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What axons tell each other: axon–axon signaling in nerve and circuit assembly☆

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A remarkable feature of nervous system development is the ability of axons emerging from newly formed neurons to traverse, by cellular scale, colossal distances to appropriate targets. The earliest axons achieve this in an essentially axon-free environment, but the vast majority of axons eventually grow along a scaffold of nerve tracts created by earlier extending axons. Signal exchange between sequentially or simultaneously extending axons may well represent the predominant mode of axonal navigation, but proportionally few efforts have so far been directed at deciphering the underlying mechanisms. This review intends to provide a conceptual update on the cellular and molecular principles driving axon–axon interactions, with emphasis on those contributing to the fidelity of axonal navigation, sorting and connectivity during nerve and circuit assembly.

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Historical backdrop

The question how myriads of neurons and their processes assemble into the functional architecture of the nervous system essentially co-emerged with Cajal's formulation of the neuron theory [1,2]. Not long after, the idea arose that this must necessarily entail interactions not only of growing neuronal processes with substrates along their trajectories or targets, but also interactions among the vast numbers of neurites proper [3]. This possibility was first experimentally tested through surgical manipulations in amphibian embryos by Hamburger and Taylor in the 1920s and 1940s, respectively [4,5], which were the precursors for a series of classical studies that decades later used essentially the same experimental rationale to study the contribution of axon-axon interactions to vertebrate and invertebrate nervous system development [6–17]. With the notable exception of sensory representation maps, however, the contribution of axon–axon signaling to neural circuit assembly has since then moved out of the center of attention. To date, insights into principles underlying axon-axon interactions comprise a relatively limited, yet steadily expanding number of instances [18–21].

