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The Emerging Role of Cranial Nerves in Shaping Craniofacial Development

Organs and structures of the vertebrate head perform a plethora of tasks including visualization, digestion, vocalization/communication, auditory functions, and respiration in response to neuronal input. This input is primarily derived from afferent and efferent fibers of the cranial nerves (sensory and motor respectively) and efferent fibers of the cervical sympathetic trunk. Despite their essential contribution to the function and integration of processes necessary for survival, how organ innervation is established remains poorly understood. Furthermore, while it has been appreciated for some time that innervation of organs by cranial nerves is regulated in part by secreted factors and cell surface ligands expressed by those organs, whether nerves also regulate the development of facial organs is only beginning to be elucidated. This review will provide an overview of cranial nerve development in relation to the organs they innervate, and outline their known contributions to craniofacial development, thereby providing insight into how nerves may shape the organs they innervate during development. Throughout, the interaction between different cell and tissue types will be highlighted.

Introduction to the peripheral nervous system and innervation of the head and neck

The peripheral nervous system

Sensory and motor nerves of the peripheral nervous system innervate all organs/tissues of the body, including the head and neck, serving to convey information to and from the central nervous system (i.e., brain and spinal cord; CNS). Afferent fibers carry sensation (touch, pressure, pain, and temperature) from cutaneous structures and mucous membranes, as well as general proprioception from somatic structures such as muscles, tendons, and joints (somatic sensory; SS), or from the viscera (visceral sensory e.g., glands; VS). In addition, the five special senses housed within the head (sight, smell, taste, hearing and balance) are innervated by so-called special sensory (SpS) fibers. Sensory neurons have cell bodies that are located outside of the central nervous system, forming sensory ganglia with no synapses. Efferent fibers provide motor innervation to skeletal muscles, with a distinction between skeletal muscles of branchial arch origin (branchial motor; BM) and those originating from unsegmented mesoderm or somites (somatic motor; SM). Both branchial and somatic motor neurons have cell bodies located within the CNS which are referred to as nuclei (they do not form ganglia). Motor neurons also provide innervation to post-ganglionic nerves of the autonomic nervous system. The autonomic nervous system is divided into two interactive branches; the sympathetic and parasympathetic system, which innervate involuntary smooth muscles, cardiac muscle and glands to unconsciously regulate the internal, and external-internal interface environments, in response to internal and external stimuli (Catala and Kubis, 2013). While parasympathetic ganglia are located close to or within the tissues they innervate, sympathetic ganglia are located close to the CNS and further from their targets. Additionally, it has been suggested that the somatic motor neurons of

the oculomotor (III) and trochlear (IV) cranial nerves (see below) be referred to as special somatic motor neurons (SSM). This reflects the uniqueness of these motor neurons from other somatic motor neurons. The reader is referred to an excellent review discussing the most recent understanding of the molecular induction of motor neurons, which forms the basis for the suggested change in nomenclature (Fritzsch, Elliott and Glover, 2017).

The cranial nerves

The head and neck are innervated by 12 pairs of cranial nerves that emerge directly from the brain (Table 1). Each of the 12 nerves perform specific, non-redundant functions and can consist of singular (e.g., sensory or motor only) or mixed (e.g., sensory and motor) fibers that innervate one or multiple structures. For example, cranial nerves I (olfactory) and II (optic) are considered purely afferent nerves since they conduct special sensory information from the olfactory region, the retina of the eye, and the inner ear structures, respectively, while the facial nerve (VII) contains somatic sensory, special sensory, branchial motor and parasympathetic motor fibers that (amongst other processes) convey taste sensations from the tongue, general sensation from skin, control the muscles of facial expression, and stimulate salivary gland secretions, respectively (Table 1). Cranial nerves that provide parasympathetic input (e.g., facial, glosopharangeal) send fibers that synapse on post-ganglionic parasympathetic ganglia located either within or nearby the organs they innervate (e.g., parasympathetic submandibular ganglion). Although sympathetic fibers may travel with cranial nerves, unlike parasympathetic, they are not derived from cranial ganglia but instead emanate from the superior cervical sympathetic ganglia (one of three cervical ganglia), the most cranial part of the sympathetic chain, located opposite the second and third cervical vertebrae. Sympathetic nerves innervate a wide variety of organs in the head, including the pineal gland (circadian rhythm), cephalic blood vessels (vasoconstriction), the choroid plexus, the eye (lacrimal gland, ocular muscles and cornea), carotid body, skin, teeth, and the salivary, sweat and thyroid glands.

Cranial Nerve Development

At the end of gastrulation, during the process of neurulation i.e., the left and right halves of the neural plate (also known as the neuroepithelium or neural ectoderm) elevate and fuse to form a neural tube; the precursor of the CNS, which also gives rise to all motor neurons. The ectoderm bordering the newly specified neural plate, the neural border zone, gives rise to two transient regions of ectoderm; the neural crest (NC; Le Douarin, 1980; Le Douarin *et al.*, 2004; Sauka-Spengler and Bronner-Fraser, 2008; Mayor and Theveneau, 2013) and the cranial placodes (Schlosser, 2010; Graham and Shimeld, 2013; Saint-Jeannet and Moody, 2014; Figure 1). These two independent cell populations are multipotent progenitors which contribute to the architecture of the face by generation of non-neuronal cell types, as well as to the formation of the

ganglia of the head and neck, with placodal cells forming exclusively sensory cells, and NCs giving rise to sensory and autonomic neurons. Here we describe these cell populations with a specific focus on the formation of the nervous system in relation to the developing organs, and structures of the head and neck. For a detailed review of craniofacial development, the reader is directed to the following reviews (Santagati and Rijli, 2003; Helms, Cordero and Tapadia, 2005; Szabo-Rogers *et al.*, 2010).

The Cranial Neural Crest and the pharyngeal arches

The NC give rise to somatic sensory and autonomic ganglia, along with peripheral glia. In the head, cranial NC cells (NCC) therefore differentiate to form the ciliary, pterygopalatine, submandibular and otic parasympathetic ganglia, and some proximal sensory neurons of the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagal (X) nerves. It should be noted that the majority of sensory neurons in the respective ganglia (Table 1) are derived from the trigeminal (ophthalmic and maxillomandibular in amniotes) and epibranchial (geniculate, petrosal and nodosal) placodes (see below). Cranial NCCs are highly migratory and multipotent, and apart from forming neurons and glia, also give rise to cartilage and bone, tendons and connective tissue, melanocytes, endocrine and adipose cells. NCCs are specified at the neuroepithelial-surface ectoderm border along the whole rostro-caudal axis of the neural tube, and are categorized as cranial, cardiac, vagal, trunk and sacral based on the axial location from where they originated, with both cell intrinsic and extrinsic (microenvironment) factors appearing important (Santagati and Rijli, 2003; Minoux and Rijli, 2010; Szabo-Rogers *et al.*, 2010; Wu *et al.*, 2017). Cranial NCCs are specified at distinct sites in the diencephalon (caudal part of the forebrain), the mesencephalon (midbrain) and the rhombencephalon (hind brain), which is divided into 7- 8 rhombomeres depending on the species; humans have 7.

