Interactions between developing nerves and salivary glands

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Our aim is to provide a summary of the field of salivary gland development and regeneration from the perspective of what is known about the function of nerves during these processes. The primary function of adult salivary glands is to produce and secrete saliva. Neuronal control of adult salivary gland function has been a focus of research ever since Pavlov's seminal experiments on salivation in dogs. Less is known about salivary gland innervation during development and how the developing nerves influence gland organogenesis and regeneration. Here, we will review what is known about the communication between the autonomic nervous system and the epithelium of the salivary glands during organogenesis. An important emerging theme is the instructive role of the nervous system on the epithelial stem/progenitor cells during development as well as regeneration after damage. We will provide a brief overview of the neuroanatomy of the salivary glands and discuss recent literature that begins to integrate neurobiology with epithelial organogenesis, which may provide paradigms for exploring these interactions in other organ systems.

Introduction

Types of salivary glands. Much of what we know about the functional innervation of the human salivary gland, in terms of both central and peripheral nervous system control has been determined in adult salivary glands from other mammalian species. The human salivary gland system can be divided into two separate exocrine groups: major and minor glands. The major salivary glands are bilateral paired glands and include parotid (PG), submandibular (SMG), and sublingual glands (SLG). The minor salivary glands are distributed in groups of hundreds in the upper aerodigestive tract mucosa but will not be the focus

of this review. The major physiological function of the salivary glands is to secrete saliva, which is essential for the lubrication, digestion, immunity, and overall maintenance of homeostasis within the body. Saliva secretion is mediated by both parasympathetic and sympathetic autonomic innervation. Recently, significant improvement has been made in our understanding of the molecular basis of salivary gland development as well as on the roles of the parasympathetic and sympathetic innervation in gland organogenesis.[1-4]

Salivary gland innervation routes. An anatomical overview of the autonomic parasympathetic and sympathetic innervation of the adult salivary glands is outlined in **Figure 1**. The innervation of the PG, occurs via the glossopharyngeal nerve (or cranial nerve IX), which carries preganglionic parasympathetic fibers from the inferior salivatory nucleus (ISN) in the medulla region of the brainstem to synapse in the otic ganglion (OG). The otic ganglion is located away from the PG just below the foramen ovale on the base of the skull, next to the mandibular division of the trigeminal nerve (or cranial nerve V). Then, postganglionic fibers exit the otic ganglion to provide parasympathetic secretory innervation to the PG via the auriculotemporal nerve of cranial nerve V for the secretion of serous-watery saliva.[5,6]

