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Optogenetic Control of the Peripheral Nervous System

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The peripheral nervous system (PNS) is highly complicated and heterogenous. Conventional neuromodulatory approaches have revealed numerous essential biological functions of the PNS and provided excellent tools to treat a large variety of human diseases. Yet growing evidence indicated the importance of cell-type-specific neuromodulation in the PNS in not only biological research using animal models but also potential human therapies. Optogenetics is a recently developed neuromodulatory approach combining optics and genetics that can effectively stimulate or silence neuronal activity with high spatial and temporal precision. Here, I review research regarding optogenetic manipulations for cell-type-specific control of the PNS, highlighting the advantages and challenges of current optogenetic tools, and discuss their potential future applications.

The peripheral nervous system (PNS) connects body organs and the brain, and is essential for numerous physiology functions and behaviors. The PNS contains two subsystems: the somatic nervous system containing sensory and somatic nerves and the autonomic nervous system, which controls involuntary physiological functions. Anatomically, the PNS is composed of cranial nerves, spinal nerves, and local neural networks around body organs (Catala and Kubis 2013; Karemaker 2017). Cranial nerves, which originate from the brain, link organs in the head and neck region, including skin, muscle, eye, tongue, ear, nose, larynx, and pharynx, with the central nervous system. Organs below the head are generally innervated by spinal nerves. Many essential specific sensory modalities such as vision, smell, taste, hearing, and balance are carried by cranial nerves. Somatosensations, including touch, pain, temperature, and visceral sensation, are also conducted by sensory fibers within cranial and spinal nerves. Spinal sensory fibers travel through dorsal roots of the spinal cord, with their cell bodies located in dorsal root ganglia (DRG). Muscle movements in the head and neck region are controlled by cranial nerves, while spinal motor fibers that directly innervate their effector muscles in the lower body exit the spine from ventral roots.

Some cranial nerves and spinal nerves are sensory or motor only, while others contain a mixture of fibers for sensory, motor, or autonomic roles. A unique member of the cranial nerves, the vagus nerve (also known as the cranial nerve X or CN X), provides both sensory

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(afferent) and motor (efferent) autonomic innervation to organs in the upper and lower body. In the head and neck region, this includes larynx and pharynx, but also many lower body organs, including lung, heart, blood vessel, esophagus, stomach, intestine, liver, kidney, and pancreas (Berthoud and Neuhuber 2000). Majority of parasympathetic preganglionic neurons that innervate organs in the head reside in cranial ganglia (Wehrwein et al. 2016; Karemaker 2017). Their downstream targets, parasympathetic postganglionic neurons, are located within or near their target organs. Vagus parasympathetic (motor) fibers innervating visceral organs project directly from the dorsal nucleus and nucleus ambiguus in the brainstem. On the other hand, sympathetic neurons are contained in spinal nerves. Preganglionic neurons originated from the spinal cord travel a short distance to synapse with postganglionic neurons with cell bodies located in bilaterally symmetric sympathetic chain ganglia or the collateral ganglia. Such postganglionic neurons then send long projections to control their target visceral organs. Another large subset of the PNS is composed by local neural networks around visceral organs, such as the enteric nervous system, which is composed by ~500 million neurons lining the gastrointestinal tract in human (Furness 2012). Similar neural networks are also described for the cardiovascular system (Armour et al. 1997). These local neural plexuses interact extensively with visceral organs as well as sensory and motor autonomic nerve fibers. They can function independently as local reflexes and work together with the sympathetic and parasympathetic nervous system to coordinately regulate organ physiology.

