growth of the nervous system and the remodeling of many of its connections, and beyond. The overall growth of the human brain by 3–4 times during childhood constitutes a challenge for the existing neuroanatomical structures as substantial tension is generated on whole brain regions, neurons, and neighboring cells. Tension is produced at the level of entire organs undergoing growth, and likely is engendered by the massive volume increase during brain maturation (Van Essen, 1997). Also, the growth of the body implies that individual neurons grow as well if they are to remain connected to their distant targets (e.g., as the limbs elongate, axons of the sciatic nerves grow such that they still reach the toe).

The addition of new cells, their growth, and migration also exert mechanical forces on neurons close by, albeit more locally. Also, the addition of new neurons, cells, and synaptic arbors profoundly alters the molecular landscape of the neurons that are acquiring new neighbors. The ensheathing of axons by Schwann cells or oligodendrocytes replaces prior contacts between neighbors

and establishes new ones, with different cell surface molecules. In the face of the mechanical and molecular pressures generated during brain maturation, at least some neuroanatomical features that developed earlier likely need to remain connected, and accommodate and integrate the novelty around them.

1.3. Challenges faced by nervous systems throughout life

The nervous system not only faces challenges of various sorts during growth and maturation, but also throughout adult life. This is because nervous systems are not fixed in a static state and remain plastic throughout adulthood, incorporating activity-dependent changes and remodeling, refining, strengthening, adding, and eliminating synapses (Sanes and Lichtman, 2001). In addition, the movements of our body parts are a source of ongoing, lifelong mechanical stress on neuronal structures. For instance, neurons of the peripheral nervous system (PNS) are subjected to tension by the actions of our hands, limbs, eyes, tongue, etc. Also, within the central nervous system (CNS), a structure like the optic nerve is under continuous mechanical stress imposed by eye movements. Thus, the mechanical and molecular challenges, local and widespread, loom throughout life. This demands a finely orchestrated balance between flexibility, and plasticity on the one hand, and the stability and maintenance of structures, on the other.

1.4. Levels of neuronal maintenance

There are two different aspects to the maintenance of structure and function of the nervous system. First, like any other cell type, neuronal cells maintain intrinsic structural and functional features. Second, on a broader scale, the overall organization of ensembles of neurons is maintained, which involves the maintenance of the correct position of neurons (soma, axon, dendrites) relative to one another and relative to other components of the nervous system, such as glial cells. Such maintenance is expected to be absolutely critical to ensure that neurons retain their ability to appropriately communicate with one another throughout periods of postnatal growth, remodeling and mechanical stress.

2. Maintenance of Cellular Features of Neurons

The most obvious and fundamental cellular aspects that neurons must maintain are (a) their gene expression programs, which define their identity and functional properties, and (b) their cellular survival. The maintenance of

gene expression programs involves the continuous activity of transcription factors, often those that at first initiate neuron-type-specific transcriptional events, but also chromatin factors that serve to maintain activated and repressed states of transcription (Brock and Fisher, 2005; Shirasaki and Pfaff, 2002). Neuronal survival is ensured through a host of well-known trophic factors such as neurotrophins (Henderson, 1996).

Moreover, the integrity and morphology of a neuron and its subcellular parts, including axons, dendrites, and synapses, require active maintenance. This is well illustrated by genetic mutant analysis, which revealed that the removal of specific genes does not affect the initial generation of specific neuronal features, but affects their continuous maintenance. A few examples include the following. In flies, the genes *warts* and *hippo* are required for maintenance of dendritic tiling (Emoto *et al.*, 2006). *warts* mutants initially have their body wall normally tiled by dendritic branches. However, from several days post-egg laying on, dendrites progressively lose branches. Dendritic arbor maintenance is also defective in mouse knockouts of the Abelson family kinases. In these mutants, dendritic arbor is normal in immature mice, but defects unfold in adult cortical neurons, only after 3 weeks of postnatal life, that is, just after the most intensive period of dendrite growth in the mouse cortex (Moresco *et al.*, 2005). Illustrating the disease relevance of neuronal maintenance, cortical neurons of Down's syndrome patients gradually lose dendritic branches after initially forming fully branched dendritic fields (Kaufmann and Moser, 2000). Neurological symptoms appear only late in development, after dendrite structure is already established, suggesting that dendrites deteriorate. Indeed, dendritic length is normal or exceedingly long in fetuses and infants with Down's syndrome, but steadily decreases in subjects older than 2 years. Even though it is not quite clear which specific molecule is involved in this Down's syndrome phenotype, there are several other molecules known to be involved in maintaining dendritic complexity, such as BDNF and TrkB (Gorski *et al.*, 2003; Xu *et al.*, 2000), integrins (Marrs *et al.*, 2006; Peng *et al.*, 2008), and collagen IV (Fox *et al.*, 2007).