In mammalian embryos, unlike in avian embryos, cranial neural crest cells migrate before the neural tube is closed (Tan and Morriss-Kay, 1985). However, in all vertebrate species, the location of induction of NCCs governs their contribution to structures of the head and neck. For example, NCCs originating in the forebrain and midbrain contribute to the frontonasal process, palate, and mesenchyme of the first pharyngeal arch, whereas neural crest cells originating in the anterior hindbrain region generate the mesenchyme of the second pharyngeal arch (Le Lievre and Le Douarin, 1975; Le Lievre, 1978; Couly, Coltey and Le Douarin, 1992, 1993; Johnston, 2005). The timing of migration also regulates outcome: in the hindbrain, the early ventrally migrating population fill the underlying pharyngeal arches and form ectomesenchymal derivatives within these structures, whereas the later-migrating NCCs do not enter the arches and form neurons and glia (Baker *et al.*, 1997). Each of the 5 pharyngeal arches is associated with a cranial nerve that serves to innervate many of the structures derived from the arch (Figure 1;

Cranial Nerve	Branches	Fiber Types	Class	Ganglia	Function
CN Ⅰ, Olfactory		Sensory	SpS	-	Sense of smell
CN Ⅱ, Optic		Sensory	SpS	-	Sense of sight
CN Ⅲ, Oculomotor	Cephalad	Motor	SSM	-	Control of skeletal muscle eye movement
	Caudad	Motor	SSM	-	Control of skeletal muscle eye movement
		Parasympathetic	PVM	Ciliary	Pupil constriction and accommodation
CN Ⅳ, Trochlear		Motor	SSM	-	Control of skeletal muscle eye movement
CN Ⅴ, Trigeminal	Ophthalmic (CN V_1)	Sensory	SS	Trigeminal	Sensation to upper face and upper nasal cavity
	Maxillary (CN V_2)	Sensory	SS	Trigeminal	Sensation to mid face and cheeks, and lower nasal cavity
	Mandibular (CN V_3)	Sensory	SS	Trigeminal	Sensation to lower face and lateral cheeks
		Motor	BM	-	Skeletal muscle of mastication
CN Ⅵ, Abducens		Motor	SM	-	Control of skeletal muscle eye movement
CN Ⅶ, Facial	Temporal			-	
	Zygomatic	Motor	BM	-	
	Buccal	Motor	BM	-	Control of skeletal muscle for facial expression
	Mandibular	Motor	BM	-	
	Cervical	Motor	BM	-	
	Posterior auricular	Motor	BM	Geniculate	Sensation to posterior external ear canal
	Chorda tympani	Sensory	SS	Geniculate	Taste sensation from anterior 2/3 of tongue
		Sensory	SpS		
		Parasympathetic	PVM	Submandibular	Sublingual, submandibular & oral gland secretion
	Greater petrosal	Parasympathetic	PVM	Pterygopalatine	Lacrimal and nasal gland secretion
CN Ⅷ, Vestibulocochlear	Vestibular	Sensory	SpS	Vestibular	Sense of balance
		Motor	SM	-	Vestibular plasticity and compensation
	Cochlear	Sensory	SpS	Spiral	Sense of hearing
		Motor	SM	-	Olivocochlear system: adaptation
CN Ⅸ, Glossopharyngeal	Tympanic	Sensory	SS	Superior	Sensation to middle ear, tympanic membrane etc.
		Parasympathetic	PVM	Otic	Parotid gland secretion
	Tonsilar	Sensory	SS	Superior	Sensation of tonsils
	Ligual	Sensory	SS	Superior	Sensation in posterior 1/3 of tongue
		Sensory	SpS	Petrosal	Sense of taste in posterior 1/3 of tongue
	Carotid	Sensory	VS	Petrosal	Carotid body/ sinus: monitor blood pressure and oxygen saturation
	Pharyngeal	Sensory	SS	Superior	Sensation in upper pharnynx

	Muscular	Motor	BM	-	The stylopharyngeus muscle
CN X, Vagus	Auricular	Sensory	SS	Jugular	Sensation from skin of the ear canal, tragus, and auricle
	Meningeal	Sensory	SS	Jugular	Sensation to dura mater at posterior of the skull
	Pharyngeal	Sensory	SS	Jugular	Sensation of the pharynx
		Sensory	VS	Nodose	Mucus membrane of the pharynx
		Motor	BM	-	Skeletal muscle of the pharynx, soft palate & 1 intrinsic muscle of tongue
	Superior laryngeal	Parasympathetic	PVM	-	Smooth muscle and glands of the pharynx
		Sensory	SS	Jugular	Sensation of the larynx
		Sensory	VS	Nodose	Mucus membrane of larynx
		Motor	BM	-	Cricothyroid muscle of the larynx
	Recurrent laryngeal	Parasympathetic	PVM	-	Smooth muscle and glands of the larynx
		Sensory	SS	Jugular	Sensation from esophagus and trachea
		Sensory	VS	Nodose	Mucus membrane of lower larynx,
		Motor	BM	-	Intrinsic muscles of the larynx except the cricothyroid muscle
	Rest of the body*	Parasympathetic	PVM	-	Smooth muscle and glands of the trachea
CN XI, Accessory		Motor	BM	-	Provides part of the motor innervation of the larynx and pharynx^
CN XII, Hypoglossal		Motor	SM	-	Supplies all intrinsic and all but 1 extrinsic muscle of the tongue

*Vagus nerve innervates organs in the thorax also

^Innervates the same structures as the branchiomotor component of the vagus nerve

- No cranial ganglia

Table 1. The 12 cranial nerves, their major branches, fiber types, ganglia and functions. Sympathetic fibers are not considered to be part of cranial nerves. However, they do run alongside cranial nerves and blood vessels and innervate cranial structures, such as the muscles for pupil constriction, and lacrimal, submandibular, sublingual, parotid, nasal and sweat glands. BM = branchiomotor; PVM = parasympathetic visceral motor; SM = somatic motor; SS = somatic sensory; SSM = special somatic motor; SpS = special sensory; SVM = sympathetic visceral motor; VS = Visceral sensory.