Dysregulation of the PNS is associated with numerous diseases, and thus neuromodulation in the PNS has been a great interest for more than a century. Surgery, pharmacology, and electrical stimulation are common approaches to modulate peripheral nerve activities. Surgical denervation of selective nerve branches is used to treat a diverse range of diseases. For example, vagotomy was one of the most popular surgeries for treatment of duodenal and gastric ulcer disease during the 1970s and 1980s and is still in use in some emergency operations (Woodward 1987; Lipof et al. 2006). Cervicothoracic sympathectomy was used to treat patients with hyperhidrosis (excessive sweating), vasospastic conditions (e.g., Raynaud's disease), and angina pectoris (chest pain) for many decades (Drott 1994). Endoscopic thoracic sympathectomy that removes part of the thoracic sympathetic nerve trunk is still a modern procedure to treat patients with focal hyperhidrosis (Cerfolio et al. 2011). Stellate ganglion block, achieved by injecting local anesthetic into the stellate ganglion, is commonly used to reduce pain and has been considered for treating arrhythmia (Nademanee et al. 2000; Jeon 2016). A targeted lung denervation procedure launched by Nuvaira is currently on clinical trial for the treatment of severe asthma (Wolter 2018). In addition, discoveries of receptors, ion channels, signaling molecules, and other proteins critically involved in PNS-related diseases over the past few decades prominently facilitated the design of pharmaceutical drugs. Recently, extensive research has been applied to investigate bioelectronic devices to achieve peripheral nerve stimulation. Surgically implanted vagus nerve stimulation (VNS) devices are clinically used to treat patients with drug-resistant refractory epilepsy and severe, recurrent unipolar and bipolar depression (Schachter and Saper 1998; Rush et al. 2000; Uthman 2000; Groves and Brown 2005; O'Reardon et al. 2006; Krahl 2012; Yuan and Silberstein 2016a). Many VNS-based clinical trials have been conducted or are currently ongoing for treating inflammatory and cardiovascular diseases (Gold et al. 2016; Koopman et al. 2016; De Ferrari et al. 2017; Bonaz 2018; DiCarlo et al. 2018). In animal studies, VNS is being investigated for treatment of numerous diseases, including acute asthma, acute kidney injury, hypertension, heart failure, inflammation, blood glucose management, and metabolic syndromes (Das 2011; Sabbah et al. 2011; Hoffmann et al. 2012; Chapleau et al. 2016; Inoue et al. 2016; Meyers et al. 2016; Ardell et al. 2017; Pavlov and Tracey 2017; Kim et al. 2018; Pavlov et al. 2018). Noninvasive VNS approaches are also being investigated, and the first noninvasive VNS therapy was approved in late 2017 for acute

treatment of cluster headache (Siefring 2017) and later for the treatment of migraine pain (Brauser 2018).

Although existing neuromodulatory approaches are powerful tools, and being used for research in animal studies and also for human therapies, multiple fiber types, including sensory, motor, or autonomic fibers, are almost always affected by these techniques. However, the PNS is a highly heterogenous system. A wide variety of neurotransmitters and neuropeptides are used in the PNS (McCorry 2007; Wehrwein et al. 2016). Parasympathetic preganglionic neurons are largely cholinergic, using acetylcholine to communicate with their postganglionic targets. The primary neurotransmitter in parasympathetic postganglionic neurons is also acetylcholine, although other neurotransmitters, including nitric oxide and vasoactive intestinal polypeptide (VIP), are active as well (Travagli and Anselmi 2016). Likewise, sympathetic preganglionic neurons rely on acetylcholine to activate nicotinic acetylcholine receptors on postganglionic neurons. Unlike the parasympathetic system, sympathetic postganglionic neurons control their target functions primarily using norepinephrine, classified as adrenergic neurons. Other neurotransmitters such as acetylcholine, epinephrine, and dopamine are also released by sympathetic postganglionic neurons for some targets (Murphy and Elliott 1990; McCorry 2007). Sensory neurons in the glossopharyngeal nerve (cranial nerve IX), the vagus nerve (cranial nerve X), the trigeminal nerve (cranial nerve V), and DRG are largely glutamatergic. Neuropeptides such as VIP, CGRP, and substance P present in subsets of sensory neurons and their physiological roles still need to be fully determined (Levine et al. 1993; Zhuo et al. 1997; de Lartigue 2014; Goto et al. 2017). To an extreme extent, more than 30 neurotransmitters are used by neurons in the enteric nervous system (McConalogue and Furness 1994; Costa et al. 2000). Cell heterogeneity in different tissues has continued to be explored over the past decades. Numerous novel cell types in regions that were previously thought to be homogenous have been identified using novel microfluidics-based, massively parallel single-cell transcriptome analysis (Macosko et al. 2015; Habib et al. 2017; Rodda et al. 2018). Cell heterogeneity in peripheral ganglia and the enteric nervous system has previously been reported, and more cell types will certainly be discovered in the PNS going forward. Yet the existing tools, including electrical stimulation, surgical perturbation, and pharmacological modulation, can hardly provide insights at a cell-type-specific resolution, calling for new neuromodulatory technologies and approaches in the PNS. Recent developments and applications for optogenetic tools in peripheral nerves are outlined below.