The need for specific mechanisms to maintain axonal integrity is well illustrated by several examples. For instance, axonal transport is crucial in maintaining the axon scaffold. Postnatal disruption of the dynein/dynactin complex that inhibits retrograde axonal transport leads to late-onset axon degeneration in mice (LaMonte *et al.*, 2002). Neuronal polarity also appears to be actively maintained. After having grown a single axon and neurites in culture, neurons convert pre-existing dendrites into axons, generating multiple axons, as a result of disrupted function of members of the serine/threonine protein kinase D that disturbs membrane trafficking in the Golgi apparatus (Yin *et al.*, 2008).

Synapses also require active maintenance, as revealed by the analysis of genes whose depletion causes a progressive loss of synaptic contacts. This has been best illustrated at the neuromuscular junction, as reviewed earlier

(Sanes and Lichtman, 2001). More recently, a series of distinct target-derived signals has been identified that organize the formation, maturation, and maintenance of motor nerve terminals. In particular, synapse-specific collagen IV chains (alpha 3–6) accumulate only after synapses are mature and are required for synaptic maintenance (Fox *et al.*, 2007).

A recurrent theme in maintaining the morphology of individual neurons is the activity-dependent suppression of sprouting of individual neuronal processes (Cao *et al.*, 2007; Huberman *et al.*, 2008). Classic drug studies have demonstrated that neurons alter their morphology in response to inhibition of synaptic transmission, resulting in the growth of sprouts, an apparent attempt of a neuron to re-establish a functional connection (Brown *et al.*, 1981). Genetic analysis has amply illustrated this point further. For example, mutations in the cyclic nucleotide-gated channel subunits *tax-2* and *tax-4* in *C. elegans*, which are required for sensory neuron signal transduction, lead to abnormal axonal branching or the failure of axons to terminate at normal positions (Coburn *et al.*, 1998; Peckol *et al.*, 1999). The time of action for these genes is strictly postdevelopmental, demonstrating the continuous need of these molecules to maintain normal structural features of a neuron (Coburn *et al.*, 1998). Postdevelopmental, supernumerary axonal branching defects can also be observed upon genetic disruption of trans-synaptic communication (Loria *et al.*, 2004; Zhao and Nonet, 2000). These observations point to as yet poorly understood retrograde feedback signals that monitor synaptic activity and signal toward morphological rearrangements upon loss of appropriate synaptic activity. Other molecules involved in maintenance of structural properties include, for example, the *unc-119* locus in *C. elegans* (Knobel *et al.*, 2001). The UNC-119 protein is located in axons and functions in neurons throughout larval stages, after neurons completed developing their morphology, where it inhibits excessive axon branching.

A completely distinct example of a molecule that maintains intrinsic features of a neuron is the *Drosophila* protein Spam. Spam forms an extracellular shield that protects mechanosensory neurons from massive cellular deformation caused by heat-induced osmotic imbalance (Cook *et al.*, 2008). This example illustrates the importance for a neuron to utilize specific molecules to cope with postnatal challenges.

3. Maintenance of the Architecture of Neuronal Ensembles

3.1. Neuron/glia interactions

Molecular mechanisms that support the maintenance of synaptic contacts suggest that a neuron must maintain not only its intrinsic cellular properties, but also the interactions with its cellular environment. In the vertebrate

nervous system, one context in which this is very clear is that of neuron/glia interactions. A major function of glia in the adult is the formation of myelin sheaths around axons thus allowing the fast conduction of signaling essential for nervous system function. Glia also maintain appropriate concentrations of ions and neurotransmitters in the neuronal environment (Jessen, 2004). Studies of neuron/glia interactions have revealed ample evidence for the involvement of specific molecules in not only generating but also maintaining specific cellular contacts and we briefly mention a few examples below.

Oligodendrocytes and Schwann cells are critically involved in the long-term survival of axons in the CNS and PNS, respectively. Two myelin membrane proteolipids, PLP and DM20, are essential for the integrity of myelinated axons. In the absence of PLP–DM20, mice assemble compact myelin sheaths but subsequently develop widespread axonal swellings and degeneration. From the age of 6 to 8 weeks, focal axonal swellings containing organelles are detected throughout the white and gray matter in all regions of the CNS. By 1 year of age, numerous axonal swellings in the optic nerve and spinal cord occur, and at older ages, the motor performance of mutant mice is altered (Griffiths *et al.*, 1998). Similarly, PLP-null mutants have only subtle myelin defects and are neurologically normal in the first year of life. At older ages, a destabilization of compacted myelin arises (Klugmann *et al.*, 1997). Mice deficient in the gene for myelin-associated glycoprotein (MAG) also develop normal myelin sheaths in the PNS, but in mutant mice older than 8 months the maintenance of axon–myelin units is disturbed, resulting in both axon and myelin degeneration. MAG thus appears to play a crucial role in the long-term maintenance of the integrity of both myelin and axons (Fruttiger *et al.*, 1995).