The innervation of both the SMG and the SLG occurs via the parasympathetic fibers carried by the facial nerve (or cranial nerve VII). The parasympathetic preganglionic fibers run from the superior salivatory nucleus (SSN) in the pons region of the brainstem passing through the nervus intermedius and into the internal auditory canal to join the facial nerve. The fibers are conveyed by the chorda tympani nerve in the mastoid and enter the infratemporal fossa. In the infratemporal fossa, preganglionic fibers join the lingual nerve (a branch of the marginal mandibular division of the trigeminal nerve), which then carries these fibers to synapse at the submandibular ganglion (SG). Short postsynaptic fibers leave the ganglion to innervate the SMG and SLG, which stimulates serous-mucous and mucous saliva secretion, respectively.[6,7] Electrophysiological studies show that inputs from multi-modal afferents (carrying general somatic, gustatory
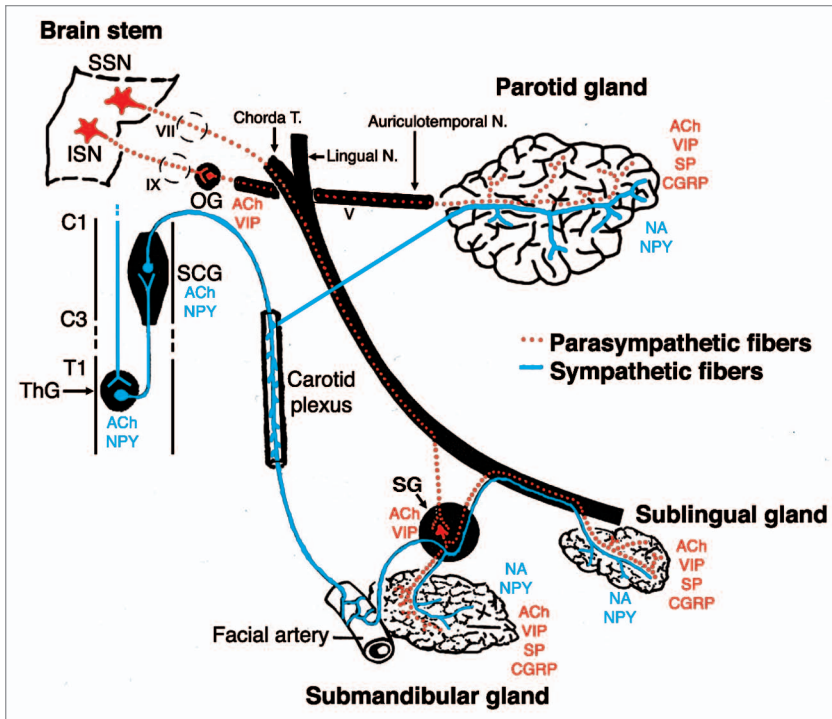
Figure 1. Model of parasympathetic and sympathetic innervation of the adult major salivary glands (in red and blue, respectively). Neurotransmitters for parasympathetic (red) and sympathetic fibers (blue): ACh, acetylcholine; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide; NA, noradrenaline; SP, substance P; CGRP, calcitonin gene-related peptide. Brain stem nuclei: SSN, superior salivatory nuclei; ISN, inferior salivatory nuclei. Ganglia: ThG, thoracic ganglion; SCG, superior cervical ganglion; OG, otic ganglion; SG, submandibular ganglion. Spinal cord: C, cervical vertebra; T, thoracic vertebra. Cranial nerves: VII, facial nerve; IX, glossopharyngeal nerve; V, trigeminal nerve.

and visceral information) and from cardiac and respiratory centers converge on the preganglionic SSN neurons projecting to the submandibular and lingual ganglia. These inputs from diverse sources converge on the SSN neurons to regulate blood flow and salivary secretion possibly through myoepithelial contraction at the SMG and SLG glands.[8]

The primary sympathetic salivary centers are located in the upper thoracic segments of the spinal cord. The paravertebral sympathetic trunk carries the ascending preganglionic fibers from the thoracic ganglion (ThG), which travel in the spinal cord to synapse at the superior cervical ganglion (SCG). Postganglionic sympathetic fibers exit the SCG to innervate upper thoracic, cervical and craniofacial regions. Sympathetic fibers target the salivary glands through the external carotid artery plexus and its branches, including the facial artery. Postganglionic sympathetic fibers from the external carotid plexus give off branches to reach all three pairs of major salivary glands.[7] Ganglionectomy of the SCG has revealed the role of the sympathetic fibers in regulating peripheral blood flow, salivary secretion, and local inflammatory and immune mediators.[9,10] For a more detailed review of the neuroanatomy of cranial nerves, the reader is referred to an anatomy atlas.[7]

Types of neurotransmitters released by autonomic nerves innervating salivary glands. There are two major types of neurotransmitters released by the autonomic nervous fibers that innervate the salivary glands: acetylcholine (ACh) and noradrenaline (NA) providing cholinergic and adrenergic signaling responses, respectively (**Fig. 1**). Other non-adrenergic non-cholinergic transmitters are released from either parasympathetic, sympathetic or both autonomic nerves in salivary glands: vasoactive intestinal peptide (VIP), enkephalin, substance P (SP), neuropeptide Y (NPY), neurokinin A, pituitary adenylate cyclase activating peptide, neuronal nitric oxide synthase and calcitonin gene-related peptide (CGRP).[11,12]