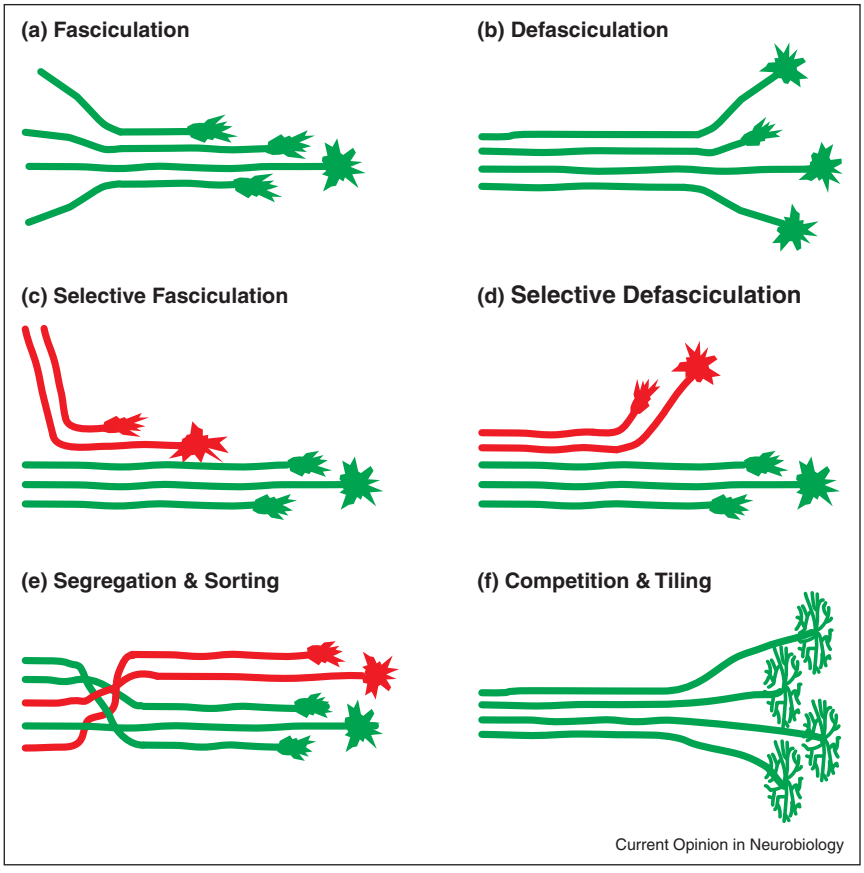
Forces at play

In the *in vivo* setting, axon–axon encounters can occur in a number of different configurations and result in a variety of outcomes (Figure 1). Before reviewing their respective contributions to nerve and circuit assembly it is helpful to first consider the main factors determining the behavior of axons towards each other. These factors are on the one hand related to the properties of axons, which may be summarized as: *adhesive code* (the expression of and responsiveness towards specific sets of molecular cell surface labels regulating adhesive force) (i) [22–25], *intracellular signaling* (triggered by membrane protein engagement) (ii) [26], *axon type* (determined by the type of the corresponding neuron) (iii) [27,28] and *axon identity* (determined by differing molecular labels on axons of the same type) (iv) [20,29]. Non-axonal factors, on the other hand, can profoundly influence likelihood, manner and outcome of axon-axon encounters, and include: *permissiveness* (of the axon growth environment) (v) [30,31], *degree of freedom* (available to axons within permissive tracts) (Figure 2a,b) (vi) [32–34] and *myelinating glia* (whose precursors associate with growing axons) (Figure 2c) (vii) [35–37]. We propose that these factors in net determine not only the specific behavior of axons towards each other, but also a generalized preference of axons to associate with other axons in most *in vivo* contexts. These considerations can have important implications for interpreting the outcome of (disrupting) axon–axon signaling mechanisms *in vivo* (Figure 2d–f).

Pioneers and followers

The prevalent pattern of both central and peripheral nerve tract assembly involves initial extension of axons that from the outset chose trajectories resembling the mature pattern of nerve pathways of the adult, and to which, in turn, later-extending axons adhere [21]. The extension of follower axons along pioneer axons was first

Figure 1



Axon–axon configurations. In the *in vivo* setting, axon–axon encounters can occur in a number of different configurations and result in a variety of outcomes. Following the original classification by Tessier-Lavigne and Goodman [103], we can distinguish four such principle configurations: **(a)** fasciculation (axon bundling), **(b)** selective fasciculation (of follower axons along pioneer axons), **(c)** defasciculation (dissolution of axon bundles) and **(d)** selective defasciculation (dissociation of a specific set of axons from a bundle), to which we can now add three more types of interactions: **(e)** segregation or sorting (of specific sets of axons from other axons within a bundle), **(f)** competition (of axons for target space) and tiling (spacing of axon terminal arborizations) [18,29,74].