The examples from tightly associated glial cells are among the best case studies for the existence of maintenance factors that keep the overall organization of the nervous system intact. Mutant analysis in simple invertebrate nervous systems, where the contribution of glial cells is less prevalent that in the vertebrate nervous system, has substantially extended our view of the cellular interactions among neurons, as well as between neurons and surrounding tissue, to ensure the proper maintenance of whole neuronal ensembles, as we discuss in the following section.

3.2. Maintaining overall nervous system architecture

Beyond the maintenance of neurons at the individual cell level or at the level of interactions with its immediate surroundings and synaptic partners, architectural aspects of the nervous system must also be maintained. By architecture we mean the spatial arrangements of multineuronal assemblages that are organized into defined neuronal fascicles, commissures, and ganglia.

This definition not only encompasses defined neuronal circuits, but the overall arrangements of circuits into suprastructural ensembles.

A priori, it would be reasonable that maintenance of such structural organization simply relies on the same cues that have initially laid down the overall organization of the nervous system. However, genetic analysis in the nematode *C. elegans* has demonstrated that dedicated mechanisms exist to maintain neuronal architecture. These mechanisms ensure that axons within fascicles and neuronal soma within ganglia retain their precise position after initially adopting their appropriate position through developmental patterning mechanisms. *C. elegans* has been a particularly well-suited organism to identify such maintenance mechanisms as the normal structure of the nervous system is known in exquisite detail, its constituents (neuronal processes, neuronal cell bodies) can be visualized with single cell resolution at defined time points, and it is easily amenable to genetic analysis. Below, we will first very briefly describe the structure and development of the *C. elegans* nervous system and then walk through individual maintenance factors.

The nervous system of the adult *C. elegans* hermaphrodite is composed of 302 neurons. They are organized into well-defined ganglia and fascicles that are apposed to specific cell types such as epidermis and muscle, from which they are often separated by dense extracellular matrix material (Fig. 6.2) (White *et al.*, 1986). The most prominent feature of the worm's nervous system is its densely clustered head ganglia (''brain''), and its ventral nerve cord (''spinal cord''), which contains motor- and interneurons and is separated by a ventral midline. Axons are not myelinated and establish chemical and electrical *en passant* synapses along the length of the axons. Most (~75%) of the adult nervous system develops embryonically, both in terms of neuron birth, axon outgrowth and synaptic connectivity, but additional neurons, mostly ventral cord motor neurons, are added during the first larval stage.

The nervous system of *C. elegans* faces the same challenges as any other nervous system, in that (a) it undergoes enormous growth (Fig. 6.1), (b) preformed circuits must tolerate and integrate the addition of new neurons throughout postembryonic, early larval stages (Fig. 6.2), and (c) it must withstand mechanical stress (Fig. 6.2). Mechanical stress results from locomotion of the worm itself, first inside the eggshell and then after hatching. Mechanical stress is also generated through the movement of internal organs, such as the pharynx, whose peristaltic pumping movements exert pressure on neighboring neurons in the main head ganglia (Fig. 6.2). Egg laying and defecation represent other repeated strains on the nervous system. In terms of growth, the worm increases in length and diameter during development, so that the body volume of an adult is 100 times larger than that of a hatchling at the end of embryogenesis, when its nervous system architecture has been laid out. This growth entails the lengthening of axons and dendrites of the embryonically born neurons.