APPLICATION OF OPTOGENETICS IN THE PERIPHERAL NERVOUS SYSTEM

Optogenetics is a powerful neuromodulatory technology that combines optics and genetics to achieve neuronal activation or inhibition in selected neurons (Boyden 2011; Deisseroth 2015). Optogenetic tools evolved rapidly since the pioneer work, which demonstrated the powerful effect of channelrhodopsoin ChR2-based light stimulation in controlling neuronal activity in mammalian neurons (Boyden et al. 2005; Li et al. 2005; Nagel et al. 2005). Nowadays, optogenetics is widely used in animal studies for research regarding neural circuits, neuronal functions, and behaviors. In general, choice of opsins, gene-targeting methods, expression levels, light delivery approaches, and appropriate controls are the major aspects that need to be considered when conducting optogenetic studies.

Opsins for optogenetics are light-gated ion channels that belong to the large family of microbial opsins (Fig. 1; Zhang et al. 2011; Deisseroth 2015). As one of the most commonly used opsins for neuronal activation, channelrhodopsin2 (ChR2) identified from green alga is a blue light–gated nonselective cation channel that offers multiple ideal features for in vitro and in vivo neural stimulation (Boyden et al. 2005; Li et al. 2005; Nagel et al. 2005). First, light stimulation of ChR2 leads to large photocurrents and potent depolarization when expressed in neurons. This is important as minimum light can be used for neuronal activation, which is espe-