also see Frisdal and Trainor, 2014). For example, the trigeminal nerve (V) is a component of the first arch which gives rise to the muscles of mastication, and also the mylohyoid, the anterior belly of digastric, tensor veli palatani and tensor tympani and the teeth – all of which are innervated by the branches of the trigeminal nerve. The facial nerve innervates all the muscular derivatives of the second arch, that is, the muscles of facial expression, stapedius, stylohyoid, platysma and the posterior belly of digastric (Frisdal and Trainor,

2014). Whether this close association between nerves and developing tissues is required for the patterning or morphological outcomes of the tissues is not known. However, the appearance of nerves often precedes the formation of the organ. An excellent and well-described example of this is the formation of the lower jaw. The first structure that develops in the primordium of the lower jaw is the mandibular branch of the trigeminal nerve. A mandibular ossification center arises adjacent to the neurovascular bundle (at 6 weeks in humans and E14 in mice) and ossification spreads around the inferior alveolar nerve with the persistence of a mandibular canal, foramen, and mental foramen (Kjaer, 1990; Ramaesh and Bard, 2003). A possible relationship between the developing mandible and alveolar nerve was first referred to more than 100 years ago by Low, (1909), but whether neuronal signals promote osteogenesis of the developing mandible remains to be determined. Furthermore, whether any or all of the cranial nerves impact the development of structures of the face derived from these arches requires investigation.

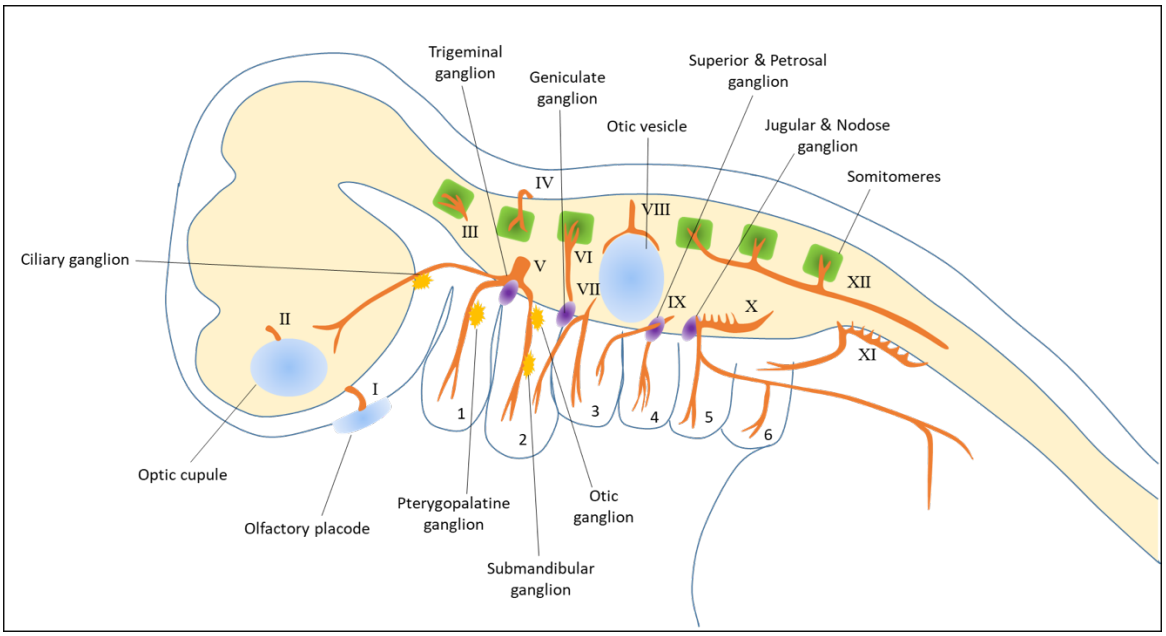

Figure 1. Early development of the cranial nerves in mouse. Cranial nerves form in a stereotypical pattern in relation to the sensory placodes and pharyngeal arches. The cranial nerves are numbered I - XII and the pharyngeal arches are numbered 1- 6. Cranial sensory and parasympathetic ganglia are depicted.

NCCs also give rise to post-ganglionic autonomic neurons. In their landmark studies, Dyachuk *et al.*, (2014) and Espinosa-Medina *et al.*, (2014) discovered that postganglionic parasympathetic neurons arise from NC derived Sox10 positive Schwann cell precursors (SCP). Parasympathetic ganglia form after the establishment of sensory and motor nerve fibers, and SCP were found to migrate towards the end of preexisting nerves and give rise to both Schwann cells and parasympathetic neurons (Dyachuk *et al.*, 2014;

Espinosa-Medina *et al.*, 2014). In response to BMP signaling, parasympathetic ganglia express ASCL1 and PHOX2B, which are essential for their development; in fact, PHOX2B is a master regulator and essential for all autonomic ganglia. The preganglionic motor neurons which synapse with the superior cervical sympathetic ganglia project axons from the cervical level of the lateral horn of the spinal cord via the ventral root. Like postganglionic parasympathetic neurons, postganglionic sympathetic neurons are NC derived, but formed from trunk, rather than head NC. As well as PHOX2B, sympathetic ganglia also require expression of ATOH1 (CASH1 in chick) and HAND2, with HAND2 required for the synthesis of catecholamines. Once established, sympathetic neurons become reliant on NGF for their maturation and survival.

The Cranial Placodes

Cranial placodes (which include the adenohypophyseal, olfactory, lens, otic, lateral line, profundal/trigeminal, and epibranchial placodes) are focal thickenings of the cranial ectoderm that give rise to the paired sensory organs and the cranial sensory ganglia generating a wide variety of cell types ranging from lens fibers to sensory receptor cells and neurons of the head (Schlosser, 2010). There is evidence that the placodes begin as a continuous pre-placodal region ventral to the rostral limits of the anterior neural plate, before neural tube closure, and subsequently regionalize into discrete placodes which contribute to specialized sensory organ development (Knouff, 1935; Couly and Le Douarin, 1987, 1990; Schlosser and Ahrens, 2004). However, there is also evidence to suggest the trigeminal and epibranchial placodes arose separately (Baker *et al.*, 1999; Begbie *et al.*, 1999; J Begbie and Graham, 2001). Similar to the NC, cranial placodal cells give rise to cell types of non-epidermal fate, albeit with more restriction. These include chemosensory, auditory, proprioceptive, mechanoreceptive, and nociceptive neurons with distinct properties such as the acquisition of bipolar morphology with a “basal” receptive process (similar to a dendrite) specialized for sensory transduction, and an apical process (an axon) for transmitting information to the central nervous system. These cells possess a range of neuronal excitability being able to generate action potentials and/or secrete neurotransmitters.