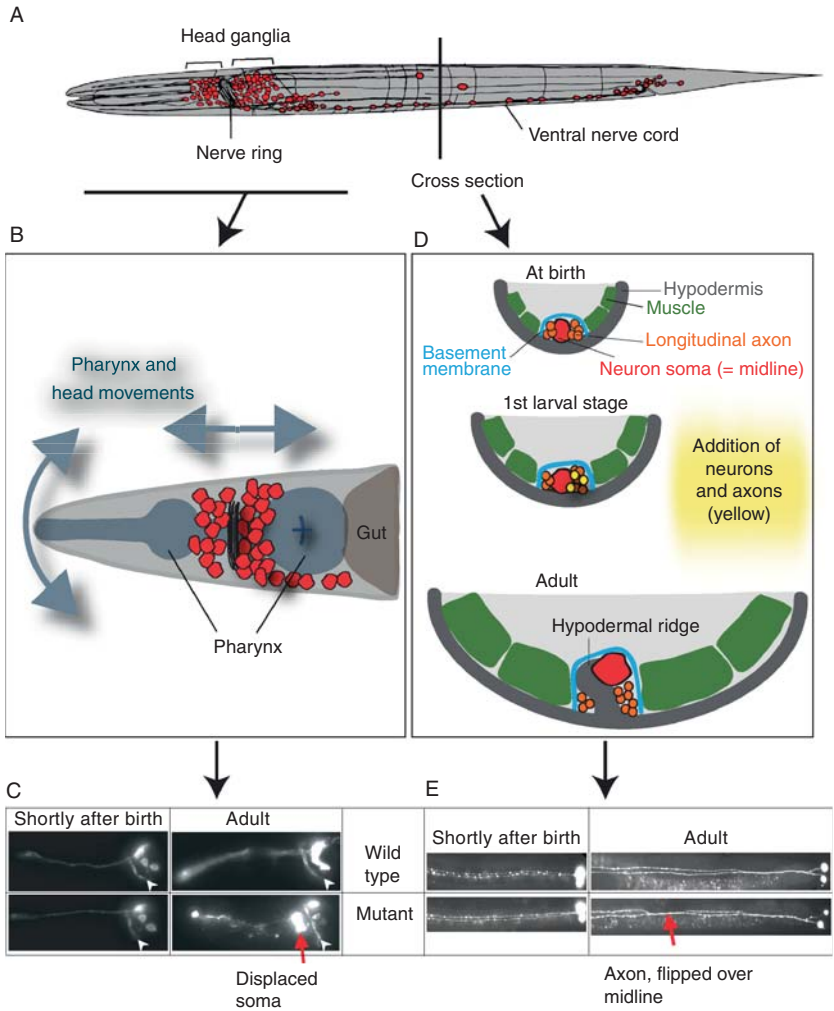

Figure 6.2 Maintenance of neuronal architecture in the nervous system of *C. elegans*. (A) The majority of the 302 neurons (in red) of the worm are organized into major ganglia in the head and tail, as well as into fascicles such as the ventral nerve cord. This organization is established during embryogenesis and retained throughout life. (B) Schematic representation of the head of a worm, with neurons in head ganglia (in red). Throughout larval and adult stages, the worm swims in its environment foraging for food and feeds through the motions of its pharynx, which pumps food toward the gut. These lifelong motions exert considerable mechanical strain on the neurons situated near the pharynx and lead to the progressive displacement of neurons in the absence of dedicated neuronal maintenance mechanisms (C). (C) Example of maintenance defects in head ganglia. In wild-type worms, the cell bodies of a subset of chemosensory neurons of the head are located posterior to the nerve ring (indicated by a white arrowhead). This chemosensory architecture develops in embryogenesis and persists throughout life. In maintenance mutants such as *sax-7* or *dig-1*, the chemosensory neurons develop

3.3. Molecules mediating neuronal maintenance in *C. elegans*

A combination of screens for mutants with defects in neuronal morphology and candidate gene approaches have revealed a number of genes that maintain nervous system architecture (Fig. 6.3). We term these genes "maintenance factors." Mutant phenotypes of the maintenance factors fall into essentially two categories. In one category, the position of axons within fascicles fails to be maintained, while in another category the relative position of neuronal soma within ganglia fails to be maintained (Fig. 6.2). Some mutants only affect axons, others only soma, while yet others affect both. Some of the molecules have developmental roles in other cellular contexts. Nevertheless in all cases, the defects observed in the respective mutants are strictly postdevelopmental. That is to say that mutant animals look indistinguishable from wild-type after the development of the neuron in question has terminated (having extended its axon, dendrites, branches, etc.), and only display defects in later stages of life. This suggests that these molecules are specifically involved in *maintaining* neuronal organization, rather than acting as developmental factors with continuous activity throughout postdevelopmental stages, as many of the cases discussed in the previous section. Lastly, maintenance defects in these mutant backgrounds can be suppressed by inhibiting locomotion (see below), suggesting that the defects are induced

completely normally and are indistinguishable form wild-type at birth, but later become progressively displaced (red arrow, anterior to the nerve ring). (D) Diagram of the ventral portion of a cross section through a worm at birth, first larval stage, and adulthood. The ventral nerve cord develops largely embryonically, with axons organized into the left and right fascicles on either side of a midline; the midline is initially constituted of motor neuron soma that are aligned along the *a/p*-axis (Boulin *et al.*, 2006) and postembryonically becomes further elaborated by an evagination ("hypodermal ridge") of epidermal tissue. The ventral nerve cord is ensheathed by basement membrane material (shown in blue). This separate organization of the fascicles of the ventral nerve cord is maintained throughout life, despite the addition of neurons and axons during the first larval stage (in yellow). However, this arrangement can be disturbed, in the absence of dedicated maintenance factors, as a result of the mechanical strain exerted by the locomotion movements of the worm (E); the failure of maintaining ventral nerve cord architecture occurs at a time right after birth (first larval stage) when additional axons and neurons are added and when the hypodermal ridge—an insurmountable obstacle in adult animals—has not yet been fully elaborated. (E) Example of maintenance defects in the ventral nerve cord. In wild-type worms, the axons of the two PVQ neurons project into the left and the right fascicles of the ventral nerve cord, during embryogenesis. These axons remain in their precise position within the ventral nerve cord throughout life, despite incessant movements of locomotion. In *zig-4*, *egl-15*, *sax-7*, and *dig-1* mutants, these axons develop normally; however, during the first larval stage, mechanical strain from locomotion leads specific axons to flip over the ventral midline to the other fascicle of the ventral nerve cord. Small white dots are background autofluorescence from the gut.

