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Dendrite enlightenment

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Abstract

Neuronal dendrites acquire complex morphologies during development. These are not just the product of cell-intrinsic developmental programs; rather they are defined in close interaction with the cellular environment. Thus, to understand the molecular cascades that yield appropriate morphologies, it is essential to investigate them *in vivo*, in the actual complex tissue environment encountered by the differentiating neuron in the developing animal. Particularly, genetic approaches have pointed to factors controlling dendrite differentiation *in vivo*. These suggest that localized and transient molecular cascades might underlie the formation and stabilization of dendrite branches with neuron type–specific characteristics. Here, I highlight the need for studies of neuronal dendrite differentiation in the animal, the challenges provided by such an approach, and the promising pathways that have recently opened.

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Introduction

Neurons can develop highly complex and neuron type–specific branched dendrites that have fascinated neuroscientists for more than 100 years [1]. The morphology of dendrites has tight functional constraints related to the localization and distribution of inputs received by the neuron and to the way in which information is integrated and processed along dendrites, defining the output of the neuron [2–4]. Not surprisingly, thus, the mechanisms that support the establishment of the neuron type–specific morphology of dendrites during development have been a major focus of research for the past three decades [5–8].

The definition of specific dendrite morphologies as well as of other neuron type–specific properties depends on intrinsic transcriptional codes that are established during development [9]. Cascades of events triggered by positional information relative to the body axes lead to the expression of combinations of transcription factors that define the morphology of the neuron. A well-studied example is afforded by the dendrite arborization (da) neurons of the *Drosophila* larva that can be subdivided into four classes (c1da–c4da) with distinctive functional and morphological properties [10]. Manipulating the relative abundance of key transcription factors shifts the morphology of a particular da neuron from one class to another [11,12]. Only a few of the downstream factors that translate the information contained in those codes into structural traits are known [13–16]. The general outcome of this transcriptional information is likely represented by the expression of several effectors, including regulators of the cytoskeleton and receptors for extrinsic signals. The activation and localization of those effectors represent the next layer of regulation requiring precise spatiotemporal information.

Owing to these intrinsic programs, some aspects of neuron type–specific morphology are preserved also in culture after the neuron has been extracted from its natural environment, its processes severed, and it has been induced to regrow in a petri dish [17]. Multiple aspects of the complex and neuron type–specific morphology of dendrites, though, depend on localized signals and specific interactions with substrates and partners. Pyramidal hippocampal neurons in culture become highly complex and multipolar [18,19]. In contrast to their *in vivo* morphology, though, they do not form a primary dendrite (S. Dupraz and F. Bradke, personal communication). Neurons in the insect central nervous system are mostly unipolar as their cell bodies are positioned outside of the neuropile [20]. Those same neurons, such as the Kenyon cells of the *Drosophila* mushroom body, acquire a multipolar morphology in culture [21]. Clearly, the context plays a central role in the definition of dendrite morphology. This is particularly important for the formation of maps of sensory inputs in the developing nervous system, such as in the barrel cortex of rodents, in which the position of dendrites sustains the formation of discrete maps [22].

Therefore, to understand how dendrite patterns are established, it is mandatory to investigate neuronal differentiation in the animal.

In this review, I will focus on the progress in understanding the cell biology of dendrite differentiation in a developing animal. I will summarize the key tools and techniques that currently advance our experimental access to the subcellular organization of neurons *in vivo*, an essential prerequisite for understanding the cell biology of differentiating neurons during animal development.

The dynamics of dendrite differentiation

Visualizing neuronal differentiation *in vivo* is challenging. This is particularly true in organisms in which the relevant developmental stages happen in the womb, but observing individual neurons over time in the same animal, while development is happening, presents a number of complications in every system. Tissues undergo morphogenic rearrangements, whereas the observed neurons migrate and change in size and shape sometimes quite rapidly, with fine process dynamics happening on the scale of minutes. The choice of the system is therefore critical. Pioneering work took advantage of transparent developmental stages of organisms such as *Xenopus laevis* tadpoles or zebrafish larvae to investigate the pattern of extension and retraction of dendrites of optic tectal neurons or the stepwise process that leads to retinal ganglion cell dendrite laminated organization [23–26]. Via DiI uptake or the expression of genetically encoded fluorescent markers, the dendrites of optic tectal neurons could be imaged by confocal microscopy over multiple days, revealing distinct phases of dendrite growth, including a period of exuberant extension and branching and a late phase of stabilization [24,25,27]. Already in those early studies, the initial observation of dendrite dynamics raised important questions about the factors that decide which dynamic branches become stabilized and which ones retract. The formation of a synaptic contact supports the stabilization of a branch favoring its maintenance [23,28–30].