While some placodes contribute non-neuronal cell types to cranial sensory organs, the neurogenic placodes that contribute sensory neurons to the PNS include the trigeminal, epibranchial, otic, and olfactory placodes (Le Douarin, Fontaine-Pérus and Couly, 1986; Webb and Noden, 1993; Baker and Bronner-Fraser, 2000; Streit, 2004; Schlosser, 2014). The dorsolateral placodes give rise to cells of the trigeminal ganglion and organs of hearing and equilibrium and the epibranchial placodes generate the distal portion of the ganglia of cranial nerves VII, IX and X. The trigeminal placodes, the ophthalmic (or profundal) and maxillomandibular, emerge at the level of the midbrain-hindbrain boundary. The ophthalmic/ profundal

and maxillomandibular/ trigeminal placodes, together with NC derived neurons, generate the sensory neurons of the profundal and trigeminal ganglia, or the ophthalmic and maxillomandibular branches of the trigeminal ganglion in amniotes (see below). These sensory neurons either innervate non-placodal cells or have free nerve endings to detect somatosensory inputs (e.g. touch, temperature and pain) from the oral cavity and upper face.

Coordinated morphogenesis of NCC and placodal cells is required for sensory structure formation

To establish the cranial ganglia, placode-derived neurons must enter the mesenchyme to co-mingle with NCCs. Several recent studies have highlighted the importance of these interactions. Using fluorescent labelling of NC and placodal cell populations in *Xenopus* and live imaging, Theveneau *et al.*, (2013) found that NC cells are attracted to epibranchial placodal cells while the placodal cells are repelled by the NC cells, both *in vivo* and *in vitro*. This "chase and run" behavior, in part mediated via the SDF1 chemokine in the placodal cells and its receptor, CXCR4, in NC cells, leads to coordinated and directional cell movements in both populations Ablation of the NC prevented distinct epibranchial placodes from forming, and blocking placode development via *Eya* morpholinos impaired NC migration (Theveneau *et al.*, 2013). Additionally, DiI and DiO labelling of the epibranchial placode and adjacent NC in chick revealed that migration of neurogenic/ neurogliogenic cells from both populations appear to overlap (Jo Begbie and Graham, 2001). Epibranchial placodes give rise to sensory neurons of the geniculate, petrosal and nodose ganglia, which must migrate and synapse with the hindbrain. Closer study of migrating placodal cells found tube-like structures of NCCs encircling placodal cells, suggesting the NCCs act as a corridor to correctly guide the placode derived neurons in both chick and mouse (Freter *et al.*, 2013). Recent studies suggest that sensory neuron populations in the olfactory and otic placodes, as well as those in the vestibular and spiral (cochlear) ganglia are entirely populated with cells expressing cranial placode-associated, rather than neural crest-associated markers (Karpinski *et al.*, 2016). However, NC specific (*Wnt1-cre*) ablation of Sox10 in mice confirmed that NC derived Schwann cells are required for the correct migration of placode derived, afferent spiral ganglion neurons and their appropriate targeting of the organ of Corti (Mao *et al.*, 2014). Earlier studies also corroborate the idea that NC cells are required for correct migration and positioning of placodal derived sensory neurons, but not their migration per se (Yntema, 1944; Kuratani, Miyagawa-Tomita and Kirby, 1991; Jo Begbie and Graham, 2001). In contrast, genetic ablation of the NC did not affect the position of the geniculate ganglion in mice (Coppola *et al.*, 2010). The remaining cranial sensory ganglia are a mosaic of cells that express placode-associated as well as neural crest-associated markers indicating the close relationship. How both placodal and neural crest derived sensory neurons form axons with stereotyped trajectories and subsequently undergo differential morphogenesis to generate key components of the cranial sensory apparatus remains poorly understood.

Influence of cranial nerves on craniofacial development

Human congenital malformations of the cranial nerves, such as congenital facial nerve palsy (Bergstrom and Baker, 1981), have long suggested that cranial nerves do not merely innervate their target organs but provide important instructive signals during organogenesis, as individuals with congenital nerve defects often present with craniofacial anomalies in target tissues in addition to paralysis. For example, a disease heavily associated with cranial nerve anomalies and craniofacial (and peripheral) anomalies is Moebius Syndrome. In this neurological disorder there is an absence or underdevelopment of the VI and VII cranial nerves, which control eye movement and facial expression, respectively, although other cranial nerves (III - V, IX, X and XII) may also be affected. As such, patients exhibit facial paralysis or weakness affecting at least one but usually both sides of the face (CN VII), and paralysis of sideways (lateral) movement of the eyes (CN IV). Intriguingly, many also develop other craniofacial abnormalities including small chin (micrognathia), a small mouth (microstomia) with malformed tongue, cleft or a high and arched palate, and dental abnormalities (missing and misaligned teeth). What causes the absence of the cranial nerves and the resulting anomalies is not known. However, the outcomes suggest cranial nerves are involved in the patterning and/or shaping of organs. Indeed, depending on the organ, it can be envisaged that loss of peripheral nerves could affect morphogenesis through loss of neuron-derived factors that regulate target tissue patterning or growth (Petersen and Adameyko, 2017). As targeted ablation of nerves during embryonic craniofacial development is technically challenging, there is a general paucity of knowledge in this area. Despite this challenge, data showing the substantial contribution of nerves to correct patterning and development of craniofacial organs is now accumulating. Here we will review the current literature as it relates to craniofacial development, with additional information from investigations into the impact of nerves on peripheral organs.

Nerves and the Salivary glands

Mammalian salivary glands receive innervation from both the parasympathetic and sympathetic branches of the autonomic nervous system. Two of the three pairs of salivary glands, the submandibular (SMG; serous and mucous acini) and the sublingual (SLG, mucous acini), are innervated by the submandibular parasympathetic ganglion which resides near or within the glands, while the parotid gland (serous acini) receives parasympathetic support from the otic ganglion located within the infratemporal fossa. Both of these parasympathetic post-ganglionic ganglia receive signals from the CNS via cranial nerves: the facial (VII) innervates the submandibular ganglion and the glossopharyngeal (IX) the otic ganglion. Sympathetic input is delivered from the superior cervical ganglion, a postganglionic ganglionic ganglion which receives preganglionic efferent fibers from the thoracic part of the sympathetic trunk. Both nerve types serve to stimulate the secretion of saliva, albeit with different outcomes: parasympathetic nerves stimulate water

secretion whereas sympathetic induce protein secretion, thereby changing the saliva content (Proctor and Carpenter, 2007). Studies over the last 150 years have demonstrated a requirement for parasympathetic innervation in salivary gland homeostasis: if parasympathetic support is removed, the organs atrophy (Kyriacou and Garrett, 1988; Zhang *et al.*, 2014). Although stimulation of sympathetic adrenergic receptors via adrenergic mimetics has been shown to promote organ regeneration after mechanical injury (Boshell and Pennington, 1980), and stimulation with alpha-adrenergic agonist before radiation aids to preserve the tissue (Norberg and Lundquist, 1988), no role has been clearly described in organ homeostasis.