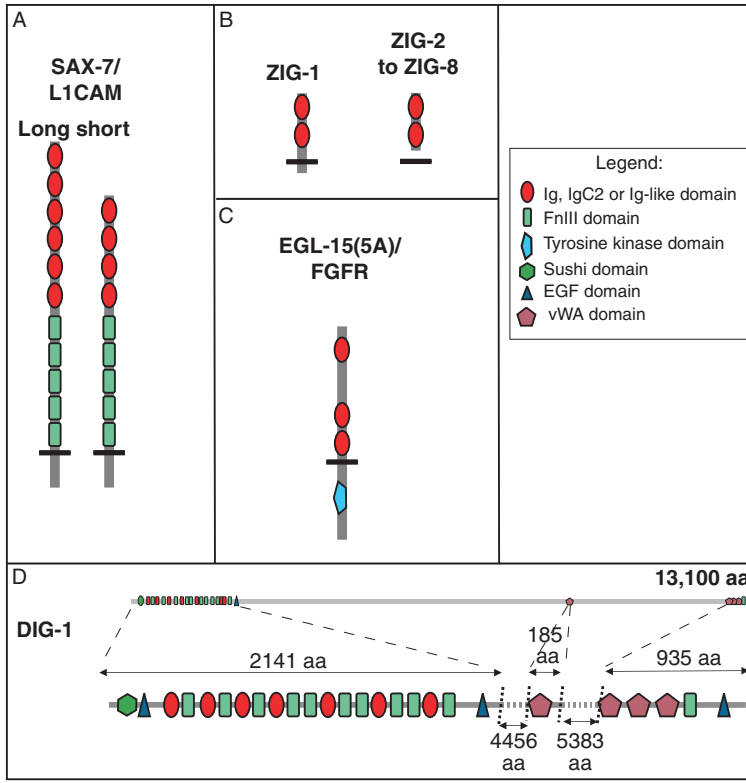

Figure 6.3 Molecules implicated in maintenance of neuronal architecture. To date, diverse members of the immunoglobulin superfamily are implicated in maintaining the precise position of axons within fascicles and neuronal soma in ganglia in *C. elegans*.

by mechanical stress and that the normal function of the maintenance factors are to counteract such mechanical impacts. We describe the individual maintenance factors in more detail below.

3.3.1. SAX-7: A human disease gene ortholog of the IgCAM family

Animals carrying loss-of-function mutations in the *sax-7* gene display a large spectrum of neuronal maintenance defects, including a failure to maintain axon positioning in the ventral nerve cord, as well as the relative position of neuronal soma in various head ganglia and ventral nerve cord (Axang *et al.*, 2007; Chen *et al.*, 2001; Pocock *et al.*, 2008; Sasakura *et al.*, 2005; Wang *et al.*, 2005; Zallen *et al.*, 1999) (Fig. 6.2). These defects are only apparent postembryonically, that is, the nervous system initially develops normally in the embryo, but embryonically patterned neuronal structures lose their normal positioning after birth (hatching from the eggshell). These defects

are partly suppressed if the worms are paralyzed, suggesting that movements lead to displacement, and that the wild-type function of *sax*-7 is to antagonize the mechanical force triggered by locomotion (Sasakura *et al.*, 2005) and also, in the case of head neurons, peristaltic pumping of the adjacent pharynx.

sax-7 encodes a broadly expressed homophilic cell adhesion molecule with several extracellular immunoglobulin (Ig) and fibronectin type III (FnIII) domains (Fig. 6.3), which acts autonomously in the nervous system (Chen *et al.*, 2001; Pocock *et al.*, 2008; Sasakura *et al.*, 2005). The intracellular domain of SAX-7 directly interacts with the cytoskeleton and this interaction is important for neuronal maintenance (Zhou *et al.*, 2008). SAX-7 is one of the two orthologues of Vertebrate L1 family members (L1, CHL1, Neurofascin, and NrCAM), which have been implicated in many aspects of nervous system function and development (Rougon and Hobert, 2003). Mutations in human L1 result in a wide spectrum of neurological abnormalities, including X-linked hydrocephalus, MASA syndrome, X-linked complicated spastic paraplegia type 1, and X-linked agenesis of the corpus callosum (Fransen *et al.*, 1997). It is not clear whether these defects are developmental, as commonly assumed, or whether they are rather a reflection of maintenance defects. It is known, however, that the specific depletion of L1 in the adult mouse brain results in behavioral abnormalities, highlighting its functional importance in the adult brain (Law *et al.*, 2003). Although no gross anatomical abnormalities have been observed in these mice, it is possible that finer defects in the relative position of neurons exist.

