Title
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Journal
Cell Adhesion & Migration, 7(4)

ISSN
1933-6918

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Publication Date
2013-07-29

DOI
10.4161/cam.25000

Peer reviewed

Cell Adhesion & Migration 7:4, 388–394; July/August 2013; © 2013 Landes Bioscience

Journey to the skin

Somatosensory peripheral axon guidance and morphogenesis

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Keywords: somatosensation, trigeminal, dorsal root ganglia, Rohon-Beard, peripheral axon, axon guidance, neurotrophin, semaphorin, Slit, LAR receptor phosphatase

The peripheral axons of vertebrate tactile somatosensory neurons travel long distances from ganglia just outside the central nervous system to the skin. Once in the skin these axons form elaborate terminals whose organization must be regionally patterned to detect and accurately localize different kinds of touch stimuli. This review describes key studies that identified choice points for somatosensory axon growth cones and the extrinsic molecular cues that function at each of those steps. While much has been learned in the past 20 years about the guidance of these axons, there is still much to be learned about how the peripheral axons of different kinds of somatosensory neurons adopt different trajectories and form specific terminal structures.

The peripheral axons of tactile somatosensory neurons are among the longest axons in vertebrate animals, projecting from ganglia just outside the central nervous system to the skin, where they detect thermal, chemical, and mechanical stimuli. As they navigate to the periphery and establish their receptive territories in the skin, these axons encounter many different tissues and signals, including other cells in the ganglia from which they originate, the mesenchyme through which they navigate, axons of other neurons with which they fasciculate, and the skin cells at their termini. This review focuses on the somatosensory neurons that innervate the skin to detect touch, but other peripheral neurons, including proprioceptive and sympathetic neurons, as well as specialized neurons of cranial ganglia, share some of the same initial axon guidance mechanisms, despite innervating different terminal tissues. Since axon guidance and branching morphogenesis is usually studied on a step-by-step basis, it is easy to lose sight of the fact that a single neurite must integrate many instructive cues emitted by various tissues as they develop. To sense multiple navigational cues, individual neurons must express a variety of receptors, each of which is deployed at precise developmental stages and some of which serve multiple distinct roles during different phases of outgrowth.

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Submitted: 04/01/13; Revised: 05/07/13; Accepted: 05/10/13
http://dx.doi.org/10.4161/cam.25000

Most vertebrates possess two main populations of somatosensory neurons, clustered in ganglia just outside the central nervous system: trigeminal neurons that innervate the head and dorsal root ganglia (DRG) neurons that innervate the rest of the body (**Fig. 1A**). Larval fish and amphibians have an additional, transient population of somatosensory neurons located in the dorsal spinal cord, called Rohon-Beard (RB) neurons (**Fig. 1B**). These neurons are typically pseudo-unipolar, projecting central axons into the spinal cord or brain that connect to downstream circuits and peripheral axons to the skin (**Fig. 1C**) that innervate dermal sensory structures or terminate as free endings in the dermis or epidermis. This review highlights a selection of findings from all of these systems to illustrate the diverse navigational decisions that peripheral axon growth cones must make along their lengthy trajectories. Somatosensory neurons fall into many different subclasses that project at different stages of development and innervate different kinds of terminals.[1,2] Thus, different somatosensory neuron types face distinct navigational challenges but all must interpret multiple signals as they develop their complex, mature forms.

Initiation of Outgrowth

Neurotrophins were the first extracellular signals identified as regulators of somatosensory neuron development—nerve growth factor (NGF), a founding member of the neurotrophin family, was discovered over 60 y ago for its role in maintaining sensory neuron survival.[3] Numerous in vitro and in vivo studies have shown that neurotrophin (NT) signaling is not only essential for the survival of sensory neurons, but also required in many other processes, such as neuronal differentiation and axon outgrowth.[4] The discovery of programmed cell death pathways provided an opportunity to separate the survival effects of NTs from their other functions. In mice with a null mutation in Bax, a proapoptotic member of the Bcl-2 family, naturally occurring neuronal death was eliminated in peripheral ganglia but gross development of the nervous system appeared normal.[5] Combining Bax knockout with knockout of NGF or its receptor, tropomyosin-related