ACh serves as the "classical" neurotransmitter between preganglionic and postganglionic neurons in both the sympathetic and parasympathetic fibers targeting the salivary glands, and between postganglionic parasympathetic neurons and effector glands. NA is another major neurotransmitter between postganglionic sympathetic neurons and effector salivary glands. VIP is produced in ACh-rich parasympathetic neurons, and was first observed in the cat SMG to increase blood flow and salivary secretion together with ACh.[13,14] In sympathetic ganglia from guinea pigs, enkephalin was found to have inhibitory effects regulating the release of ACh and substance P, and in cats, NPY possesses inhibitory and stimulatory effects on blood flow (vasoconstriction) in the SMG.[15,16] Sympathetic and parasympathetic nerve bundles also carry sensory nerve fibers containing SP and/or CGRP which target the main duct, small ducts, and blood vessels in rat SMGs.[17]

Clinical conditions affecting salivary gland function. Major causes of irreversible salivary gland hypofunction and associated xerostomia include systemic diseases such as Sjögren's syndrome, granulomatous diseases, Graft-versus-host disease, cystic fibrosis, Bell's palsy, uncontrolled diabetes, amyloidosis, human immunodeficiency virus infection, thyroid disease, late-stage liver disease, as well as irradiation (IR) treatment for head and neck tumors.[18] Sjögren's syndrome (SS) is the major systemic autoimmune disorder affecting salivary gland function. The etiological agent in SS is not known and there is much debate in the literature as to whether there is a neurological component to the primary damage of the gland, which might cause a secondary inflammatory response.[19] We will not discuss SS herein and the reader is referred to reviews on this subject.[19-21]

The other major clinical cause of salivary hypofunction (xerostomia) is due to injury after radiotherapy for head and neck cancer, where the salivary glands are in the field of IR.[22] IR-induced xerostomia can be permanent and is hypothesized to be multifactorial, involving damage to salivary gland epithelial cells, the blood vessels, and the associated nerves.[22,23] Salivary glands consist of two types of epithelial parenchyma cells: 80% acinar and 20% ductal. Following IR, patients lose the majority of acinar

cells, and surviving parenchymal cells are primarily ductal, which irreversibly impacts salivary secretion and causes inflammatory damage and fibrosis.[22,24,25]

Ductal ligation has also been used as a model to study salivary hypofunction and regeneration. When salivary ducts are blocked by salivary stones or ligated in an experimental animal model, the gland undergoes rapid atrophy. When the blockage or ligation is removed from the gland duct, damage to the gland is reversible. Initial studies used this ligation-induced atrophy plus deligation model in the rat SMG to study the function of nerves on the regeneration of salivary glands.[26] These studies show that if duct ligation is done with a pre-ganglionic parasympathectomy, gland regeneration is reduced by 50% after 4 weeks of deligation. SMG secretion, as evaluated by whole body cholinergic stimulation (methacholine-mediated), revealed that denervated and deligated glands only secreted ~60% of saliva compared with normally innervated deligated glands.[1] A similar role for parasympathetic and sympathetic nerves has also been found in the developing gland. Parasympathectomy at birth reduced gland weight by 60% and greatly reduced the development of the myoepithelial cells surrounding acini.[27] In addition, sympathectomy at birth caused a significant reduction in salivary gland size by nine weeks postnatal.[28] Therefore, the autonomic nervous system can regulate salivary gland secretion and may have a role in organogenesis and glandular regeneration.[1,2] In the next subchapters, important differences between sympathetic and parasympathetic neuronal development in salivary glands will be discussed.

Parasympathetic Nerve Development during Gland Morphogenesis

Early studies using chick-quail transplants revealed that neurons from autonomic ganglia (parasympathetic and sympathetic) derive exclusively from neural crest cells.[29,30] The mechanisms controlling the development and maintenance of the mouse SMG parasympathetic ganglia (PSG) have also been investigated during fetal development.[31,32] During early fetal stages at embryonic day 12 (E12), parasympathetic gangliogenesis and innervation occur alongside the developing SMG epithelium, and innervation is directed by the epithelium.[2,3,31] Whereas, the OG does not develop alongside the PG, and branching morphogenesis does not begin until otic postganglionic neurons reach the initial PG epithelial bud.[33] The developing PG initiates as a single end bud on a long duct that continues to elongate until the end bud is innervated and branching begins.