Mammalian systems are in general less amenable to *in vivo* imaging during development. In adult rodents, live longitudinal imaging of individual neurons has been pioneered already in the early 2000s through the injection of cell-permeant indicators or the use of transgenic mice expressing cytoplasmic green fluorescent protein (GFP) in random subsets of defined neuronal populations [31,32]. Implanting permanent cranial windows in these transgenic mice and performing 2-photon imaging revealed dendrite and spine dynamics in superficial layers of the adult cortex and later even in deeper regions such as the hippocampus [33–38]. In spite of this progress, access to the developing nervous system has been particularly challenging, as parts of neuronal differentiation happen while the embryo is in the womb. Nevertheless, in the rat and even in the mouse, cranial windows were used at neonatal stages to investigate the dynamics of dendritic spines in somatosensory cortex neurons after sensory deprivation or the interplay between intrinsic and stimulus-driven activity in shaping the refinement of the circuits in the mouse barrel cortex [39–41]. Cerebellar granule neurons (CGNs), the most abundant cell type in the cerebellum, elaborate their dendrites in postnatal stages and are therefore more approachable for imaging early steps of dendritogenesis [42]. CGNs display on average four dendrites, each terminating with a claw that enwraps a mossy fiber axonal bouton, and respond to the coincident activation of multiple mossy fiber boutons [43–45]. To achieve this very specialized dendrite organization, multiple exuberant processes are initially formed, of which only 3–5 become stabilized and form a claw in the course of about five days [46]. Claw formation is not necessary for the selection of the branches that will be stabilized, but once the claw is formed, the branch will remain stable [46]. In addition, the refinement of barrel organization in the barrel cortex happens in early postnatal stages in the mouse, and it involves the asymmetric distribution of the dendrites of stellate neurons [47]. By imaging the differentiation of individual stellate neurons *in vivo* every few hours using 2P microscopy, it became clear that the asymmetric arborization of those dendrites toward the center of the barrel is obtained by differential stabilization of the branches. In fact, dendrite branches oriented toward the outside of the barrel are formed, but are very likely to retract [48]. Thus, longitudinal imaging of dendrite growth, even at low time resolution, can help to elucidate the logic underlying the establishment of specific dendrite morphologies.

A major boost in the understanding of the molecular genetics of dendrite differentiation followed the establishment of *Drosophila* lines that express cytoplasmic GFP in a subset of neurons of the peripheral nervous system of the larva [49]. The four classes of multidendritic dendrite-arborization neurons or da neurons, mentioned previously, develop their dendrites in a 2-dimensional field underneath the transparent cuticle of the fly larva and are thus ideal for *in vivo* imaging by confocal microscopy. In this system, genetic tools to visualize and manipulate individual neurons were soon combined with large-scale mutagenesis screens to identify core and novel factors involved in regulating neuronal morphology [6]. Findings obtained through those screens over the years have helped to define the molecular players of multiple key phenomena, including 'self-avoidance' and 'tiling', central conserved rules for the establishment of dendrite trees, reviewed elsewhere [7,50]. Thanks to the accessibility of these neurons, it has been possible to image dendrite elaboration in the same neuron every few hours, similarly to what