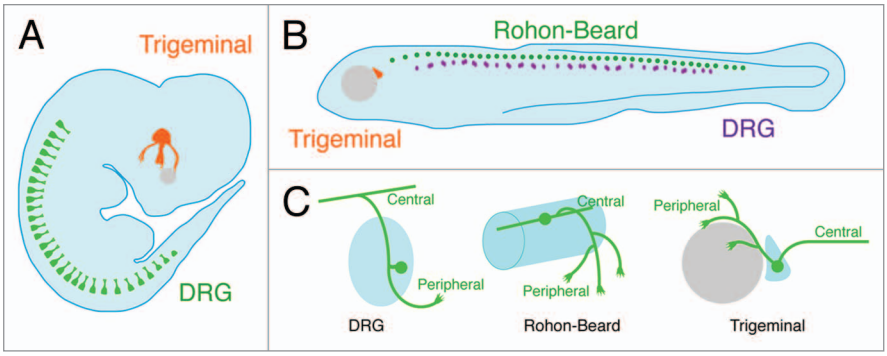

Figure 1. Anatomy of vertebrate somatosensory neurons. (**A**) Diagram of an ~E11 mouse embryo showing the location of the trigeminal ganglion (orange) and dorsal root ganglia (green) with growing peripheral axons. (**B**) Diagram of a zebrafish at ~3 d post-fertilization showing the location of the trigeminal ganglion (orange), Rohon-Beard neuron cell bodies (green), and incipient DRGs (purple). At this stage trigeminal neurons innervate the skin of the head and Rohon-Beard neurons innervate the rest of the body. DRGs, which at this stage consist of just a few cells each, arborize below the developing muscles; eventually, Rohon-Beard neurons will die and DRG neurons will innervate the skin. (**C**) Structure of DRG, Rohon-Beard and trigeminal neurons. DRG and trigeminal neuron cell bodies are located in ganglia and Rohon-Beard neurons are located in the spinal cord. All of these neurons project central axons into the central nervous system and peripheral axons into the skin.

kinase A (TrkA), allowed NGF/TrkA-dependent DRG neurons to survive. The central projections of these axons extended collateral branches into the dorsal horn of the spinal cord but their axon branches in the dermis and epidermis of the hindlimb were absent.[6] Axon counts performed in the saphenous nerve suggested that peripheral axons either never entered the major cutaneous nerve branches or could not be maintained. These experiments demonstrated that, for at least a subset of TrkA-dependent neurons, NT signaling is required at early steps of peripheral axon outgrowth. Not all NTs and NT receptors play analogous roles in the early outgrowth of their respective subtypes, but all regulate aspects of axon morphogenesis.[4]

Once a peripheral axon begins extending from a sensory neuron cell body, it must choose its initial outgrowth trajectory, a particular challenge for these pseudo-unipolar neurons since central and peripheral axons project in different directions, implying that the two axons respond to different cues. A study of RB neuron axon outgrowth in zebrafish larvae demonstrated that central and peripheral axons respond differently to the guidance cue Semaphorin 3D (Sema3D). Sema3D is expressed in the roof plate of the spinal cord, between the two rows of RB cell bodies.[7] In Sema3D-deficient embryos, fewer peripheral projections exited the spinal cord,[8] suggesting that they might normally be propelled toward the periphery by repulsive Semaphorin cues. Live in vivo imaging showed that peripheral, but not central, growth cones were repelled by ectopic Sema3D. Conversely, in transient axonal glycoprotein-1 (TAG-1)-knockdown embryos, central axons were defasciculated and apparently shorter, but peripheral axons were normal. Live imaging revealed that the overall advance of central, but not peripheral, growth cones was slower after TAG-1 knockdown. Together, these experiments indicated that axon guidance of central and peripheral axons can be specified by differential activation of receptors on these neurites.