Several studies using knockout mice show a significant role for the glial cell line-derived neurotrophic factor (GDNF) family during peripheral innervation of the salivary glands, which suggest that neuronal-epithelial communication is critical for SMG function.[34-37] The GDNF family ligands consist of four members, GDNF, Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN), which are involved in the survival, proliferation and differentiation of neuronal populations in the central and peripheral nervous systems.[38] The receptors for these ligands include the glial cell-derived family receptors α (GFRα1–4), which determine ligand specificity. As such, GFRα1 through 4

are the preferential co-receptors for GDNF, NRTN, ARTN and PSPN, respectively, however alternative ligand-co-receptor interactions can also occur. Following homodimeric ligand binding to the cognate GFRα, the RET tyrosine kinase co-receptor becomes dimerized and phosphorylated, and activates downstream pathways such as Ras-mitogen-activated protein kinase (MAPK), the phosphatidylinositol-3 kinase (PI3K)-Akt, the phospholipase c (PLC)-γ ανδ the Src signaling pathways.[38] NRTN and GDNF are crucial neurotrophic factors with different temporal effects on the prenatal and postnatal development and survival of the SMG parasympathetic ganglion (PSG) by binding the GFRα 1, 2 or 3, and the RET co-receptor.

An important aspect of these studies is that NRTN and its receptor GFRα2 are critical for the survival and maintenance of parasympathetic neurons.[34,37] Adult mice lacking NRTN or GFRα2 display a dramatic reduction of parasympathetic innervation to the SMG, the SLG and PG, as well as lacrimal glands, which may cause their reported increase in frequency of water intake when compared with wild type.[34,36,37] Moreover, the co-receptor tyrosine kinase RET, is required for the proper development of several cranial PSG, including the otic and sphenopalatine ganglia, which target the lacrimal gland. The complete absence of the sphenopalatine and otic ganglia in RET^{-/-} mice prompted the investigation of other neurotrophic factors in the formation of these ganglia. Consequently, GDNF^{-/-} mice were analyzed since GDNF was reported to have neurotrophic activity on cultured parasympathetic neurons.[39] Mice lacking GDNF^{-/-} exhibited deficits in PSG numbers in the SMG, which were reduced by 36% when compared with wild type. However, otic PSG numbers decreased by 86% and sphenopalatine PSG were almost completely eliminated (a phenotype similar to that observed in the RET^{-/-}).[40] In addition, mice depleted of the GFRα1 gene, a primary co-receptor responsible for the GDNF-mediated activation of the RET kinase, had a similar phenotype to GDNF^{-/-} mice with moderate neuronal loss (33%) in the SMG PSG.[41] They also showed that GDNF signals in vivo through its preferred receptor, GFRα1, to control the development of parasympathetic neurons. The defects observed in GDNF^{-/-} mice largely accounted for the severe deficits found in RET^{-/-} mice. However, in the SMG PSG, losses found in GDNF^{-/-}, NRTN^{-/-} and RET^{-/-} mice were essentially equivalent (36%, 41% and 30%, respectively). In summary, these knockout studies demonstrate that, along with NRTN, GDNF is a crucial factor for parasympathetic neuronal development. Since these knockouts did not result in the absence of SMG PSG, they suggest that other factors may be involved in PSG formation.