described previously for the mouse experiments [51]. In addition, the larvae can be immobilized, and da neuron dendrites can be imaged every few seconds, allowing approaching the cell biological mechanisms of branch site selection, extension, retraction, and stabilization [52–57]. The c4da neuron high-order branches extend and retract in an exploratory fashion, changing their direction of growth. In contrast, fascin-enriched high-order branches of c3da neurons tend to extend and retract, at a velocity of approximately 0.4 μm/min, in a linear trajectory [13,56]. Finally, the initial formation of higher-order branches in c1da neurons is stochastic, and their velocity of growth varies with developmental time [52]. The main branches of da neurons extend at early stages of development that have been less accessible to imaging. Only recently, studies have followed the detailed elaboration of dendrites starting from the embryonic stages in which the primary branches become defined [51,52,55]. Importantly, these studies have delivered the primary data for computational models describing neuronal dendrite differentiation. Through the early phases of primary branch elaboration of the complex and space-filling c4da neurons, the c3da neurons just as the sparse and selective c1da neurons follow a highly conserved pattern of branching that is random and simply based on the parsimonious usage of cable length [51,55,56]. The c4da neurons follow these simple conserved rules throughout their differentiation stages to fill in their entire receptive field [51]. In contrast, the proprioceptive c1da neurons transition to a phase of branch loss that is stochastic and molecularly driven by *dscam1* and that results in comb-like–shaped dendrites, in which second-order branches are parallel to the direction of body contraction [52,55]. This is important because, owing to their orientation, these branches experience a large curvature rise during contraction, which is suggested to increase the opening probability of mechanogated ion channels initiating the cell response [55,58,59]. Finally, the differentiation of c3da neuron dendrites includes a second, divergent phase in which local rules dominate over the basic limitation of cable length [56].

Some of the da neurons completely shed off their branches approaching metamorphosis and reform dendrite branches with renewed patterns during the pupal stages [60,61]. Visualization of primary branch growth is more approachable in those stages, which thus represent an interesting option [57]. Furthermore, because da neurons do not receive direct synaptic input, few laboratories turned to investigating dendrite dynamics in the central nervous system of *Drosophila* [62,63].

Similar to the da neurons, dendrites of the somatosensory PVD neuron of *Caenorhabditis elegans* are also ideal for live imaging because they form an orthogonal array of superficial branches with a highly reproducible pattern, right under a transparent cuticle [64–66]. This has allowed a detailed description of the stepwise elaboration of dendrites in this neuron, supported by the dynamic extension and withdrawal of branches organized by contact-dependent self-avoidance [66].

In summary, the chance of gaining a glimpse into the early stages of dendrite differentiation *in vivo* currently strictly depends on the model organism, on the developmental stage, and on the particular neuronal type. Thus, the dynamics of the morphological steps leading to dendrite maturation have been carefully described in a limited selection of neurons. Nevertheless, the neurons of choice have very distinct morphologies, from the space-filling c4da neurons of the *Drosophila* larva to the highly selective CGNs of the mammalian cerebellum, allowing asking general versus specific aspects. Based on these data, dendrite elaboration appears to start with the formation of exuberant processes that are dynamic and extend and retract over the course of minutes. A second phase might follow, in which neuron type-specific branches are formed or in which subsets of branches are stabilized while others are eliminated. The proportion, timing, and properties of these events depend on the particular neuronal type analyzed.

Subcellular localization of molecular effectors *in vivo*

The morphological description of dynamics combined with genetic and molecular analysis has pointed to an increasing number of key factors that control dendrite differentiation *in vivo* [5,8,67]. Nonetheless, the dynamic cellular context in which those factors operate *in vivo* might remain elusive. Indeed, biochemical reactions are controlled in cells to a large extent by the specific subcellular localization of the molecules involved [68]. For cellular events to take place, the local concentration of multiple relevant factors might need to reach a certain threshold related to their specific chemical properties. In broad terms, these concepts have important implications for how we think of dynamic molecular cascades of events happening during cellular differentiation. In fact, if the subcellular localization of molecular players and their local concentration are key elements of the regulation of cellular processes, it is of essential importance to reveal when and where molecular interactors come together to initiate a cellular phenomenon. This is particularly relevant for highly polarized cells, such as neurons, that form dendrites arborizing with specific morphological characteristics and dynamics in the complex context of the developing nervous system.

Achieving the temporal and spatial resolution required to reveal the dynamics of subcellular localization of molecules *in vivo* in defined neuronal populations is challenging. For instance, the main approach to visualize

the subcellular localization of a molecule *in vivo* requires generating transgenic animals that express a fluorescently tagged version of this molecule in a defined subset of neurons. The impact of the tag on the function and localization of the molecule, as well as the effect of potential overexpression, needs to be carefully controlled. Regardless, for certain molecular contexts, for instance the molecular control of actin dynamics, tools to achieve this in cultured motile cell types or dissociated neurons undergoing differentiation have been actively developed for years [69]. The complex dynamics of actin can be revealed via fluorescent molecules that reversibly associate with filamentous actin in those contexts or by GFP-tagged actin (e.g. [70,71]). In addition, the localization of GFP-tagged actin regulators helps to infer the local dynamics of actin filament nucleation, elongation, or severing [53,69,72,73]. In the past few years, super-resolution imaging of actin even in the thin dendrites of superficial layers of the cortex in transgenic mice could be achieved using *in vivo* 2-photon stimulated emission depletion (2P-STED) microscopy. This permitted a direct view into actin dynamics within dendrite spines, which is related to the functional potentiation of individual synapses [74,75].