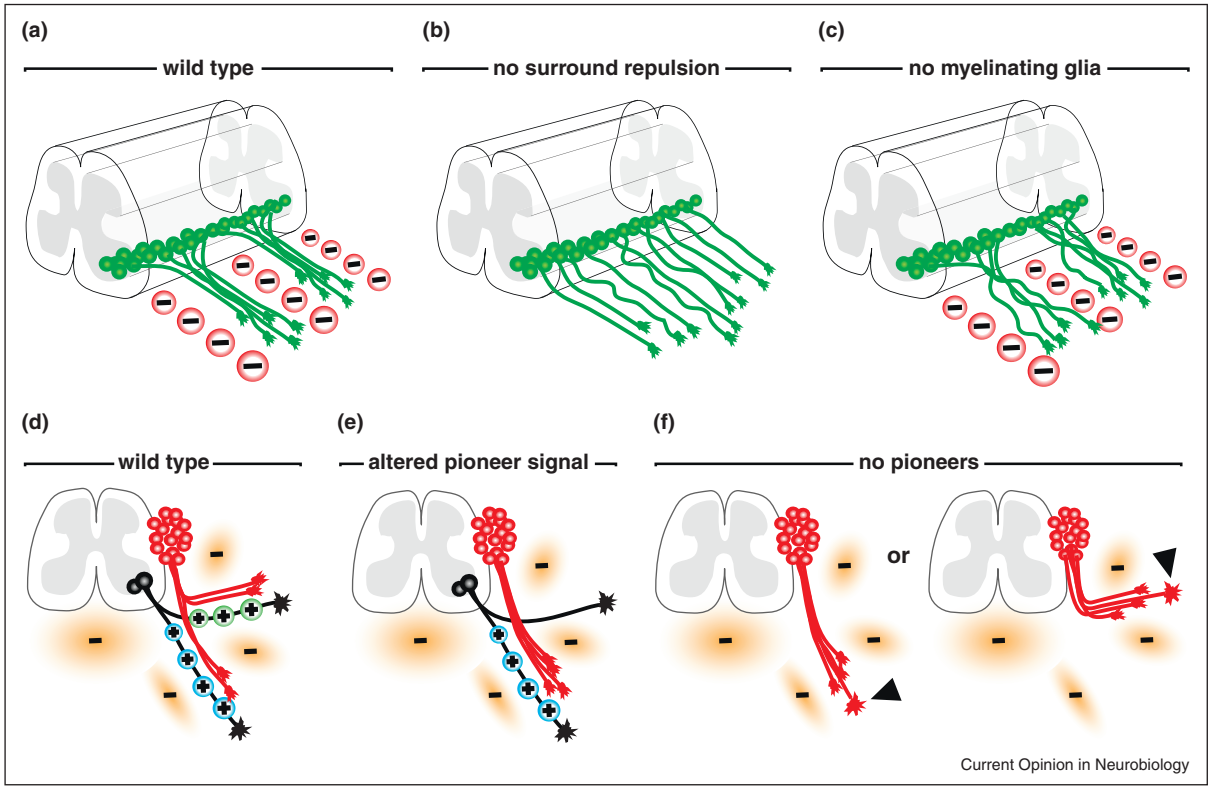
suggested by Paul A. Weiss to involve contact-dependent axon–axon interactions, for which he introduced the term 'selective fasciculation' [3,38]. Dramatic examples of selective fasciculation can be observed in insect and fish embryos, involving the generation of dedicated pioneer neuron populations that seek out specific trajectories, and which are eliminated once they served their purpose of guiding follower neurons [11,39]. Ample evidence in both invertebrates and vertebrates support the principle importance of pioneer axons for establishing accurate follower axon projections [6–9,12–17,21,39]. In many instances involving homotypic interactions between axons originating from the same neuron type, however, it is less clear to what extent pioneer axons indeed differ from follower axons other than their timing of outgrowth [40]. Here, we will distinguish between such relatively non-selective fasciculation of pioneer with follower axons (primarily driven by homotypic axon–axon adhesion) and selective fasciculation (driven by discriminating axon–axon interactions).

Selective fasciculation

Heterotypic selective fasciculation. Selective fasciculation provides an attractive model for how the growth trajectories of different axon types become coordinated as a prerequisite to their incorporation into common nerve tracts and circuits. Vertebrate peripheral nerves, for instance, accommodate several axon types that are part of the circuits providing efferent control over skeletal muscle or organ function, as well as somatosensory afferent input to the nervous system [41]. At an even higher level of complexity, the vertebrate brain is crisscrossed by white matter tracts comprising axons from hundreds of different neuron types linking distant neural territories as parts of higher order functional assemblies [42].

Sequential extension and heterotypic guidance. The first axons to populate peripheral nerve tracts are motor axons [43,44,45••], which from the outset possess a remarkable capacity for choosing trajectories towards correct muscle targets [46–48]. Most available data suggest that dorsal