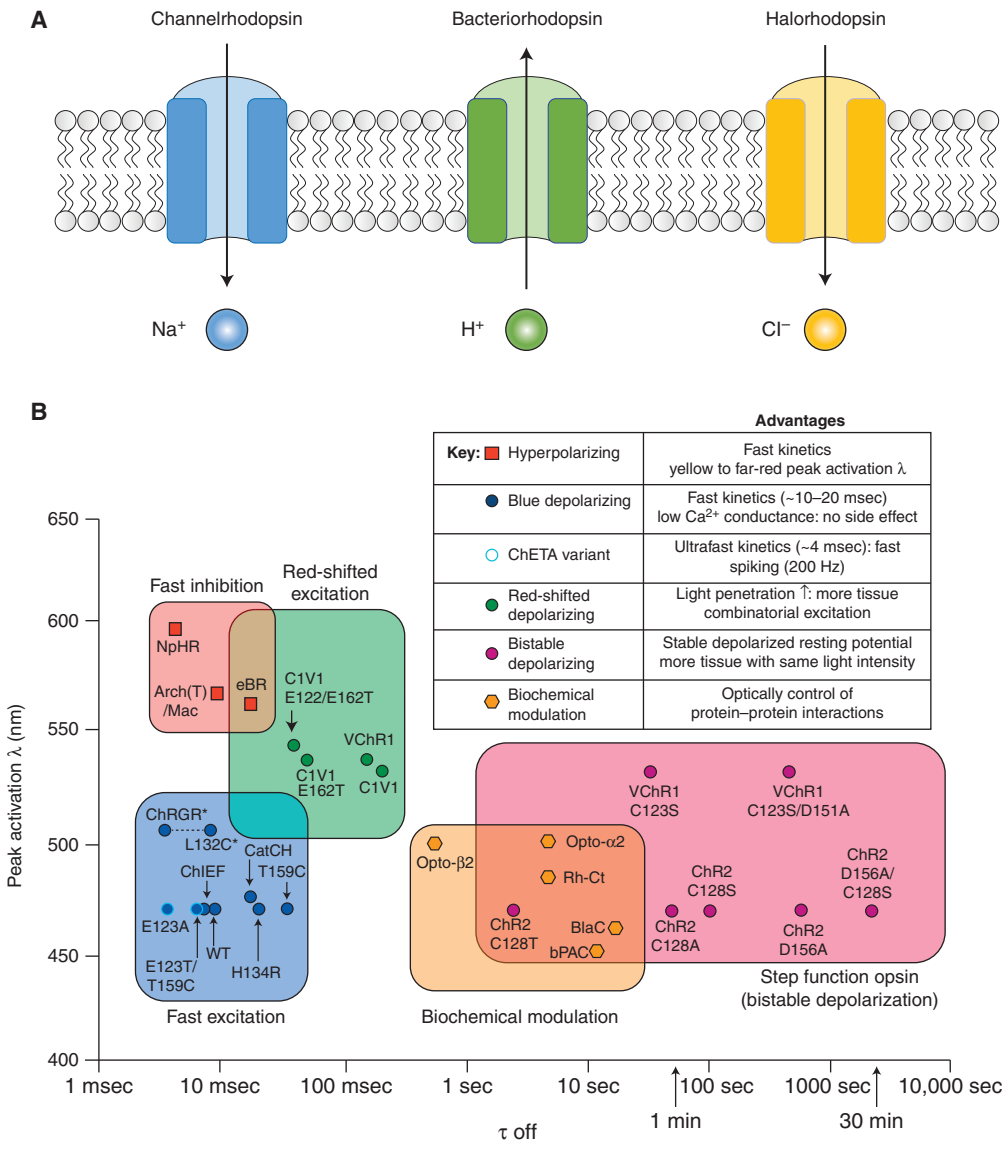

Figure 1. Light-activated microbial opsins in optogenetics. (*A*) Schematic illustration of the three major classes of microbial opsins for neuronal inhibition and activation. (*B*) Summary of most available opsins for optogenetic studies. (Panel *B* from Gerits and Vanduffel 2013; reprinted, with permission, from Elsevier © 2013.)

cially critical for in vivo applications. Second, because of the fast activation and relaxation kinetics of ChR2, neuronal activation can be achieved repeatedly in a temporal precision manner at a millisecond scale. Today, a large number of channelrhodopsin variants with improved photocurrents, kinetics, light sensitivity, and trafficking efficiency are available. ChR2 (H134R), a ChR2 variant with a single amino acid mutation, has an improved channel conductance and photocurrents with minimal sacrifice of kinetics and is widely used for a variety of in vitro and in vivo applications (Nagel et al. 2005; Madisen et al. 2012). Chronos, a channelrhodopsin discovered from *Stigeoclonium helveticum* that has a superior temporal resolution

with super-fast on and off kinetics is ideal for high-frequency neuronal stimulation (Klapoetke et al. 2014). As optogentic tools get better, a next goal is to achieve better tissue penetration for in vivo applications and to expand the optimal light stimulation spectrum for independent activation of multiple opsins simultaneously at different wavelengths. A number of channelrhodopsins with red-shifted action spectrum were developed for these purposes. VChR1, the first reported red-shifted channelrhodopsin discovered from *Valvax*, is maximumly excited with green light around 545 nm and can still be activated with orange light at 589 nm (Zhang et al. 2008). Nevertheless, photocurrents of VChR1 is small and its trafficking efficiency in neurons is not ideal. A few engineered variants with similar wavelength sensitivity but enhanced photocurrents and trafficking efficiency were later developed, including C1V1 and ReaChR (Yizhar et al. 2011; Lin et al. 2013). These improved opsins are great for deep tissue stimulation; however, as their wavelength sensitivity is quite broad, they can still be activated with blue light and are not ideal for multiwavelength stimulation with ChR2 for activation of distinct neuron types. Chrimson, a more red-shifted channelrhodopsin discovered from *Chlamydomonas noctigama* with maximum activation at 590 nm was developed (Klapoetke et al. 2014). Chrimson has large photocurrents at far-red 660 nm and is minimally activated at 470 nm compared to other channelrhodopsin variants. Now, it is possible to combine ChR2 and Chrimson together to achieve independent activation of two neuron populations.

A variety of microbial opsins are also developed to inhibit neuronal activities, including variants of halorhodospins, light-activated chloride pumps discovered from halobacteria, and archaerhodopsins, light-activated outward proton pumps found in Archaea. As both chloride and proton currents decrease neuronal excitability, halorhodopsins and archaerhodopsins are inhibitory. NpHR, a halorhodopsin from *Natronomonas pharaonic*, and Arch/ArchT, proton pumps from *Halorubrum sodomense* and *Halorubrum* strain TP009, respectively, are widely used for inhibitory optogenetics (Han and Boyden 2007; Zhang et al. 2007; Gradinaru et al. 2008; Chow et al. 2010; Madisen et al. 2012). A series of revisions with improved photocurrents and trafficking efficiencies have been developed. While the most current versions, eNpHR3.0, eArch3.0, and eArchT3.0, all have large photocurrents and fairly wide sensitivity across green, yellow, and red wavelengths, eNpHR3.0 is more red-shifted than the Archs (maximum activation at 590 nm vs 535 nm) (Mattis et al. 2012). All three are effective in neuronal silencing and have been applied for a variety of neuron types in vitro and in vivo, although it is worth noting that all three opsins require a relatively high light power density and thus may not be ideal for silencing a large volume of tissue. Another concern for inhibitory opsins is that unlike gain-of-function experiments that involve pulsed light stimulation, loss-of-function experiments often require chronic illumination, which may lead to current decay, depletion of ion sources, and tissue damage. Compared to channelrhodopsins, efficiencies of inhibitory opsins in neuronal silencing are more cell-type-dependent.