Parasympathetic nerves have also been shown to play a role in salivary gland morphogenesis. Salivary gland formation begins with a thickening of the oral epithelia, which subsequently invaginates into a condensing mesenchyme to form an end bud (pre-acinar) connected to the proximal duct (from E11-12 in mouse). This epithelial bud undergoes multiple rounds of branching from E12.5 (Patel, Rebustini and Hoffman, 2006; Knosp, Knox and Hoffman, 2012) to form a network of interconnected lumenized ducts and terminal saliva synthesizing acini that are fully functional by birth (Knosp, Knox and Hoffman, 2012; Mattingly, Finley and Knox, 2015). Innervation of the developing epithelium begins upon establishment of the submandibular parasympathetic ganglia at the proximal duct at E12, a process that requires WNT signals released by KRT5+ progenitors (Knosp *et al.*, 2015), and as the epithelium undergoes branching functional acetylcholine (ACh)-producing nerves extend along the ductal system to envelope the newly forming end buds (Coughlin, 1975; Knox *et al.*, 2010). This unidirectional migration of nerves is mediated by the release of the neurotrophic factor neurturin from KIT+ end bud cells, which binds GFRα2 receptors on the axons to promote neurite growth and interaction with the SMG epithelia (Knox *et al.*, 2013) (Lombaert *et al.*, 2013). In a series of studies using both ex vivo and in vivo models, Knox and colleagues demonstrated that the innervating nerves were far from passive during development as previously thought (Coughlin, 1975), but coordinated specific morphogenic features of organogenesis. In the absence of the nerves (performed by mechanical removal) the developing gland showed a significant reduction in the reservoir of keratin 5 positive (KRT5+) epithelial progenitors and aberrant epithelial branching, with a loss in the number of end buds (Knox *et al.*, 2010). They further showed that acetylcholine activation of epithelial muscarinic (CHRM1) receptors and transactivation of EGFR was able to largely rescue both progenitor cells and end bud number (Knox *et al.*, 2010). Subsequently, parasympathetic nerves were shown to be establishing two essential cellular features of salivary glands: secretory acini and the ductal network. Emmerson and colleagues showed that an absence of nerves not only led to the loss of acini and progenitor cells but a reduction in aquaporin (AQP)5+ acini, suggesting innervation is necessary for producing the acinar lineage (Emmerson *et al.*, 2017). They further showed that nerves control the production of the AQP5+ acinar lineage via SRY (sex determining region Y)-box 2 positive (SOX2): glands

deficient in epithelial *Sox2* have a severe reduction in acinar cells and SOX2 expression is modulated by acetylcholine/muscarinic signaling (Emmerson *et al.*, 2017).

Parasympathetic nerves are also involved in multiple aspects of ductal tubulogenesis, this time through the actions of the neuropeptide vasoactive intestinal peptide (VIP). Salivary duct development is similar to the pancreas in requiring duct elongation, followed by microlumen formation and fusion to create a contiguous lumen that must then expand. Nedvetsky *et al.*, (2014) showed that VIP signaled through VIP receptor 1 (VIPR1) on epithelial cells to increase proliferation of KRT19+ ductal progenitor cells (and thus duct elongation) and promoted formation of a contiguous lumen in a cAMP/ PKA dependent manner. Furthermore, they showed that lumen expansion was CFTR-dependent, thereby linking CFTR function to parasympathetic innervation (Nedvetsky *et al.*, 2014). Intriguingly, neurturin also increases the expression of *Vip* in parasympathetic ganglia (Nedvetsky *et al.*, 2014), thereby mediating a positive feedback loop between nerves and epithelia allowing for organ expansion and tubulogenesis.

Nerves and Teeth

The dentition of the mandible is highly innervated by sensory fibers of the mandibular branch of the trigeminal nerve, and also by sympathetic fibers from the superior cervical ganglion. Studies in fish and more recently in mice demonstrate that peripheral nerves are required for aspects of tooth development and homeostasis in some species. Tooth development begins with budding of the dental epithelium into the underlying mesenchyme. During the following cap and bell stages, the epithelium undergoes folding morphogenesis. Each new tooth receives innervation by sensory nerves of the trigeminal ganglion as well as autonomic axons from the sympathetic nervous system. In non-mammalian vertebrates including multiple fish species, *de novo* formation of tooth germs occurs throughout life, with the tooth germs developing into replacement teeth that erupt and shed their predecessors, providing a model to examine tooth development in an experimentally amenable adult animal. For more information on tooth development see (Thesleff, 2003; Mitsiadis and Graf, 2009). Tuisku and Hildebrand (1994) performed unilateral denervation of the mandibular branch of the trigeminal nerve in the cichlid *Tilapia mariae*. The authors reported that turnover of teeth ceased, and soft-tissue tooth primordia was absent on the denervated side, but intact on the non-operated side, indicating neuronal input is necessary for ontogenesis.

Although the requirement for innervation for tooth development in mammals has been more difficult to delineate, studies have shown that tooth germ formation and innervation from the maxillary and mandibular branch of the trigeminal nerve are tightly choreographed spatially and temporally. Early studies report innervation to precede germ formation in human, rat and mouse (Pearson, 1977; Kollar and Lumsden, 1979;

Lumsden, 1982; Mohamed and Atkinson, 1983), with mandibular nerves present in the dental mesenchyme below the area into which the dental epithelium will bud, but excluded from the mesenchyme which will condense around the bud. The nerves form a plexus around the periphery of the tooth follicle, and only begin to invade the dental papilla mesenchyme postnatally after onset of dentin and enamel formation (Pearson, 1977; Mohamed and Atkinson, 1983; Løes *et al.*, 2002; Kettunen *et al.*, 2005). Neurotropic factors such as NGF, NT-3, NT-4 and BDNF are expressed by the dental mesenchyme and epithelia early in bud initiation stages, and may have non-neural roles in tooth morphogenesis (Mitsiadis and Luukko, 1995). The neurorepellent, SEMA3A, is secreted by the condensing/ condensed mesenchyme, and to a lesser extent, by the budding epithelia, which mediates repulsion of axons from the germ area (Kettunen *et al.*, 2005).