Figure 2

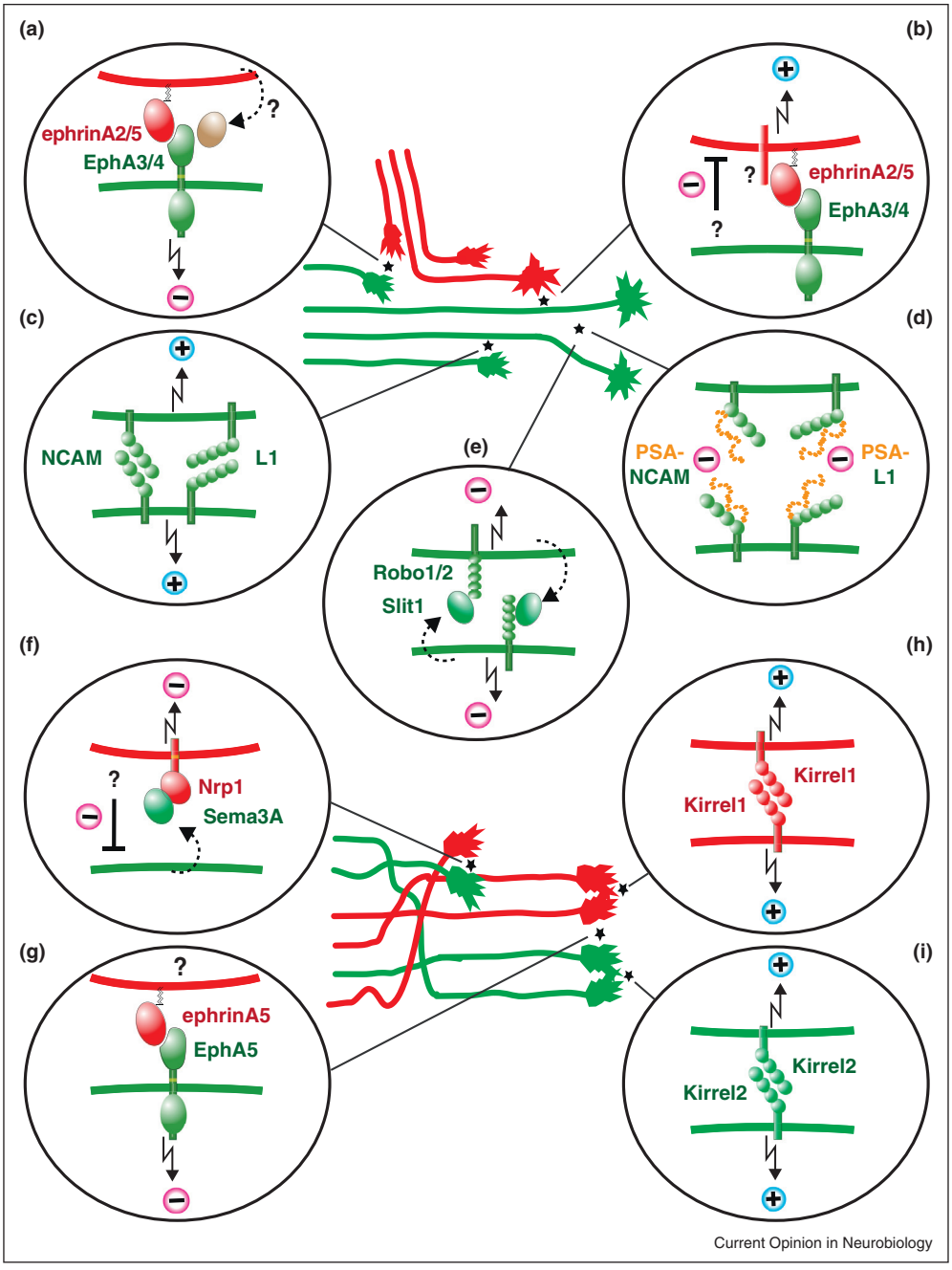


Non-axonal factors and axon–axon interactions. Influence of non-axonal factors on generalized **(a)**–**(c)** and selective **(d)**–**(f)** axon–axon fasciculation illustrated by mutations disrupting vertebrate peripheral nerve organization. (a) During normal development, peripheral motor axon extension is confined to narrow permissive tissue corridors, surrounded by segmental repulsive tissue blocks [32–34]. (b) Mouse mutants devoid of repulsive sclerotomal tissue blocks allow axons to initially extend as an almost continuous defasciculated sheet, instead of forming discrete peripheral nerve segments [104,105]. (c) Mouse mutants lacking peripheral myelinating glia (Schwann cells) exhibit pronounced axon defasciculation, while overall nerve segmentation is preserved [36,37]. (d) Normal subdivision of peripheral nerve involves selective fasciculation of follower (sensory: red) along pioneer (motor) axons (green) [45••], patterned by extrinsic permissive and repulsive signals [32–34]. (e) Upon loss of signal (EphA3/4) from one subset of pioneer axons, follower axons preferentially chose other pioneer fascicle [45••]. (f) Wholesale absence of pioneers results in follower axons randomly choosing a single trajectory in an all-or-nothing fashion; presumably driven by initial randomized trajectory choice by first-extending sensory axons (arrowheads), followed by fasciculation of later-extending axons [45••].

root sensory axons, which generally lack a predetermined preference for specific trajectories or targets [49,50], rely on the association with preceding motor axons to establish appropriate peripheral connectivity patterns [6–10]. Our own recent data suggest that this involves contact-dependent heterotypic interactions that prompt sensory growth cones to change course and commence tracking along preceding motor axons (Figure 3b) [45••,51]. These events rely in part on attractive signaling provided by EphA receptor tyrosine kinases (acting in a kinase-independent manner) that engage cognate ephrin-A proteins on sensory growth cones and directly or indirectly collaborate with additional homo and heterotypic axon–axon mechanisms (Figure 3a–e) [45••].