3.3.2. DIG-1: A giant multidomain extracellular matrix protein

dig-1 mutants display phenotypes similar to those observed in *sax*-7 mutants, namely a failure to maintain axonal position within fascicles and relative position of neuronal soma in head ganglia. A large number of neuronal soma and axonal projections acquire their proper position during development, but become displaced after birth of the animal (Benard *et al.*, 2006; Zallen *et al.*, 1999). Defects are suppressible by inhibition of movement, indicating that like *sax*-7, the *dig-1* gene serves to counteract mechanical stress exerted on neuronal structures (Benard *et al.*, 2006). The neuronal defects of *dig-1* mutants are not a consequence of gross developmental or morphological defects of neighboring muscles or epidermis, as these tissues appear completely normal. Also, the maintenance defects of axon and soma for a given neuron are not correlated.

DIG-1 is a huge secreted protein of 13,100 amino acids and contains numerous conserved domains implicated in extracellular protein interactions (Fig. 6.3). In its N-terminal region, DIG-1 contains Ig, FnIII, Sushi, and EGF domains, which are frequently part of proteins implicated in cell adhesion, and in components of the extracellular matrix. An enormous

central region is composed of a large number of repeats, rich in β-strands that may form individual, globular domains. This central repetitive region harbors many Ser–Gly peptide motifs in an acidic context, which could be glycosaminoglycans attachment sites (Burket *et al.*, 2006; Lindahl and Hook, 1978). In the C-terminal region, DIG-1 contains Ig, EGF, and several von Willebrand factor A (vWA) domains, again commonly involved in cell adhesion and cell–cell interactions. This overall arrangement of domains and motifs in the DIG-1 protein make it akin to the hyalectan class of proteoglycans, with N- and C-terminal Ig, EGF, and Sushi/CCP domains and a central glycosaminoglycan attachment region (Bandtlow and Zimmermann, 2000). Theoretically, the DIG-1 predicted polypeptide could extend over 100 nm in length, if one takes into account the approximate size of each of its domains and assumes that domains are ordered like beads on a string. This size would be sufficient to bridge the extracellular matrix that separates neurons from adjacent muscle cells from which *dig-1* has been shown to be secreted through mosaic analysis. With cell adhesion and cell–cell interaction domains at either end of the protein, DIG-1 could straddle the basement membrane and interact with neurons on one end of the protein, and muscle and/or epidermal cell surfaces on the other end. DIG-1 may therefore be viewed as a scaffold that keeps individual neurons organized. As mentioned above, this putative scaffolding function is not required during development of neurons, but rather required to maintain structural organization.

3.3.3. EGL-15(5A): A specific isoform of the FGF receptor mediates axonal maintenance

egl-15 encodes the *C. elegans* fibroblast growth factor receptor tyrosine kinase and harbors three immunoglobulin domains in its extracellular domain. A specific splice form of the receptor, defined by the inclusion of an additional extracellular segment between the first and second Ig domain (Fig. 6.3), termed EGL-15(5A), is specifically required for maintenance of the position of axons, and has no role in axon guidance like other EGL-15 isoforms (Bülow *et al.*, 2004). In contrast to *dig-1* and *sax-7*, neuron soma position is unaffected and a more restricted number of axons are affected in the ventral nerve cord, compared to *sax-7* and *dig-1* mutants. This observation reveals an important feature of maintenance factors—they appear to be cell-type specific, with some neurons requiring all known maintenance factors and others only a subset (plus perhaps other as yet unknown factors) (Pocock *et al.*, 2008).

The maintenance function of EGL-15 is independent of its canonical signaling role, as neither its cognate FGF ligands nor its kinase domain are required for its maintenance role (Bülow *et al.*, 2004). Together with its focus of action in the epidermis, which underlies the ventral nerve cord, it is conceivable that EGL-15 may act in an adhesive function, possibly providing some adhesive substratum that keeps axons in place. It is likely

that in such a role, EGL-15 is part of a larger adhesive complex, since, curiously, the extracellular domain of EGL-15 alone is sufficient to provide a maintenance role (Bülow *et al.*, 2004). An alternative scenario would be that EGL-15 is—like Eph-type receptor tyrosine kinases (Klein, 2009)—involved in some "inside–out," reverse signaling events with unknown receptors localized, for example, on axonal surfaces.