The requirement of nerves for tooth initiation and development in mice and rats has been investigated using mandibular explant cultures and grafting studies. Tissue was explanted from E9-10, before innervation of the tissue is believed to occur. Although several studies suggested a role for nerves in tooth development (Pourtois, 1964; Thiebold and Karcher-Djuricic, 1972; Kollar, 1976), Lumsden and Buchanan (1986) conducted extensive *in vitro* cultures and grafting studies and concluded that tooth initiation and development was independent of nerves, in agreement with other reports (Ruch, Karcher-Djuricic and Gerber, 1973; Gerber, Karcher-Djuricic and Ruch, 1974). Additionally, deletion of *Sema3a* in mouse resulted in early innervation of the pre-condensing and condensed mesenchyme and misaligned fibers, but no defects were observed in tooth formation (Kettunen *et al.*, 2005). In light of the discrepancy in findings, it would be worthwhile to revisit this question using the genetic tools and mouse lines currently available. For instance, *Neurogenin 1* (*Neurog1*) is required for proximal sensory ganglion formation and *Neurog1* null mice do not form the trigeminal, vestibular and spiral ganglia (Ma *et al.*, 1998). These mice are found to have changes of the inner ear (below), but there are no reports on tooth development.

Similar to the studies in fish, denervation has a profound effect on the continuously growing murine incisor. That is, when the sole sensory nerve innervating the adult lower incisor is severed, the tooth appears thinner, shorter and narrower, consistent with a decrease in epithelial and mesenchymal proliferation and aberrant mineralization (Chiego et al., 1981; Chiego et al., 1983; Kubota et al., 1985; Kerezoudis et al., 1995; Zhao et al., 2014). Zhao and coworkers recently identified a mechanism by which these nerves regulate mesenchymal stem cells (MSCs) residing in the dental pulp (Zhao et al., 2014). They show that periarterial Gli1+ MSCs (thought to contribute to all mesenchymal derivatives) require neuronally-derived sonic hedgehog (Shh), as ablation of nerves or inhibition of Shh signaling reduces the number of cells. Interestingly, the authors found increased Schwann cells within the mesenchyme, suggesting an expansion of glia in response to denervation. Subsequent exciting work by Kaukua and coworkers (2014) suggests

that Sox10+ Schwann cells and Schwann cell precursors give rise to all mesenchymal cell types in the pulp including dentin-making odontoblasts, thereby providing a new source of these cells (Kaukua et al., 2014). Whether Sox10+ Schwann cells are precursors for Gli1+ MSCs, and if Shh+ from sensory nerves (or Schwann cells) regulates their behavior, remains to be investigated.

Nerves and cochlear development

Like parasympathetic innervation in the salivary gland, sensory innervation regulates ductal morphogenesis in the mouse cochlear; a coiled sensory end organ of the inner ear necessary for hearing in mammals, albeit by differing mechanisms. The cochlear is derived from the otic placode. Once the otic placode has been induced, it invaginates to form the otic cup and then upon closure, the otocyst. This structure gives rise to the inner ear and specialized epithelia (including endolymph-producing and other secretory cells, supporting cells and mechanosensory hair cells), including the cochlear (detects sound) and the vestibular apparatus: the utricle, saccule and semicircular canals (spatial orientation, motion and equilibrium). The otic placode also forms sensory neurons which migrate to the vestibular and spiral ganglia and innervate hair cells. The loss of either afferent and/ or efferent innervation impairs growth of inner ear organs. Mice null for *Neurog1*, which do not form the vestibular and spiral ganglia and therefore whose inner ear does not receive either afferent or efferent fiber innervation, have significantly smaller dimensions of the cochlea, saccule, and the anterior, posterior and horizontal canals of the semicircular system (Ma, Anderson and Fritzsch, 2000). Due to the smaller dimension of the mutant cochlea, they were found to have a 61 % reduction in total hair cells despite seeing an increase in the number of rows of hair cells in some areas (Ma, Anderson and Fritzsch, 2000). Innervation is also required for maintenance of hair cells in both the Organ of Corti and the vestibular sensory epithelia. Genetic deletion of the neurotrophins BDNF and NT-3 in mice results in complete loss of afferent and efferent innervation after 3 weeks, with near complete loss of outer hair cells and some loss of inner hair cells in the organ of Corti by 4 months (Kersigo and Fritzsch, 2015). The authors also report that complete loss of innervation to the vestibular canal cristae, and limited innervation of the utricle, resulted in smaller areas of the utricle and canal cristae and changes to the number and stereocilia of hair cells in the utricle (Kersigo and Fritzsch, 2015).

The spiral ganglion develops in concert with the cochlear duct and sequentially controls the timing of terminal mitosis and differentiation. The organ of Corti is unusual in that cell differentiation is decoupled from the order in which cells exit the cell cycle – i.e. cell cycle exit proceeds in an apical to basal wave but differentiation begins at the mid-base and spreads bi-directionally. Conditional deletion of Shh in the spiral nerve fibers using the Cre/LoxP system results in mild to severe loss of cochlear duct length and premature exit of the cell cycle exit by hair cell precursors (Bok *et al.*, 2013). Additionally, instead of a normal bi-

directional path, differentiation of hair cells followed cell cycle exit in an apical-to-basal direction in mutant mice with the greatest reduction in *Shh* expression in the spiral ganglion. In these mice, differentiation occurred promptly after terminal mitosis implying that the gradient formed by apically secreted, nerve-derived Shh delays differentiation to mature hair cells. Thus, Shh from the spiral ganglion maintains or promotes cell proliferation as the cochlear duct elongates, and the spatial gradient of Shh controls the temporal differentiation of mechanosensory epithelial hair cell precursors into mature hair cells (Bok *et al.*, 2013). These changes are likely to affect frequency discrimination by the organ of Corti.

A role for nerves in inner ear development has also been reported in fish. In neonatal swordtail fish, the growth of the otolith, a structure of gelatinous matrix and calcium carbonate particles in the saccule and utricle of the inner ear of all vertebrates, was found to be dependent on vestibular nerve innervation. In particular, the uptake of calcium by the otolith appeared to be neurally regulated, but whether this was by afferent of efferent fibers was not determined (Anken, Edelmann and Rahmann, 2002).

Nerves and Muscle

The skeletal (branchiomeric) muscles for facial expression receive innervation primarily from the facial (VII) cranial nerve, which develops alongside these muscles, while the oculomotor (III), trochlear (IV) and abducens (VI) nerves innervate the extraocular eye muscles that control eye movement and lift the eyelid. The facial muscles are derived from cranial paraxial mesoderm, while the extraocular muscles (six in total) are thought to be derived from both the cranial paraxial mesoderm and the prechordal mesoderm. Cranial skeletal muscle formation is unique from trunk and limb skeletal muscle formation in that it occurs in unsegmented mesoderm and without epithelialization. The core gene network (*Myf5, Myf4, Myod*) which regulates myogenic commitment is common between all skeletal muscles, however, the genetic hierarchy upstream of the core network is distinct between trunk, branchiomeric and extraocular muscles (Sambasivan, Kuratani and Tajbakhsh, 2011). In the craniofacial region, immature myoblasts develop in close proximity to the nerves which will innervate them and begin to associate before migrating to their final anatomical positions (Gilbert, 1957; Gasser, 1967).