In the invertebrate model systems that I described previously, genetically encoded fluorescently tagged constructs are frequently used to investigate the subcellular spatiotemporal distribution of molecules [76–78]. Transient actin patches were thus correlated in dendrites of c4da neurons of the *Drosophila* larva with the initiation of collateral branch formation [53,54]. Those patches are locally initiated by the activation of Arp2/3 complex-mediated nucleation of branched actin filaments triggered by the WAVE complex (Figure 1). The transient recruitment of Arp2/3 complexes can be detected as a punctum that predicts the site at which a novel branch will form [53] (Figure 1A and B). The site of activation of this cascade in *C. elegans* PVD neurons is positioned by a multiprotein ligand–receptor complex that thus defines the branching site accurately [79]. A combination of genetic analysis and time-lapse imaging can thus help to clarify the role of actin regulatory proteins in dendrite differentiation [56]. The actin-bundling protein fascin is enriched only in the high-order branches of c3da *Drosophila* neurons, but not in the other da neurons, and it accumulates in particular onto elongating branchlets. In the absence of fascin, these branches become bent and branched. This led to the suggestion that fascin bundles and stabilizes actin filaments in the extending branchlets, stiffening them and guaranteeing their typical straight elongation [13].

These types of experiments address the dynamic localization of proteins in the cell. However, many proteins can be rather ubiquitously localized in the cell, whereas their activation is tightly restricted to spatially defined domains. Thus, the specific location of the active protein gives relevant insight. Multiple tools to detect activated signaling cascades in neurons are described in an accompanying review [80], and genetically encoded tools are used routinely for recording neuronal activity, using transient surges in Ca^{2+} concentration [81]. An important set of conserved molecules in regard to the establishment of neuronal dendrite morphology are the small GTPases of the Rho, Rac, and Cdc42 families [82]. An approach to reveal the subcellular domains of Cdc42 activation *in vivo* took advantage of the reversible binding of Cdc42 to a specific peptide (CBD) on activation. Binding of the activated Cdc42 to a co-expressed CBD could be revealed by fluorescence resonance energy transfer (FRET) [83]. These constructs helped to correlate the localized, Pak1-dependent, activation of Cdc42 in *Drosophila* motor neurons during embryogenesis with the site at which dendrite branches form [63,83].