3.3.4. ZIG proteins

Eight genes in the *C. elegans* genome, named the *zig* genes, encode small proteins with two immunoglobulin domains (Fig. 6.3). All except one (ZIG-1) contain no transmembrane domain and are predicted to be secreted, likely from a unilateral neuron in the ventral nerve cord, called PVT (Aurelio *et al.*, 2002). Loss of individual *zig* genes or combinations of *zig* genes result in a diverse set of phenotypes. Loss of *zig-4* function leads to defects in axon maintenance in the ventral nerve cord, but has no effect on the position of neuronal soma (Aurelio *et al.*, 2002). The initial position adopted by the axons during the establishment of the ventral nerve cord in the developing embryo is normal, underscoring that the wild-type *zig-4* gene is dedicated to maintaining axons in their appropriate position. Consistent with its restricted role in maintenance, and in contrast to the molecules discussed above, *zig-4* appears to be expressed only postembryonically, supporting its apparently dedicated role as a maintenance factor. The spectrum of phenotypic defects of *zig-4* mutants is much more restricted than that of *dig-1* and *sax-7* and overlapping but still distinct from that of *egl-15* mutants, underscoring the cell-type specificity of axon maintenance mechanisms.

Other *zig* genes also appear to function in maintenance of neuronal architecture. For example, *zig-3* displays similar defects in ventral cord axon positioning as *zig-4* mutants, suggesting that these two molecules may act together (C. Bénard and O. Hobert, unpublished results). In contrast, a double knockout of the *zig-5* and *zig-8* genes results in neuronal soma position defects in head ganglia, mimicking those observed in *sax-7* and *dig-1* mutants (C. Bénard and O. Hobert, unpublished results).

Numerous proteins characterized by two-Ig domains exist in other metazoans such as flies and mammals, which could possibly be functional homologues. In flies, there are at least 20 genes encoding two-Ig domain proteins (Rougon and Hobert, 2003). The secreted Beaten path or Beat Ia, and a subset of 13 transmembrane Beat-like proteins, function to regulate fasciculation during the development of the *Drosophila* nervous system (Fambrough and Goodman, 1996; Pipes *et al.*, 2001). Possible maintenance functions of the Beat proteins have not yet been explored. Another group of ~20 small two-Ig proteins, some with, some without a transmembrane domain, is the Dpr-Ig family, defined by the protein Dpr1 (defective in proboscis extension response) (Nakamura *et al.*, 2002). Dpr1 is required for proper behavioral response to salt (Nakamura *et al.*, 2002; Woo *et al.*, 2008).

Whether these defects are due to maintenance defects has not been investigated yet. Mammalian genomes contain a multitude of genes coding for two-Ig domain-containing proteins, whose function in nervous system maintenance is also unexplored.

3.3.5. F-spondin/SPON-1: An extracellular matrix protein

Spon-1, the worm homolog of the vertebrate F-spondin protein, is a component of the basement membrane of the worm's body wall muscles and pharynx. *spon-1* is required for axons of ventral cord neurons to maintain their position (Woo *et al.*, 2008). In contrast to the factors mentioned above, *spon-1* also has functions during axonal development. In *spon-1* mutants, developing axons are misguided and there are major fasciculation defects, making it more difficult to separate the worsening of developmental defects from subsequent maintenance failure. *spon-1* also display complex genetic interactions with *zig-4* and *egl-15*, which are not presently understood, but further corroborate the importance of the extracellular matrix in maintaining neuronal structures.

4. Discussion

Genetic analysis in *C. elegans* has revealed a surprisingly complex machinery that serves to maintain nervous system architecture. The normal function of maintenance factors is to counteract specific mechanical challenges exerted onto a nervous system. As such, their importance only becomes apparent if the entire nervous system is looked at as a whole, on the system level, rather than at the isolated cell level. Notably, though, while movement is *required* to induce defects in the absence of maintenance factors, it is not in all cases *sufficient* as maintenance defects become apparent only at stages where mechanical stress coincides with remodeling of the nervous system. That is, even though mutant embryos that lack maintenance factors already move vigorously right before hatching, most axonal and soma maintenance defects become apparent only by the first larval stage, when new neurons and their axons are added to the ventral nerve cord and when the underlying epidermis gets remodeled through cell fusions. It is therefore a combination of various external, destabilizing influences (mechanical stress, remodeling, growth) that necessitate the existence of maintenance factors. Thus, maintenance factors may not only be viewed as cell-specific, mechanical glues, but also as molecules that shield pre-existing neuronal structures from potentially disruptive influences of guidance cues that are required to integrate newly generated neurons into pre-existing circuitries.