More recently, useful tools have been made available to investigate when gene expression associated with developing nerves occurs during salivary gland organogenesis. The Salivary Gland Molecular Anatomy Project (SGMAP) provides a searchable public database of microarray gene expression analysis during mouse salivary gland development (http://sgmap.nidcr.nih.gov/sgmap/sgexp.html). The temporal expression patterns of genes are available for SMG and SLG from embryonic day (E) 11.5 through postnatal and adult stages, and spatial expression patterns are provided for selected developmental stages from both
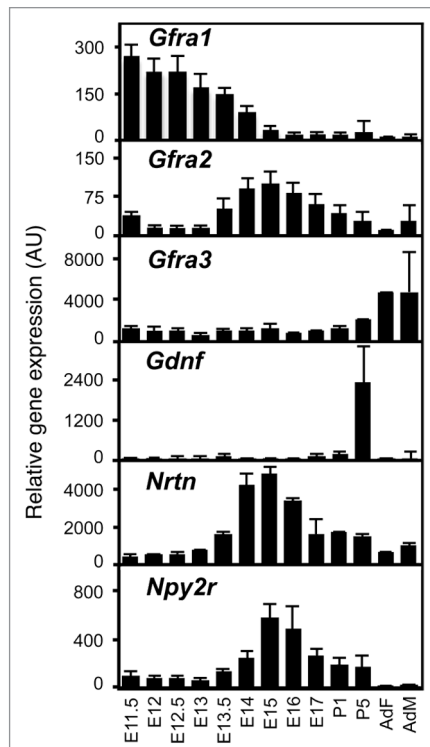
Figure 2. Differential expression of genes associated with parasympathetic and sympathetic innervation during salivary gland development from SGMAP (http://sgmap.nidcr.nih.gov/sgmap/sgexp.html) Gene expression was measured by microarray with global normalization, over a developmental time course from E11.5 to adult (Ad). AdF, adult female; AdM, adult male; E, embryonic day; P, postnatal day.

microdissected tissue at E13 and laser-capture dissected cells at specific stages of development.[42] Using this database we can analyze the temporal expression of receptors in the developing SMG. The expression of GFRα1 decreases after the SMG ganglia is formed (E11.5-E12) until E16 when expression levels remain relatively constant through adult stages. In contrast, during a similar embryonic period GFRα2 is upregulated dramatically from E13-E15 and then decreases from E15 to adult stages (**Fig. 2**).

Early in development, during the shift from neural precursors to neurons (from E11.5-E12), the receptor expression pattern observed in the developing otic PSG show that GFRα1 expression is downregulated, and that GFRα2 becomes the predominantly expressed receptor after ganglion formation.[41] NRTN gene expression follows a similar trend to that of GFRα2, but GDNF has a very low and stable gene expression through embryonic life and only increases at birth and postnatally (**Fig. 2**). Taken together, it appears that during the shift from neural precursors to neurons during PSG development, cells switch their dependency from GDNF to NRTN.[41] Interestingly, earlier studies have shown that neurons of the sphenopalatine and otic PSG require NRTN for proper differentiation and maintenance rather than survival since they have been found in normal numbers in adult NRTN^{−/−} mice. However, our lab recently demonstrated that NRTN also promotes neuronal survival in isolated SMG PSG and restores parasympathetic innervation.[3]

An important advance in the field came with the discovery that the developing parasympathetic nervous system in the SMG influenced the developing epithelium.[2] At early stages of development, the parasympathetic ganglion develops from the neural crest and associates with the SMG duct, and gland innervation occurs in parallel with epithelial branching morphogenesis. Salivary gland epithelial morphogenesis likely depends on the size of the epithelial progenitor pool during development. In these studies, we used ex vivo dissociation, recombination, and culture of the tissue components, including the epithelium, mesenchyme, and PSG. After recombination in the absence of the PSG there was reduced gene expression of the epithelial progenitor cell markers cytokeratins-5 (K5) and -15 (K15), as well as aquaporin 3 (Aqp3), and a reduction in the number of K5+ cells in the epithelium. Importantly, ACh from the nerves stimulated proliferation of the K5+ epithelial progenitor cells. This was dependent on the muscarinic receptor (Chrm1) and the epidermal growth factor receptor (EGFR). Thus, the PSG maintained the K5+ epithelial progenitor cell population and induced ductal differentiation into K19+ cells, which was critical for further organogenesis. Developing SMGs contain 9.6 ± 1.3% of K5+ cells mainly in the end buds and ducts.[2] We also showed that muscarinic and EGFR signaling were important in adult salivary glands in explant culture for the maintenance of K5+ cells, and that a similar mechanism occurs in the mouse ventral prostate.