Timing of extension and heterotypic guidance. In the developing mammalian brain, cortical and thalamic axons meet in the subpallium to form the internal capsule, a major white matter tract, before projecting in opposite directions to thalamus and neocortex, respectively [42]. Experimental conditions that selectively eliminate or alter thalamocortical axon trajectories perturb corticothalamic projections and *vice versa*, suggesting that both axon types rely on mutual interactions for proper navigation [42,52]. These heterotypic axon–axon interaction appear to be subject to tight temporal regulation provided by transient release of repulsive cues by subpallium neurons, which stall early extending corticothalamic axons and thus facilitate their rendezvous with later extending thalamocortical axons [53••]. While molecular and cellular mechanisms underlying the corresponding axon-axon interactions remain largely unresolved, recent genetic evidence suggest the intriguing possibility that this involves selective expression of the cannabinol receptor CB_1R by

Figure 3



Axon–axon signaling mechanisms in nerve and circuit assembly. Examples of identified axon–axon signaling mechanisms driving sequential stages of peripheral nerve **(a)**–**(e)** and olfactory sensory map assembly **(f)** and **(g)**. (a) Heterotypic motor (green) and sensory (red) axon segregation via repulsive ephrin-A/EphA forward signaling and postulated additional EphA-activating factor [27]. (b) Heterotypic selective fasciculation driven by switch to attractive EphA/ephrin-A reverse signaling (via yet unknown co-receptor) at later stages; postulated additional repulsive activity [45••]. (c) Homotypic fasciculation driven by NCAM and L1 [59,60]. (d) Localized defasciculation by polysialylation (PSA) of NCAM and L1 at axon choice point [59,60]. (e) Overall degree fasciculation balanced by juxtaparacrine and/or autocrine repulsive Slit/Robo signaling [69•] (note: action of depicted factors in same subsets of axons is conjectural). (f) Pre-target sorting of olfactory sensory axons via Sema3A/Nrp1 repulsive signaling (for simplicity, only two axon identities are drawn) [78••]. (g) Segregation of axon termini of different identities via repulsive (bi-directional?) EphA5/ephrin-A2 signaling [82,106]. (h) and (i) Convergence of axon termini with matching type II identities on same glomeruli via Kirrel1 or Kirrel2 homophilic adhesion [82] (for simplicity, early and late developing type I and type II axon identities are depicted in same schematic).

corticothalamic axons and its activation by 2-arachidonoyl glycerol (2-AG) synthesized by thalamocortical axons [54•].

Homotypic selective fasciculation. Cell adhesion molecule (CAM)-mediated axon–axon adhesion seems to be a primary motor for the selective fasciculation of homotypic follower and pioneer axons in both *Drosophila* and *C. elegans*. In the former, homophilic axon–axon adhesion by neural cell adhesion molecule (NCAM) isoforms promote selective fasciculation of follower axons with longitudinal fascicles [55,56], while in the latter, the cadherin Flamingo seems to mediate general association of follower with pioneer ventral nerve cord axons [57•]. Similarly, gain-of-function experiments in chick suggest that cadherin-mediated homophilic axon–axon adhesion could underlie the association of axons expressing certain cadherins with specific white matter tracts [58].

Selective defasciculation. Localized defasciculation at discrete choice points can play vital roles in axon navigation by allowing subsets of axons to dissociate from larger fascicles, to respond to novel guidance cues and to enter target-bound trajectories [21,31]. In chick, for instance, post-translational polysialylation of the CAMs NCAM and L1 is thought to underlie defasciculation of motor axons at the limb plexus, a major peripheral choice point, by reducing homotypic axon-axon adhesion (Figure 3c,d) [59,60]. In *Drosophila*, repulsive Sema1a/PlexinA signaling or the action of CAM-like receptor tyrosine phosphatases appear to antagonize NCAM-mediated homotypic axon–axon adhesion, thus promoting localized defasciculation of subsets of motor axons from transiting intersegmental nerves and facilitate intrasegmental muscle innervation [55,56,61–63]. Such transient dissolution of axon–axon adhesive bonds and its eventual transition to renewed fasciculation may involve delayed-acting intracellular pathways that shut off repulsive guidance receptor signaling [64•]. Repulsive signals provided either by axons or surrounding tissue appear to frequently regulate the overall degree of fasciculation (Figures 2a,b and 3e) [32,33,65–68,69•], but whether this similarly involves direct crosstalk with mechanisms driving axon–axon adhesion remains to be explored.