Maintenance factors affect a distinct, though largely overlapping spectrum of axons and neuronal cell bodies, and are supplied by distinct cell types.

ZIG-4 is secreted from the PVT interneuron to affect the maintenance of several sets of axons (Aurelio *et al.*, 2002); EGL-15 acts in the epidermis on which the axons are positioned (Bülow *et al.*, 2004); DIG-1 is secreted from muscle (Benard *et al.*, 2006); and SAX-7 acts within neurons to affect axon position (Pocock *et al.*, 2008; Sasakura *et al.*, 2005). These neuronal maintenance factors, all bearing Ig domains, as well as possibly a number of unidentified factors, likely interact in different, cell-type-specific combinations to build adhesive complexes that anchor neurons and their projections in their appropriate environment. They might directly mediate, or regulate through signaling events, direct cell–cell interactions among neurons, between neurons and other neighboring cells, or with the extracellular matrix. DIG-1 may be a central component to all distinct maintenance mechanisms as its loss affects the most types of neurons. In light of its size and large number of interaction domains, DIG-1 may nucleate the assembly of cell-specific maintenance complexes. Secreted proteins such as ZIG-4 may contribute in building or facilitating the building of such complexes.

Loss of activity of different maintenance factors leads to neuron-specific defects, at different time points during the animal's life, reflecting the precise requirements of different cells in particular temporal and tissue contexts. The time of occurrence and degree of displacement of a neuron might depend on the age of a neuron and on the strength of adhesion or attachment to its environment, as well as on the developmental or mechanical events affecting the neuron, and the specific tissue topology. Two broad categories of temporal requirements for architectural maintenance factors can be distinguished, one relating to specific stresses during critical time windows, and the other relating to general long-term stability throughout life. The first temporal requirement is that of a critical period or time window in development or maturation of the nervous system, where a neuron becomes displaced in the absence of a maintenance factor, losing the proper position it had initially acquired. The state of affairs remains fixed after this time window passes. This is best illustrated by the axon that flips-over in the ventral nerve cord due to body movements during a time when instability is generated in the vicinity of the axons by the addition of motor neurons and the remodeling of the epidermis. After this time, while the strain from body movements of locomotion persists, the remodeled epidermis constitutes a physical barrier that prevents further axon displacement (Fig. 6.2D). The second temporal requirement for architectural maintenance is manifested throughout life and ensures long-term architecture stability. A lack of this continuous maintenance action relates to the age-dependent progressive accumulation of defects. This is well illustrated by the displaced chemosensory neurons in the head ganglia in *dig-1* and *sax-7* mutants, which show the first signs of displacement in the second larval stage and worsen over time with the continued movements of the animal. Nervous system structures that show later onsets of progressive maintenance

defects might be better anchored in their surrounding environment, therefore taking more time to be displaced by mechanical stress.

It is important to emphasize that the discovery of dedicated maintenance factors is contrary to a perhaps more parsimonious model in which one may have assumed that factors that help develop a system merely "stick around" to ensure that neuronal ensembles, once established, maintain their correct architecture. The existence of factors that appear to be entirely dedicated to a maintenance role obliterates such a simplistic model. The need for dedicated maintenance mechanisms, rather than a reutilization and/or continuous use of developmental cues, could be explained by a possibly disruptive impact that continuously expressed guidance cues may have on newly developing neurons. Indeed, molecules involved in axon guidance are often expressed transiently during development. It is, in fact, the transient, dynamic, and localized expression of a limited set of guidance cues that is thought to generate complex patterns of neuronal connectivities. Continuous expression to maintain circuitries may therefore be undesired. Whether this is the rule or the exception remains to be determined, as several molecules known to maintain cell-intrinsic features of a neuron or appropriate contacts with neighbors, such as synaptic adhesion molecules (Sanes and Lichtman, 1999), have roles in development as well.

A distinction between molecular mechanisms that "initiate" and "maintain" a biological process is a concept that is not unique to the regulation of structural aspects of the nervous system. In the field of gene regulation, it is also known that given genetic loci require distinct factors (DNA-binding transcription factors) for the initiation of a specific expression pattern and a distinct set (chromatin-modifying factors and other transcription factors) for ensuing maintenance (Brock and Fisher, 2005). Such separations may abound in other biological processes as well.

An understanding of maintenance mechanisms holds the prospect of uncovering new clues about neurodegenerative disease. It is conceivable that some neurodegenerative diseases, for which the causes are unknown, may be the result of postdevelopmental failures in the maintenance mechanisms. Conceivably, the displacement of neurons and axons results in subsequent demise of neurons, loss of their function, eventually bringing about neurological symptoms. Human homologs of some of the genes identified in *C. elegans* may be candidates for the diagnosis and treatment of some neurodegenerative disorders.

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