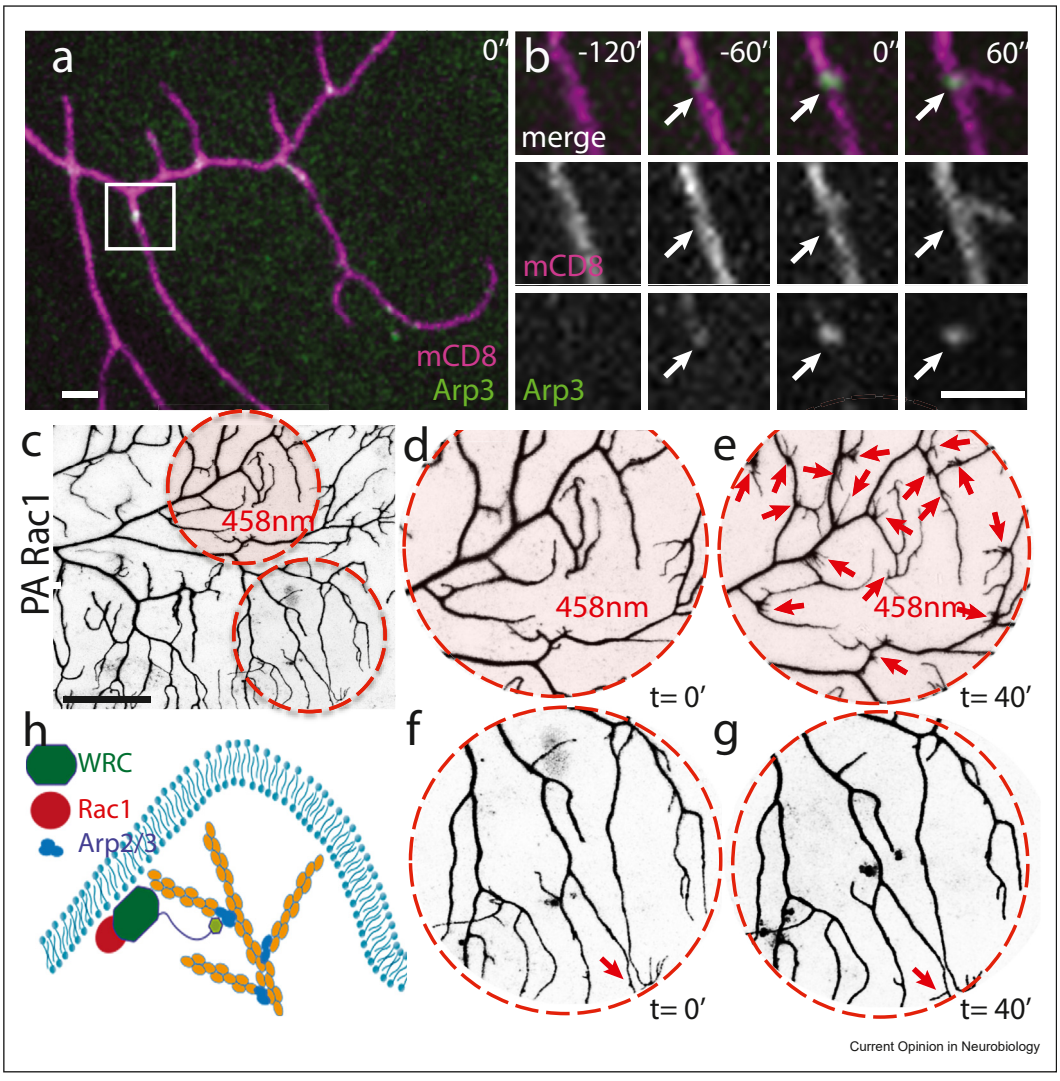
Taken together, via a combination of fluorescent markers and rapidly improving microscopy solutions, the subcellular localization and in specific cases also the localized activation of various types of molecules can be detected. This allows inferring where and when biochemical cascades are in action to induce morphogenic cellular changes.

Manipulating key molecular players to understand dendrite differentiation

Over the years, the knockout of individual genes has led to the discovery of important pathways controlling dendrite branching *in vivo* [5,7,67]. Nevertheless, the analysis of full knockdown of genes that might have multiple functions in different tissues during development can be complicated by functional compensation or by pleiotropic phenotypes confounding or even making the analysis of dendrite differentiation impossible. Conditional knockouts in defined neuronal populations or even generating individual mutant neurons help overcome multiple of these issues [47,84]. Regardless, the elaboration of dendrites is a rather late step during neuronal differentiation, after migration, polarization, neurite formation, axon extension. Important cellular regulators are likely to have functions early during cellular differentiation that hamper the interpretation of dendrite phenotypes. As an example, although cofilin is required for dendrite establishment, hippocampal neurons isolated from mice mutant for actin depolymerizing factor (ADF)/cofilin do not even form neurites [54,85,86].

One possible solution to this issue is to develop tools for acute activation or inactivation of proteins *in vivo*. The technical aspects behind these approaches in developmental biology have been extensively reviewed recently [87,88]. For *in vivo* applications in the nervous system, the constructs need to be expressed in transgenic animals in subsets of neurons. Furthermore, activation or inactivation should be ideally obtained by noninvasive