Segregation and sorting

Axon segregation and sorting typically involve an interplay of homophilic adhesive and repulsive axon–axon interactions that cooperatively promote the confinement of heterotypic axons into discrete fascicles, the self-organization of homotypic axons into orderly arrays or the convergence of certain subsets of axons onto, and the exclusion of others, from discrete target zones (Figure 3f–i).

Anatomical and functional segregation. The pooling of multiple axon types into common nerve tracts is a pervasive feature of nervous system functional architecture. This side-by-side arrangement of different axon types may be inherently vulnerable to conditions that promote illicit intermingling of functionally disparate neural pathways and circuits [70]. In the vertebrate peripheral nerve, for instance, different axon types segregate into discrete fascicles soon after emerging from neural tube or dorsal root sensory ganglia [27,71], which is recapitulated by the mutual segregation of cultured motor and dorsal root sensory axons in the absence of other cellular components [27]. The apparent self-organization of both axon types into discrete fascicles involves contact-dependent repulsive axon–axon signaling, in part elicited by EphA receptors on motor axons and cognate ephrin-A proteins on sensory axons (Figure 3a), whose inactivation in mouse embryos arrogates the anatomical and functional segregation of motor efferent and somatosensory afferent pathways [27,45••]. In the corpus callosum, the major white matter tract connecting both hemispheres of the brain, similar contact-dependent axon–axon interactions effectively segregate axons originating from anterior or posterior cortical neurons; a process likewise involving repulsive EphA/ephrin-A signaling [72•].

Pre-target sorting and mapping. Perception and proper classification of external stimuli relies on the orderly representation of sensory modalities carried by the axons of primary sensory neurons into the nervous system [73–75]. In *Drosophila* and mouse, for instance, primary olfactory axons expressing the same odorant receptors converge on common stereotypically positioned target foci (glomeruli) in antennal lobe or olfactory bulb, respectively [73]. While axon–target interactions play a role in setting up these odorant representation maps, their assembly seems to a large degree reliant on axon–axon interactions [18,76,77]. In mouse, olfactory sensory axons appear to self-organize into orderly arrays that forecast their eventual mapping order across the anteroposterior axis of the olfactory bulb; a process driven at least in part by axon–axon repulsion involving varying levels of Sema3A or its receptor Nrp1 in olfactory sensory axons expressing different odorant receptors (Figure 3f) [78••]. Similar pre-target sorting of axons occurs in the developing visual system of vertebrates [79,80], but whether pre-target sorting in the optic nerve would predominantly rely on axon-axon interactions remains unclear [81].

In-target segregation and mapping. In the olfactory systems of mouse and *Drosophila*, parallel homophilic axon–axon adhesion and contact-dependent repulsion seem to drive the eventual convergence of matching axon termini onto specific glomeruli or protoglomeruli and the exclusion of non-matching axons [82]. In *Drosophila*, this involves Sema1a expression by early extending olfactory sensory axons repelling late arriving axons expressing its cognate receptor Plexin-A [83,84]; events that cooperate with parallel homophilic axon–axon adhesion by N-cadherin to confine early and late arriving axons to discrete

protoglomeruli [83]. In mouse, differential regulation of the CAMs Kirrel2 and Kirrel3, as well as EphA5 and ephrin-A5, by neural activity results in the complementary expression of these proteins by subsets of olfactory sensory axons [82]. Neural activity-driven regulation of adhesive codes may thus effectively establish a range of axon identities extending from sensory neurons tuned to specific odorants — which, for instance, results in the segregation of EphA5$^+$ and ephrin-A5$^+$ axons (Figure 3g), paralleled by homophilic adhesion of Kirrel2$^+$ or Kirrel3$^+$ axons and their convergence onto discrete glomeruli (Figure 3h,i) [82]. Similar mechanisms based on co-action of axon–axon adhesive and repulsive forces may be reiterated in other olfactory axon identities targeting different glomeruli [85,86].