Guidance to the Skin

Peripheral sensory axons often travel toward the periphery alongside motor axons before branching from the nerve trunks and approaching the skin. How are sensory peripheral axons guided to skin rather than muscle? In vitro assays and embryological experiments suggest that cues in the developing skin attract them. For example, axons from *Xenopus* DRG neurons projected toward skin explants in vitro, a phenomenon that appeared to be independent of NTs.[9,10] To test whether the skin regulates sensory axon guidance in vivo, Martin and colleagues ablated patches of chick dorsal wing ectoderm with UV irradiation. This treatment eliminated the cutaneous nerve plexus and its branches that should target the damaged field.[11] This finding supported the idea that a long-range signal from the ectoderm triggers divergence of cutaneous nerve branches from the deep mixed nerve. Since irradiation of ectoderm also damages underlying dermal tissue, Honig and colleagues used a surgical approach to remove patches of ectoderm from the chick hindlimb at various stages and assessed the consequences on peripheral nerve guidance.[12] These experiments showed that cutaneous nerves failed to form when ectoderm was removed at specific developmental stages. Together these studies suggest that the skin produces a long-range attractant for sensory axons that has yet to be identified.

Studies in trigeminal neurons provided another example for how a target-derived attractant might guide axons to the skin, while also contributing to the formation of specific patterns of innervation. Trigeminal neurons segregate into three branches (ophthalmic, maxillary, and mandibular) that project to distinct regions of the face. It has long been known from co-culture experiments that explanted maxillary or mandibular tissues can stimulate the directed outgrowth of trigeminal sensory axons, implying the existence of a target-derived attractant, termed "Maxillary Factor."[13] More than a decade later, the NTs brain-derived neurotrophic factor (BDNF) and Neurotrophin-3 (NT-3) were identified as the molecular components of Maxillary Factor in co-culture experiments.[14] However, these factors are expressed by both the target epithelium and the pathway mesenchyme of the maxillary and mandibular processes, arguing that NT-3 and BDNF may act as short-range signals instead of directional cues to instruct initial axon migration into the maxillary process. Moreover, mice deficient in both NT-3 and BDNF adopted the normal trajectory of trigeminal axons.[14] This finding hinted that multiple, redundant cues likely work together to guide axons, but left open the question of whether a long-range target-derived signal guides sensory axons to the skin.

Recent studies of zebrafish RB neurons identified another signaling system that regulates sensory axon guidance to the skin. Simultaneously knocking down two members of the leukocyte

common antigen-related (LAR) family of receptor tyrosine phosphatases in RB neurons, or inhibiting their function with dominant negative proteins, disrupted skin innervation by peripheral sensory axons.[15] Time-lapse imaging indicated that peripheral axon guidance, rather than outgrowth or maintenance, was defective in LAR-deficient neurons. The identification of LAR receptor tyrosine phosphatases as axonal receptors required for peripheral guidance raised the possibility that heparan sulfate proteoglycans (HSPGs), which guide axons in other systems via activation of LAR family members,[16-18] might be involved in skin innervation. Indeed, peripheral axons were misrouted in *dackel* mutants, which are defective in HSPG production.[19] Additionally, axons avoided HSPG-depleted areas created locally by injecting the enzyme heparinase III.[15] Together, these results support a model in which skin-produced HSPGs are attractive ligands for LAR receptors on RB neurons. Since the expression of LAR receptors in somatosensory neurons is conserved,[20] it is possible that they are also involved in innervation of the embryonic skin in other vertebrate animals. RB peripheral axons navigate a short distance from the cell body to the skin, and HSPGs can be membrane-bound or secreted, so it is not clear whether contact-dependent or diffusible HSPGs activate LAR guidance receptor proteins on peripheral growth cones. Identifying the specific HSPG core proteins that serve as attractants would help answer this question.

Positive Cues Contribute to Branching and Patterning in the Skin

Not all skin is the same: once in the periphery, some sensory neurons preferentially innervate specific regions of the skin. This distinction is most obvious for regions of the periphery innervated by axons that grow in stereotyped patterns, like the three branches of the trigeminal, and regions of skin that are innervated by different classes of neurons, such as hairy and glabrous skin in mice.[21,22] Regions of skin can also differ in the quantity, rather than the quality, of innervation. For example, there is a striking difference in the density of sensory fiber innervation between the hand and digit tips of humans, a pattern that correlates with differential sensitivity to mechanical and painful stimuli.[23] At least two mechanisms are used to create regionalized patterns of innervation. First, long-range or local cues attract or repel growth cones, thus steering sensory axons toward specific regions of the periphery. Second, factors that regulate the degree of axon branching in the skin influence the density of terminals, as well as territorial patterning, since axons that branch more have larger receptive territories. Guidance and branching cues thus together determine the characteristics of sensory innervation in specific regions of skin.