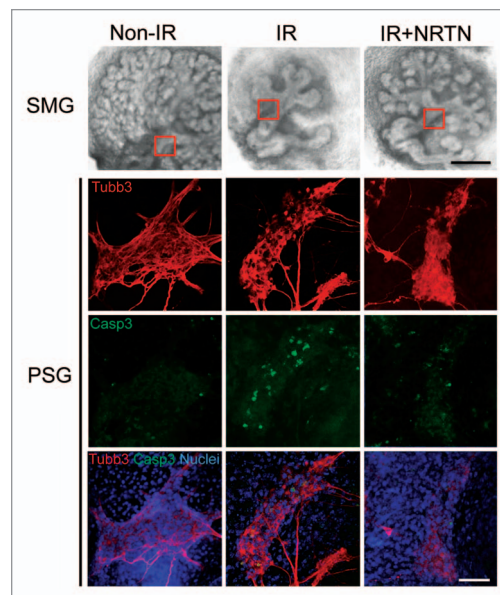## Sympathetic Nerve Development during Gland Morphogenesis

Chronic sympathectomy experiments (for 12 weeks) in the adult rat PG caused a significant reduction in the gland's weight with a specific reduction in the synthesis of proline-rich proteins.[43] Likewise, nine weeks of continuous sympathetic denervation after birth produced a significant gland hypoplasia with a reduction in secretory granule content.[28] The sympathetic nerves are presumed to innervate the gland near birth by following the blood vessels into the gland while pro-acinar and terminal ductal cells are maturing. Although, earlier studies indicated that sympathetic innervation in the SMG is postnatal, around P3–P5.[44]

The SCG is the sympathetic ganglion that contains the neuronal cell bodies of the postganglionic fibers that directly innervate the SMG. RET ligands may play essential roles in the development of the SCG. Interestingly, NRTN was first identified based on its ability to promote the survival of the rat SCG, although the SCG of NRTN^{−/−} mouse has a normal appearance. This observation was particularly surprising because the SCG is completely absent in mice lacking RET[−/−][45] and GDNF^{−/−} or GFRα1^{−/−} mice have normal or moderately affected SCG (35% smaller), suggesting that RET ligands, other than NRTN, may provide the critical survival signals for SCG neurons in vivo.[46-48] Another RET ligand, Artemin, is likely to be important for SCG sympathetic neuron survival. To support this idea, Artemin was found to be trophic for SCG neurons and Artemin's preferred co-receptor GFRα3 is expressed in the SCG.[49] However, in the SMG, this effect on sympathetic neuronal survival may be occurring after birth, since gene expression levels of GFRα3 remain steady from

neuronal progenitor migration to complete ganglia formation in the embryonic mouse SMG (**Fig. 2**). When we view GFRα3 expression throughout SMG development using the SGMAP, it has increased expression after birth, suggesting an involvement in postnatal innervation. However, certain SCG sympathetic receptors have increased gene expression in the mouse during early SMG development when branching morphogenesis occurs (E13.5-E14). For example, neuropeptide Y (NPY) receptor 2 (NPY2r), a receptor with preferential binding to neuropeptide Y is present in sympathetic SCG[50] and its expression significantly increases before birth in the SMG (**Fig. 2**). NPY is also active at angiogenic sites, exerting stimulatory effects on endothelial cell migration, proliferation, and differentiation into capillary tubes in vitro, and it significantly enhances in vivo angiogenesis.[51] Antagonists for receptor NPY2r block NPY-induced endothelial cell migration that may occur in early organogenesis.[51] Moreover, recent studies on dopamine neurons have suggested a neuroprotective role of NPY, which is preferentially mediated via the Y2 receptor and implicates the activation of both the MAPK and the Akt pathways.[52] Similarly, MAPK and Akt signaling are also downstream of NRTN/GFRα2/RET tyrosine kinase and have been shown to promote neuronal survival.[53] As such, NPY-positive neurons may also be involved in neuronal survival during the late stages of SMG prenatal development. These NPY-positive neurons carry sympathetic and sensory information.