Figure 1



Initiation of dendrite branchlet formation in c4da neurons of *Drosophila* larvae. **(a)** Time-lapse imaging of differentiating c4da neurons of second instar *Drosophila* larvae expressing Arp3-GFP (green) and mCD8cherry (magenta) imaged every 60 s. **(b)** Three-minute time lapse of the region boxed in A. Arrows indicate the sites where a new branchlet will form. Scale bars: 10 μm. **(c–g)** Images from time-lapse recordings of a c4da neuron expressing membrane-tagged red fluorescent mCherry to visualize their membrane and photoactivatable constitutively active Rac1 (PA Rac1). The red circles represent a region of interest (ROI) illuminated with 458 nm light to activate PA Rac1 (orange background) and a ROI that was not illuminated. **(d–g)** Images of those two ROIs before (D, F; t = 0′) and after 40 min of photoactivation (E; t = 40′) or 40 min of no illumination (G; t = 40′). The arrows indicate some of the newly formed branchlets or groups of branchlets. **(h)** Schematic representation of the cascade of events leading to the formation of a dendrite branchlet. This starts with local activation of the WRC complex by Rac1, which in turn yields a local recruitment of the active Arp2/3 complex and thus the formation of a patch of branched actin filaments (Adapted from Stürner et al., 2019).

means. In non–warm-blooded animals, temperature-sensitive mutations represent a classical answer to these needs. The *Drosophila* gene *shibire* encodes for the conserved protein dynamin that forms multimers catalyzing the pinching off of endocytic vesicles [89]. Constructs carrying a temperature-sensitive *shibire* mutant can be expressed in specific tissues or neuronal subsets in *Drosophila* [90]. A shift in temperature yields a reversible block of endocytosis in these flies that can also quickly arrest neurotransmitter release. The systematic design of potential temperature-sensitive mutations could be noticeably accelerated by improvements in protein structure prediction [91]. A more common approach takes advantage of light (optogenetics) to modulate cellular functions and was pioneered to control activity in neurons [92]. For more broad cell biological approaches, domains derived from light-sensitive plant proteins were co-opted to modulate the regulation, clustering, or localization of proteins [87,88,93]. Such domains, as the light–oxygen–voltage

(LOV) domain, undergo conformational changes on blue light illumination with low intensity [94,95]. By positioning the LOV domain in a critical position within the structure of the target protein of interest, it is possible for instance to occlude an active domain in a light-dependent, reversible manner. Such an approach has been used to generate an optically switchable constitutive active form of the small GTPase Rac1 [96]. Localized activation of Rac1 obtained with this tool in c4da neurons of the *Drosophila* larva inducted a molecular cascade leading to activation of WAVE and recruitment of the Arp2/3 complex, thus locally initiating the formation of dendrite branchlets [53] (Figure 1C–H). LOV domains, as well as cryptochrome 2 (CRY2)/N-terminal domain of cryptochrome-interacting basic-helix-loop-helix 1 (CIB1), are used to sequester proteins to defined subcellular locations or to induce protein oligomerization [97–100]. In *C. elegans*, constructs including an LOV domain were used to display *in vivo* the role of different motor proteins in polarized trafficking in neurons [101]. Such acute manipulations of protein activity or localization are very exciting as they allow direct testing of causal relationships between certain molecular functions and cellular processes leading to dendrite differentiation. Nonetheless, these methods have many technical limitations. In the case of the LOV domain, the range of activating light is broad, although recently improved, making it difficult to restrict temporally its activation in the living animal. Depending on the wavelength of activation, penetration in deeper tissues might be problematic. Achieving a tightly localized activation, in certain cells or cell domains, such as few dendrites, requires careful titration [102]. In fact, there is no toolbox that works for all proteins. Instead, for each protein of interest, a potential solution needs to be identified and the obtained constructs then carefully tested in cells and finally in the animal. Despite these complications, the advantages of acute protein manipulation to dissect cellular processes during development are so evident that we can expect a large expansion of the toolbox in the coming years.

Conclusions and future perspectives

The systematic analysis of the cellular regulation of dendrite morphology establishment *in vivo* is coming of age, thanks to developments in protein visualization and manipulation combined with rapid advancement of microscopy solutions. These will allow asking more systematically important questions such as how are the sites of de novo dendrite branch formation defined, the sources of guidance signals for directed growth, or the cellular mechanisms that lead to selective branch stabilization. When observing the cellular phenomena that control the establishment of the variety of dendrite trees *in vivo*, a major question will be the extent to which common pathways and mechanisms are used. Is there a basic modular set of molecular pathways that can be used in various combinations to achieve neuron type–specific dendrite morphologies, or are *ad hoc* solutions used to solve specific tasks? Such fundamental questions for our understating of the elaboration of those varied morphologies need to be addressed in the appropriate context after all, the developing animal.

Conflict of interest statement

Nothing declared.

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* of special interest
** of outstanding interest

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