NGF was one of the first extrinsic factors found to stimulate branching of sensory axons. NGF is expressed in many areas of the skin at early stages of development, when DRG and trigeminal sensory neurons are extending axons, and its expression persists into adulthood.[4] In vitro studies demonstrated that NGF could promote the branching of sensory axons. For example, NGF-coated beads triggered directed collateral sprouting from nearby axons of cultured embryonic chick DRG neurons.[24] Studies of regeneration and collateral sprouting of cutaneous sensory nerves in rats provided early in vivo evidence for NGF's role in regulating terminal sensory axon branching. Diamond and colleagues isolated the receptive field of individual sensory nerves (emanating from a particular DRG) innervating the dorsal skin of the rat by removing surrounding nerves.[25-27] The nociceptive components of isolated nerves frequently expanded their sensory fields by sprouting collaterals into the neighboring, denervated skin. This process was completely halted by daily administration of antiserum to NGF.[25] Interestingly, regeneration of isolated sensory nerves following nerve crush was unaffected by blocking NGF signaling, indicating that NGF is essential for stimulating collateral sprouting of sensory axons but not for their guidance to the skin during regeneration.[27]

Mouse genetic studies have demonstrated that NGF promotes branching not only after injury, but also during development. For example, overexpressing NGF in mouse skin during development promoted increased innervation of the mouse mystacial pad, presumably due to more axon sprouting, as increased survival of trigeminal neurons alone could not account for the excess innervation in animals overexpressing NGF.[28] Conversely, as described above, Patel and colleagues observed reduced dermal and epidermal sensory innervation in the distal hindlimbs of NGF/Bax and TrkA/Bax double knockout mice.[6] However, there were also dramatically fewer axons in the saphenous nerve of double knockout mice, suggesting that reduced sensory innervation may be attributable to fewer axons reaching the skin, as opposed to a deficit in collateral branching at the axon terminals. A more recent study found that a majority of sensory axons were able to coarse into the limb buds of embryonic NGF/Bax double mutant mice, but failed to innervate and branch normally within the target territory.[29]

Similar to NTs, Slit/Robo signaling also promotes axon branching in the periphery. The Slit family of secreted proteins has been extensively characterized as repulsive signals for growing axons, most notably commissural interneurons in the developing spinal cords of mammals and ventral nerve cords of flies,[30] but Slit proteins appear to also positively regulate somatosensory axon branching. This function was first demonstrated by a series of biochemical purifications that isolated the N-terminal fragment of Slit2 for its collateral branch-promoting activity in dissociated rat DRG neuron cultures.[31] In vivo experiments in embryonic zebrafish supported a role for Slit in promoting axon branching: Global overexpression of Slit2 increased the branching and elongation of peripheral axons from trigeminal[32] and RB neurons.[33] Surprisingly, PlexinA4, commonly known for its role as a Semaphorin receptor, was required for the branch-promoting activity of Slit2 in zebrafish sensory neurons.[33] This function of Slit appears to be conserved in mammals, since in Slit2/Slit3 or Robo1/Robo2 double mutant mice branching of trigeminal axons surrounding the eye was reduced.[34] This branching defect was limited to the ophthalmic branch of the trigeminal nerve that innervates skin just above the eye, while peripheral innervation patterns of the maxillary and mandibular branches, as well as DRG axons, appeared largely normal. This finding illustrates the

principle that different peripheral targets produce unique molecular signals to stimulate innervation by appropriate sensory fibers.