Congenital fibrosis of the extraocular muscles (CFEOM) is an autosomal dominant congenital disorder which results from hypoplasia of the oculomotor nerve, and may also affect the abducens nerve to a lesser extent, which innervate the extraocular muscles (Heidary, Engle and Hunter, 2008; Cheng *et al.*, 2014). CFEOM was first described as a disorder in which normal contractile muscle tissue was replaced by fibrotic tissue (Apt and Axelrod, 1978; Harley, Rodrigues and Crawford, 1978). It was not until more recently, using both magnetic resonance imaging (MRI) and autopsy analysis, that the oculomotor nerve was found

to be hypoplastic, and it was proposed that the muscle pathology was secondary to the nerve pathology (Engle *et al.*, 1997). Most notably, a profound reduction in the superior division of the oculomotor nerve results in severely diminished to apparently missing superior rectus and levator palpebrae superioris muscles which are contacted by the nerve. A milder reduction in volume has been found in some of the other extraocular muscles also (Demer, Clark and Engle, 2005). The authors found no fibrotic replacement of tissue in these muscles, and suggested this may be an artifact of biopsy sampling. They did, however, describe an increase in internal nuclei and abnormal aggregations of mitochondria in myofibers, although the consequences of this were unknown (Engle *et al.*, 1997). These findings suggest that innervation of these muscles is not required for their initial commitment and migration. More careful studies in mouse models of CFEOM (Cheng *et al.*, 2014) are required to understand how much of the muscle phenotype found in human CFEOM patients is due to aberrant development, a failure in early maintenance or atrophy.

Moebius Syndrome is another congenital cranial dysinnervation disorder in which the absence or underdevelopment of the abducens (VI) and facial (VII) nerves leads to facial muscle weakness or paralysis. Mice deficient in *Hoxb-2* also present with loss of the facial nerve and share many of the craniofacial characteristics of Moebius Syndrome (Barrow and Capecchi, 1996). However, the muscles themselves in both mice and humans have not been examined, and so whether dysinnervation causes developmental abnormalities or simply atrophy is unknown. Given the paucity of information in cranial nerves and their impact on muscle development, we have discussed the role of nerves in other skeletal muscles below.

Mice deficient in neuronal neuregulin-1 type III lose phrenic nerve innervation of the diaphragm and exhibit aberrant diaphragm muscles at P0, with a 50% reduction in muscle fibers and abnormal, centrally located nuclei (Wolpowitz *et al.*, 2000). In the mouse mutant peroneal muscular atrophy (pma), in which the extensor digitorum longus (EDL) muscle is aneural (pma), primary myotube numbers are greater than in control at E12/ E13, but by E15, numbers are reduced by approximately half due to degeneration (Ashby, Wilson and Harris, 1993). The formation of secondary myotubes is initially normal, but by E18 the numbers were significantly reduced and consequently fiber numbers were also reduced (Ashby, Wilson and Harris, 1993). These studies suggest that innervation is not required for initial establishment of primary and secondary myotubes, but is required for their early maintenance.

Nerves are also important in regulating the development of invertebrate muscle. Multiple studies in Drosophila melanogaster have demonstrated that motor nerves regulate the development of indirect flight muscles by controlling the number of myoblasts available to generate muscles, as well as the formation of specific muscle fibers (Fernandes and Keshishian, 1998, 1999, 2005). Indirect flight muscles are highly ordered muscles of the thorax and consist of two types, dorsal ventral muscle (DVM) and dorsal

longitudinal muscle (DLM), that develop by two distinct modes. DLMs develop through the fusion of myoblasts into persistent larval muscle fibers (also referred to as muscle scaffolds), whereas DVMs develop de novo. Denervation of indirect flight muscles, by severing the mesothoracic (motor) nerve in the embryo, results in retardation of DLM fiber formation in the pupa and prevents formation of the DVMs (Fernandes and Keshishian, 1998, 1999). For both muscle types myoblast proliferation was found to require innervation (Fernandes and Keshishian, 2005). In addition, correct sequestering of DVM myoblasts into muscle primordia is dependent on motor nerves: in the absence of these nerves, the DVM myoblasts remain unpatterned, and the muscles failed to form (Fernandes and Keshishian, 2005). Thus, motor neurons can regulate the development of de novo forming fibers and modulate the final size of adult muscle fibers by influencing the size of the myoblast pool.

Nerves and the vasculature.

One of the most striking discoveries showing the importance of nerves in controlling tissue architecture during development is in the patterning and maturation of the vasculature. It is well known that nerves and blood vessels intimately interact to produce a highly branched network reaching every organ of the body. Similar to the limb, there is a tight association of nerves and vasculature during craniofacial development, beginning with the pharyngeal arches. Within each of the pharyngeal arches are the developing aortic arches and, specific for each arch, cranial nerves. The most cranial arches (1st and 2nd) contribute to the vascularization of the derivatives of the 1st and 2nd pharyngeal arches (mandible, facial muscles etc.), the 3rd arches contribute to the development the main source of blood supply to the head i.e. carotid arteries and the 4th to the aorta. During the phase of development of the pharyngeal apparatus, nerves and arteries exhibit a tight relationship even during extensive transformations of aortic arch arteries. Indeed, the location lateral to the dorsal aorta is retained for the nerves of the first three arches, so nerves V, VII, and IX are found lateral to the internal carotid artery. However, whether the blood vessels pattern the nerves, or vice versa during this development is unclear.

Studies in embryonic skin of the developing limb indicate that nerves and associated Schwann cells (support cells essential to neuron survival) can regulate blood vessel architecture. Sensory and motor neurons invade the embryonic skin of the limb at approximately E13.5, after a primary capillary plexus is established. Subsequently, the pattern of sensory/motor axons provides a spatial template for the pattern of arterial vessel branching (Mukouyama et al., 2002; Li et al., 2013). In Neurogenin1/Neurogenin2-deficient embryos, which lack sensory nerves (and Schwann cells) in the limb skin, the progressive branching pattern of vessels is disrupted (Mukouyama et al., 2002; Mukouyama et al., 2005). Recently, Li and colleagues (2013) identified Schwann cells as the mediators of this patterning (Li et al., 2013). Schwann cell secretion of Cxcl12 promotes the migration of Cxcr4 expressing vascular endothelial cell of the capillary plexus to

align with axons. Once aligned, axonal secretion of VegfA induces arterial differentiation of the blood vessels through Flk-1/Npn1. In this way, the nervous signals not only direct migration and patterning, but also the temporal and spatial dynamics of arterial differentiation in limb skin.