Figure 3. NRTN treatment improves epithelial morphogenesis after IR by reducing apoptosis of the PSG neurons. Top panels are brightfield images of E13 SMGs with or without IR and pre-treatment with recombinant NRTN. SMGs were visualized after 96 h of culture. Red boxes show the approximate location used to image the PSG below. Scale bar: 500 μm. Lower panels are confocal analysis of the PSG. Immunostaining for PSG with neural tubulin antibody (Tubb3 red), for apoptotic cells with Caspase 3 (Casp3, green), for nuclei with Hoechst. The bottom panels are merged images. Scale bar: 10 μm.

Targeting the Nerves after Gland Damage Could Induce Salivary Gland Regeneration

Every year approximately 500,000 new head and neck cancer patients undergo therapeutic IR worldwide. When salivary glands lie in the radiation field, gland damage and permanent moderate to severe dry mouth occurs in 64% of cancer patients.[22,54] The consequent salivary gland hypofunction has a dramatic impact on the oral health of patients and significantly reduces their quality of life.[22,54] Recently, our research group found that stimulating the irradiated-fetal mouse SMG with recombinant NRTN can significantly decrease the IR-induced PSG apoptosis, thus improving the maintenance of K5+ epithelial progenitors and rescue branching morphogenesis (**Fig. 3**).[3] As a result, NRTN may provide a promising in vivo tool to regenerate a damaged irradiated-gland by (1) either inducing K5+ progenitor cells or (2) by stimulating the nerves and remaining epithelium to proliferate and migrate. In IR human SMGs, K5+ progenitor cells are present, and NRTN expression in the remaining acinar cells was reduced by approximately 60%. Progenitor cell markers K5 and keratin 19 were increased similar to the IR mouse embryonic SMG. Other downregulated genes in IR human SMGs included the muscarinic receptors Chrm1 and Chrm3, aquaporin 5 a marker of acinar differentiation, and fibroblast growth factor receptor 2, which is involved in branching morphogenesis. On the other hand, sympathetic innervation was increased in IR adult human SMGs, where expression of the sympathetic markers tyrosine hydroxylase and adrenergic receptor 2B increased.[3] In the adult, sympathetic innervation regulates salivary secretion and may have inhibitory effects on epithelial regeneration, as

sympathetic signaling blocks muscarinic-induced EGFR activation.[55] Whether IR causes PSG apoptosis in humans, as occurs in IR fetal and adult mouse SMGs, remains yet to be determined. Additionally, other GDNF neurotrophic factors, ARTN and PSPN, may also be useful to stimulate RET downstream neuronal survival cascades, like MAPK and Akt pathways, although no studies have yet been done in the fetal mouse IR model with these neurotrophic factors. We speculate that in the SMG increased sympathetic activity may result in reduced progenitor cell self-renewal. For example, in the liver, blocking sympathetic function increases hepatic progenitors and liver regeneration.[56] Therefore, increased sympathetic innervation combined with reduced parasympathetic function after IR may negatively influence organ regeneration. A future area of research will be to investigate the balance between parasympathetic and sympathetic inputs during salivary gland regeneration.

Future Studies

In conclusion, understanding how the developing nervous system influences organogenesis is an exciting but understudied research area. Epithelial organ repair or regeneration can occur after injury if parasympathetic innervation is maintained. Without functional innervation and in the absence of factors that maintain neuronal-epithelial communication, progenitor cell regeneration and tissue repair do not occur. Our preliminary data suggests that neurotrophic factors such as NRTN may

protect parasympathetic neurons and/or increase their function, which would prevent or reduce tissue damage from insults such as IR and improve regeneration with or without additional stem cell therapy. It will be important to investigate the function of innervation during development in other tissues and organs with parasympathetic innervation that also express NRTN, including the gastrointestinal tract, trachea, cornea, exocrine pancreas, lacrimal glands, and hair follicles.[57] In terms of a clinical pipeline, NRTN is a potential candidate for gene therapy to protect, repair, or regenerate the remaining epithelium in the salivary glands after IR damage. A NRTN-expressing adeno-associated virus is currently in clinical trials to improve motor function and coordination in patients with Parkinson disease.[58,59] Other classes of neurotrophic factors including RET ligands could potentially regenerate damaged salivary glands; although further studies are required.

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