A big advantage of optogenetic tools compared to conventional neuromodulatory approaches (e.g., electrical, surgical, pharmacological, thermal, etc.) is the compatibility with genetics. In model organisms such as fruit fly and mouse, microbial opsins can be easily delivered to target neuron populations using a variety of genetic approaches to achieve cell-type-specific manipulation. In particular, diverse transgenic fly and mouse models exist and are publicly available. In mice, opsins can be transgenically expressed under a specific gene promoter (Arenkiel et al. 2007), be inserted in situ to either replace the target gene or after the target using an internal ribosome entry site (Wang and Zylka 2009; Smear et al. 2013), or be introduced using DNA recombinase-based technologies such as the *Cre-LoxP* system (Madisen et al. 2012; Zeng and Madisen 2012). A few things need to be considered before generating a transgenic mouse tool. First, expression of foreign genes in genetically targeted cells is usually more accurate in knockin animals compared

to transgenic. Second, the expression level is usually not a concern unless using a weak endogenous promoter, and is relatively stable across animals. Third, although the *Cre-LoxP* system has enabled optogenetics in diverse neuron types, and many mouse lines for Cre-dependent optogenetics are publicly available, spatial and temporal specificity is usually a concern. Viral delivery is another common strategy for both in vitro and in vivo studies (Deisseroth 2015; Montgomery et al. 2016). Compared to transgenic mouse models, viral delivery requires less mouse-generating efforts, is suitable for more cell types, and may give better temporal and spatial specificity. The expression level of introduced opsins can usually be controlled by adjusting (1) amount of virus used, (2) titer of the virus, (3) promoter, and (4) time after infection. Promoters commonly used for neurons include EF1α, CamKII, and hSyn. High-expression levels can be achieved by using different promoters such as CAG, which can be beneficial for some cases. On the other hand, viral delivery usually requires surgery, which introduce additional complications. Viral infection may elicit immune responses and cause more cell damage. Viral delivery may introduce additional variance among experiments, such as variabilities in number of infected cells and expression levels of opsins. In particular, overexpression can be problematic and may lead to cell sickness. Cell health usually needs to be closely monitored and assessed after viral infection. Some examples will be discussed in the following sections.

Lasers or high-powered, light-emitting diodes (LEDs) are great options for light stimulation in vivo. Within the brain, light delivery is usually achieved using an optic fiber cannula placed right above target neurons through a hole drilled through the skull (Sparta et al. 2012). The cannula is secured onto the skull with dental cement to ensure stability. This approach is widely used in both anesthetized and awake, freely behaving animals. As an optic fiber is needed to connect the light source with brain cannula, the animal is tethered in this strategy. Within the PNS, light delivery is easier to achieve for in vitro or ex vivo applications as well as for in vivo anesthetized animal preps using an externally positioned light source (Maksimovic et al. 2014; Chang et al. 2015; Williams et al. 2016; Nonomura et al. 2017), but is considerably harder for most studies that require awake animals (Fig. 2). Transdermal light illumination is widely used for optogenetic stimulation of peripheral nerves with nerve endings on or beneath the skin (Ji et al. 2012; Daou et al. 2013, 2016; Boada et al. 2014; Draxler et al. 2014; Iyer et al. 2014; Park et al. 2016). A few optic cuff-based strategies were developed for light illumination of large peripheral nerve trunks inside animal bodies (Towne et al. 2013; Michoud et al. 2018). In these applications, light delivery was achieved using an optic cuff made with optic fibers, wire-coupled LEDs, or wireless LEDs (Fig. 2). Nevertheless, these optic cuff-based devices function very well in larger animals with thicker nerve trunks but present significant challenges in small animals like mice because of surgical difficulties and tiny nerve size. An alternative strategy is to illuminate nerve or nerve endings on the organ surface using LED meshes (Samineni et al. 2017a). Specificity and efficacy are usually the major factors that need to be considered when using this approach. Recent advances in red-shifted opsins provide an opportunity for noninvasive deep-tissue stimulation, although their applications in peripheral nerves deep inside visceral organs still need to be demonstrated. Genetically encoded light sources like luciferase-based bioluminescence that generate light in response to externally administered substrates may provide effective internal illumination. Such tools basically convert optical stimulation to chemical stimulation. In particular, by fusing bright luciferase and red-shifted channelrhodopsins together, light stimulation efficacy can be significantly improved. Such bioluminescence tools have been applied to a few neuron types for in vivo stimulation (Land et al. 2014; Berglund et al. 2016). Although still in its early stage, a bioluminescence-based approach may provide powerful light sources for internal optogenetic stimulation in the future. Overall, light delivery approaches for awake animal studies in the PNS are still in a relatively early development stage and need to be examined in more applications.