Competition and tiling

Regular spacing of axons and their terminal arbors is a common feature of topographic sensory representation maps and columnar circuit architecture in general [29]. Axon spacing entails repulsive (competitive) axon–axon interactions similar to those involved in axon segregation, but which effectively result in the mutual avoidance of axons of the same identity [20,29]. Such isotypic axon–axon repulsion can further result in the establishment of non-overlapping terminal arborization zones (tiles) [20,29]. Axon competition and tiling can operate independent of or together with neurotrophin-based and/or neural activity-based mechanisms [29,87–90].

Competition, tiling and columns. In the visual system of *Drosophila*, individual R7 or R8 photoreceptor and L1–L5 laminar axons form terminal arborizations and connections that are restricted to single columns in the medulla [91]. Establishment of these columnar connectivity patterns relies on both competitive axon spacing and tiling of axon terminal arbors. For instance, isotypic axon-axon repulsion mediated by the transmembrane protein Golden goal and the cadherin Flamingo in R8 axons seems to counterbalance cadherin-mediated axon–axon adhesion; thus driving axon spacing prerequisite for innervating discrete medullar columns [92,93]. The columnar tiling of axon terminal arbors by L1 or R7 axons is eventually achieved by isotypic axon-axon repulsion mediated by Dscam2 (Down syndrome cell adhesion molecule 2) or the Ig superfamily protein Turtle, respectively [94,95•]. In addition to these axon contact-dependent mechanisms, columnar tiling of R7 axons further seems to involve autocrine action by the secreted TGFβ family protein Activin, apparently cooperating with contact-dependent repulsion by Turtle [96]. Even more fine-grained isotypic axon competition is thought to be achieved by a vast array of Dscam isoforms generated by alternative splicing in *Drosophila* mushroom body neurons [97]. This entails axon–axon repulsion triggered exclusively between axons expressing the same Dscam isoforms, effectively keeping axon branches apart that extend from the same neurons [97].

Competition and continuous mapping. In the vertebrate visual system, retinal ganglion cell axons continuously map across the tectum or superior colliculus in a manner that accurately mirrors the relative positioning of ganglion cells across the retina; effectively creating a point-to-point representation of visual space in the brain [19,74]. The establishment of this retinotopic map involves axon–target interactions, as well as spontaneous waves of retinal ganglion cell firing [89,90,98], but its key features are most parsimoniously explained by models evoking competitive axon–axon interactions [99,100]. The extent to which retinotopic map formation relies on competitive axon–axon interactions, however, seems to vary between different vertebrate species [101], possibly as a function of body size [19]. Moreover, while axons originating from opposite extremes of the retinal axis repel each other in culture [102], the cellular and molecular mechanisms underlying these long-postulated axon–axon interactions *in vivo* remain to be tackled.

Concluding remarks

Mechanisms driven by signal exchange between axons facilitate alignment or segregation of functionally analogous or distinct axon types, the sorting of axons into separate target-bound fascicles, the self-organization of axonal arrays during establishment of topographic projection maps, as well as the confinement of axons and their terminal arbors to non-overlapping target zones and postsynaptic partners. Axon–axon recognition, as a precursor to all of these events, can simply involve expression of complementary adhesive codes — but can also entail intricate neural activity and second messenger-controlled differences in adhesive codes that effectively generate large assortments of axonal identities, each extending from a neuron with a distinctive functional profile. Understanding such self-organizing properties of neurites may eventually prove key to a more complete understanding of how the functional architecture of the nervous system assembles during development, and how some of its features could be restored in the adult. Since in most *in vivo* contexts axon–axon interactions will operate alongside non-axonal signals, a major challenge lies in delineating their respective contributions to nerve or circuit assembly, and how the different signaling inputs are integrated by growing axons. Also, most evidence for axon–axon interactions *in vivo* so far have been indirect, with the underlying cellular mechanisms essentially remaining a black box. Tackling these challenges should become increasingly attainable by exploiting the steadily expanding genetic toolkit for visualizing and manipulating discrete neuronal identities, combined with advances in imaging techniques, to resolve both: the molecular and cellular events driving axon–axon interactions.

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