Perhaps a clearer example of a signal that promotes innervation of specific peripheral targets is provided by the neurotrophin BDNF, which is required for innervation of *Xenopus* cement glands and mouse mammary glands. In *Xenopus* tadpoles, the cement gland expresses BDNF and receives mechanosensory innervation from the mandibular branch of the trigeminal nerve. Mandibular axons projected aberrantly following cement gland ablation and failed to innervate transplanted cement glands with reduced BDNF expression. Moreover, swapping out the cement gland for ectoderm overexpressing BDNF stimulated mandibular fibers to target and arborize within the ectopic tissue.[35] In contrast to its apparent guidance role in cement gland innervation, BDNF was recently shown to influence sensory innervation of mouse mammary glands by regulating axon survival. The sensory innervation of mammary glands is sexually dimorphic; while both male and female mammary glands receive innervation during embryonic development, at later stages it is rapidly lost in male embryos, prior to mammary gland regression. Androgen receptor signaling triggers this sensory pruning, since treatment of male embryos with androgen receptor antagonists preserved sensory innervation, and treatment of female embryos with testosterone caused loss of innervation, similar to that seen in males. This process was BDNF-dependent, as knocking out BDNF or its receptor TrkB similarly reduced sensory innervation in female embryos. The mechanism underlying this process of sensory denervation requires androgen-dependent overexpression of a truncated form of TrkB (lacking the intracellular tyrosine kinase domain) in the mammary gland mesenchyme. This truncated receptor presumably sequesters extracellular BDNF, thereby preventing signaling that maintains sensory axons. Indeed, knocking out expression of this truncated form of TrkB significantly increased sensory fiber density in male embryonic mammary glands.[36]

Molecular signals can also selectively stimulate particular subtypes of somatosensory axons to innervate appropriate targets. One example of this phenomenon comes from studies of the Neurturin (NRTN) protein in the development of mouse nociceptive neurons. NRTN is a member of the glia cell line-derived neurotrophic factor (GDNF) family of ligands and binds specifically to a signaling complex composed of the common GDNF receptor tyrosine kinase Ret and the coreceptor GDNF family receptor α 2 (GFRα2).[37-40] Nociception is mediated by peptidergic and nonpeptidergic unmyelinated C-fibers that terminate as free nerve endings.[1] Expression of GFRα2 is restricted primarily to a subpopulation of nonpeptigeric C-fiber neurons[41,42] and its ligand NRTN is expressed in the epidermis beginning at embryonic stages.[43,44] Knocking out GFRα2 dramatically reduced the density of nonpeptidergic free nerve endings innervating the footpad but had no effect on peptidergic nerve endings in the same area.[41] Importantly, this effect was not due to decreased survival or axon outgrowth, since the number of neurons in mutant DRGs, as well as unmyelinated axons in the saphenous nerve, did not change.[41,45] These results indicate that NRTN signaling through the GFRα2 receptor complex is important for stimulating terminal innervation by nonpeptidergic nociceptive neurons. Further supporting this idea, NRTN overexpression in the skin caused a corresponding increase in the density of nonpeptidergic, but not peptidergic, free nerve endings in the epidermis of the mouse footpad.[46] Overexpression of NRTN in the skin also increased expression of sensory ion channels and made animals more sensitive to mechanical pressure, cooling and menthol exposure, demonstrating that peripheral cues can contribute to specifying the functional properties of specific somatosensory neurons.

Negative Cues Contribute to Branching and Patterning at the Target

In addition to positive factors, repulsive cues restricting axon guidance and branching in certain regions of the periphery also contribute to creating patterns of somatosensory innervation. Semaphorins are the most extensively characterized negative regulators of sensory axon development and are perhaps best known for their role as repellents during axon guidance. Seminal experiments characterizing Sema1A (previously known as fasciclin IV) function in the development of Ti1 sensory neuron axons in the grasshopper limb bud showed that semaphorins also regulate branching. Blocking Sema1A signaling with monoclonal antibodies caused not only axon guidance defects, but also induced ectopic axonal branching of Ti1 axons.[47] This role for semaphorins in branching is broadly conserved, since mouse Sema3A inhibits the branching of peripheral sensory axons. Mutant mice lacking functional Sema3A displayed increased branching of peripheral axons from both trigeminal ganglia and DRGs.[48] Additional genetic studies demonstrated that Sema3A-mediated negative regulation of axon branching requires neuropilin and plexin coreceptors located on growing peripheral axons. Knocking out the gene encoding Neuropilin-1, or mutating its Semaphorin binding domain, eliminated Sema3A-mediated axon repulsion of DRG neurons in culture and increased peripheral branching in vivo, similar to Sema3A mutants.[49,50]

In addition to limiting branching, regionally expressed repulsive cues contribute to differential patterning of innervation territories by funneling axons into particular regions of the periphery. For example, the repulsive semaphorins Sema3A and Sema3F are expressed in specific patterns in the face. Knocking down both of their receptors, PlexinA3 and A4, caused the three trigeminal branches to defasciculate and become severely disorganized.[51] The ophthalmic branch was the most affected, misprojecting in multiple directions and invading regions from which it is normally excluded. At E12.5, heavily branched ophthalmic axons in the double mutant covered the entire face, including the eyes, demonstrating that repulsive cues pattern sensory territories by excluding innervation from certain regions of the skin.