While the elegant blood-nerve alignment in the limb skin is one of the best known mechanisms of nerves orchestrating development, the role of nerves and Schwann-derived Cxcl12 cannot be generalized for all blood vessel development, ass not all blood vessels align to nerves. Cxcl12 is not required for proper patterning and differentiation of major vessels of the forelimb, trunk vasculature, heart endocardium, and myocardial trabeculation (Li et al., 2013). Borden and colleagues (2013) observed normal microvasculature of the pancreas in the absence of sympathetic nerves (Borden et al., 2013). Thus, alignment of blood vessels by nerves may be limited to smaller peripheral arteries that infiltrate some end-organs.

Nerves and Taste Buds.

Taste buds have distinct nerve supplies: taste bud cells located in the fungiform papillae of the anterior two-thirds of the tongue are innervated by the facial (VII; chorda tympani) nerve while taste bud cells that reside in the circumvallate papilla and foliate papillae in the posterior tongue are innervated by the glossopharyngeal (IX) nerve. Taste buds consist of three types of chemoreceptive taste cells (types I, II and III), basal cells which are putative taste cell progenitors and/ or transit cells, and supporting cells (Kapsimali and Barlow, 2013; Krimm, Thirumangalathu and Barlow, 2015).

In mice, the tongue primordium arises from the lingual swelling contributed by pharyngeal arches (from first to fourth) around E11–E11.5, with taste placodes appearing in specific locations on the anterior tongue surface by E12.5. These taste placodes subsequently invaginate and give rise to taste bud cells in the distinct taste papillae, namely, fungiform papillae, circumvallate papilla, and foliate papillae, starting from E14.5. Of interest, while gustatory nerves reach the epithelial surface of the tongue as early as E11.5, and by E13.5 axons have reached the fungiform placodes, but innervation of taste buds does not begin until E14.5. By E15.5, innervation of the taste placodes is complete (Barlow, 2015). BDNF is specifically expressed by fungiform placodes during development and is necessary for appropriate innervation. Ablation of *Bdnf* in mice results in increased branching of the chorda tympani and mis-targeting of fungiform papillae (Ma, Anderson and Fritzsch, 2000), while overexpression of *Bdnf* along the entire lingual epithelium resulted in aberrant innervation of filiform non-taste regions of the tongue preferentially (Lopez and Krimm, 2006). In keeping with the relatively late temporal innervation of the taste buds in rodents, the initial expression of basal cell markers, Shh, and Sox2, is observed with or without innervation in *Bdnf; Ntf3* double knockout mice (Ito, Nosrat and Nosrat, 2010), demonstrating that taste bud initiation in nerve independent. However,

by E15, both the number and size of fungiform papillae are significantly reduced compared to control mice, with the largest difference seen at P0, highlighting the importance of innervation for maintenance and maturation (Ito, Nosrat and Nosrat, 2010). Taste buds continue to mature postnatally in rodents, and in rats, it has been found that the number of neurons that innervate a taste bud at P10 determines the size of the taste bud at P40 (Krimm and Hill, 2000). In contrast, the initiation and differentiation of taste buds in amphibians appears to be independent of nerves (Barlow, Chien and Northcutt, 1996).

In adult mammals, the average lifespan of a taste bud is estimated to be 10-14 days, with the generation of new taste bud cells throughout life. After denervation, Shh signal is lost within 6 hour, suggesting a dependency on nerves (Miura *et al.*, 2004), and taste buds degenerate (Hosley, Hughes and Oakley, 1987; Huang and Lu, 1996; Oakley and Witt, 2004). On the other hand, when the glossopharyngeal nerve is crushed, taste buds regenerate when innervation is reestablished (Hosley, Hughes and Oakley, 1987; Oakley and Witt, 2004). Additionally, misexpression of Shh in the lingual epithelium is sufficient to drive the taste bud program in the absence of innervation (Castillo et al., 2014), indicating that nerves play a vital role in adult taste bud production by maintaining Shh expression. Future studies are required to determine the mechanism and identity of the neural signal(s) by which nerves maintain taste bud progenitor Shh expression.

Conclusion

While there is great species-specific variation in the morphology of the head and its appendages, it is remarkable that innervation of these structures is highly conserved. Craniofacial nerve development is tightly coordinated with the development of their target tissues, and studies to date have demonstrated that this interaction during development, as well as during homeostasis of adult tissues, forms part of the niche required for correct progenitor cell maintenance, proliferation and patterning. Given that nerves are present at the very beginnings of organ formation and can produce a plethora of activating or inhibiting factors, and the craniofacial complex is heavily innervated, it is highly likely that they regulate multiple processes of organ development, either directly through nerve-organ interactions or indirectly via e.g., control of blood vessel growth/patterning. Yet, despite our increased understanding of the influence of nerves on craniofacial morphogenesis over the past decade, our knowledge on the impact of these reciprocal interactions on organogenesis remains poor at best. This stems, in part, from the current paucity of information on nerves themselves: what is their full repertoire of ligands, receptors and secreted molecules? Moreover, despite craniofacial anomalies often being reported in syndromes in which nerves targeting organs and structures of the head and neck are perturbed, there has been little investigation of those organs in question. In addition to Moebius Syndrome described above, children with Familial Dysautonomia (FD), an autosomal recessive

disorder resulting in abnormal development and progressive degeneration of the sensory and autonomic nervous systems, exhibit multiple craniofacial phenotypic malformations including retrognathia of the mandible (i.e., lower jaw is set further back than the upper jaw) and horizontal mandibular growth. However, in most cases, the target organs of patients with such disorders or the mouse models generated to recapitulate these diseases (e.g., Ikbkap-deficient mouse for FD (George *et al.*, 2013)) have either not been analyzed or have been examined at low resolution, leaving open the question of whether those organs have undergone incorrect or aberrant development. There is also the question of whether other nerves deviate to compensate for loss of a cranial nerve and if this in turn results in a phenotypic alteration in the target tissue. Investigation of developing organs in humans and/or in mouse models of these neurological disease would significantly aid in identifying role for nerves in tissue patterning and morphogenesis.

Another obstacle to overcome is our inability to effectively target and ablate specific nerves that innervate distinct tissue types. Techniques such as optogenetics (light-mediated alterations in neuron activity) and Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to stimulate or inhibit neurons have been restricted to adult studies and on the whole to the CNS. However, in order to specifically target neuronal subtypes, we require a more extensive understanding of the neurons within each ganglia to determine whether different ganglia possess unique markers that can be targeted for genetic manipulation. Current studies in the vagus have generated tools for targeting vagal nerves (Chang *et al.*, 2015), suggesting that specificity is possible. As such, we envisage that applications such as these or similar technologies to cranial nerves will bring a wealth of knowledge to help understand the role of individual nerves in creating tissue specific niches as well as deciphering the underlying causes of some craniofacial congenital malformations and create meaningful therapies for treating the next generation of patients.

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