Tiling of Axon Terminals in the Skin

Partitioning the skin into discrete sensory receptive fields is critical for animals to accurately detect and localize stimuli along the surface of the body. Each sensory neuron projecting to the periphery must coordinate the location of its peripheral projection with neighboring terminals to achieve an orderly arrangement of

sensory fields. This may be an easier task for those axons that innervate discrete structures in the dermis that are already spaced in an organized manner, such as hair follicles, Merkel cells, and various corpuscles, but poses a challenge to axons that invade the epidermis and terminate as free endings.

During embryonic stages interactions between growing neurites appear to play a role in arranging the territories of free endings with respect to one another. Studies in developing frog and fish embryos demonstrated that axon arbors of trigeminal neurons segregate from one another to form a "tiled" arrangement, promoting comprehensive innervation of the target territory while minimizing redundant innervation by neighboring arbors. In both systems, ablating the trigeminal ganglion on one side of the head allowed sensory axons from the contralateral ganglion to cross the midline, presumably due to removal of contralateral neighbors that compete for innervation territory.[52,53] Although time-lapse imaging in zebrafish suggested that contact-dependent repulsion between growing axons is the main mechanism limiting overlap between neighboring arbors,[8,53] competition for a limiting positive factor (such as an NT) may also contribute to tiling. The collateral sprouting and expansion of receptive fields observed by Diamond and colleagues following peripheral nerve isolation in rats suggests that somatosensory innervation in mammals is also governed by competitive innervation between somatosensory axons in the skin and requires NGF, at least as a permissive factor.[54,55] Intriguingly, one study of human patients who received trigeminal sensory root section to treat trigeminal neuralgia—effectively eliminating sensory innervation to one side of the face—observed similar expansion of mechanosensory and nociceptive receptive fields across the facial midline.[56] This receptive field expansion was presumably due to collateral sprouting of intact sensory arbors from the contralateral side of the face. Together these studies indicate that tiling is a conserved strategy for arranging sensory territories of free nerve endings.

Diversity in Somatosensory Axon Morphogenesis

Studies of peripheral sensory axon guidance during the past 20 y have collectively identified the key navigational challenges encountered by many peripheral somatosensory axons—for example, whether to grow toward the periphery or the central nervous system, when to exit from nerve bundles and grow toward the skin, and where and how much to branch in particular regions of the skin. Although there are surely guidance cues still to be discovered, these studies have identified many of the major players, some of which (most notably NTs and semaphorins) function at multiple stages of axon pathfinding and morphogenesis. Many of these cues affect particular populations of somatosensory axons, but are not limited to a single subtype. One of the major challenges for the future will be to identify the cues that make the axon morphologies of each kind of somatosensory neuron subtype different from one another.

Somatosensory neurons are a diverse group of cells, reflecting the heterogeneity of the chemical, thermal, and mechanical stimuli that they sense. Differential responsiveness to some of the guidance cues discussed in this review helps explain how different populations of sensory neurons adopt distinct trajectories, but responses to those cues alone are unlikely to generate the impressive diversity of somatosensory neuron morphologies. This morphological diversity is most apparent at the axon terminals in the skin. For example, some free nerve endings, which are often referred to as "unspecialized", form intimate structural associations with epidermal cells[57] and can display distinctive, subtype-specific termination patterns at particular strata of the epidermis.[58] The axon endings innervating dermal corpuscles and hair follicles are perhaps even more striking, forming unique, stereotyped terminals onto their targets. The intricate association between axons and these sensory organs suggests that corpuscles and hair follicles provide instructive molecular cues that sculpt terminal axon morphologies, but virtually nothing is known about the nature of those cues. The recent creation of genetic tools for visualizing specific classes of axon terminals[59-62] will make it possible to study their development and identify the molecular interactions that allow them to adopt their elegant morphologies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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