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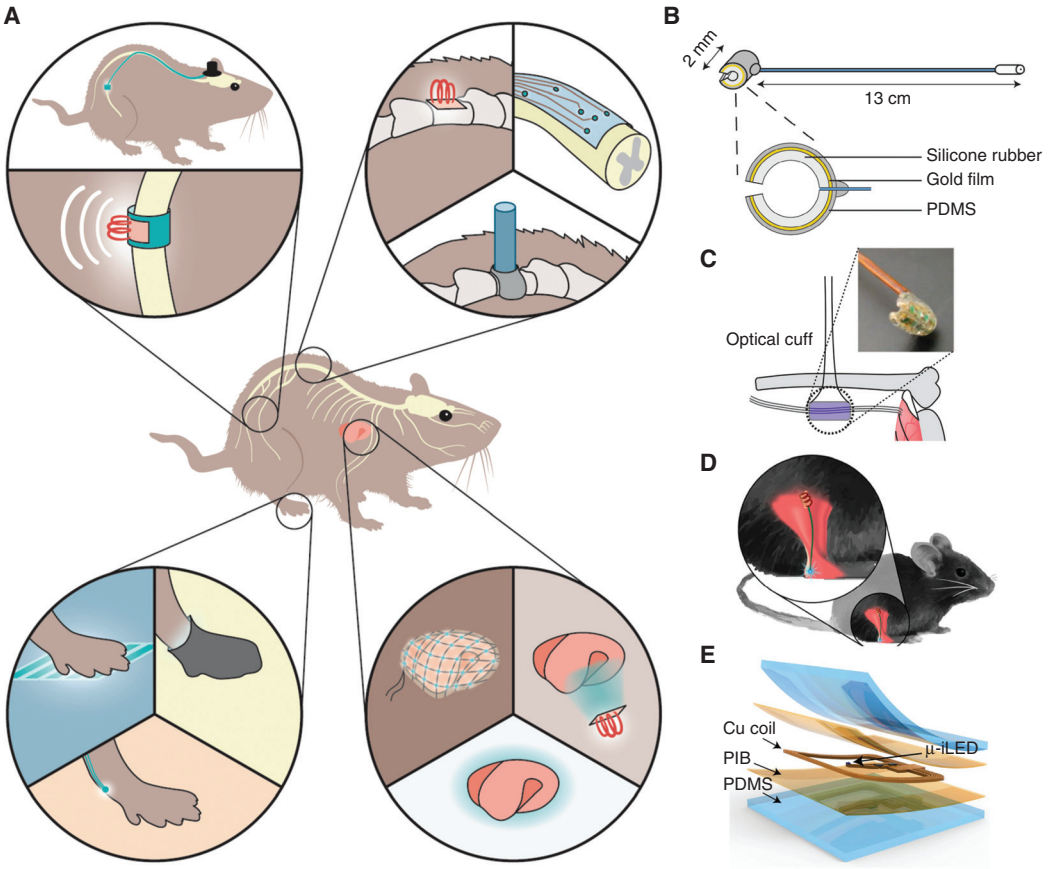

Figure 2. Light delivery methods for optogenetic studies in awake, freely moving rodents. (*A*) Schematic illustration of super soft and flexible optic cuff for optogenetic neuromodulation of large peripheral nerves, the spinal cord, nerve endings, and internal organs. (Panel based on data in Montgomery et al. 2016.) (*B*) Illustration of an optic fiber-based optic cuff implant for sciatic nerve stimulation in awake, freely moving mice. (Panel reprinted from Michoud et al. 2018 courtesy of IOP Publishing in conjunction with Creative Commons Licensing.) (*C*) Illustration of a micro LED-based optic cuff implant for sciatic nerve stimulation in anesthetized mice. (Panel based on data in Llewellyn et al. 2010.) (*D*) Illustration of a micro LED implant for illumination of sciatic nerve endings in awake, freely moving mice. (Panel reprinted from Montgomery et al. 2016 with permission from The American Association for the Advancement of Science © 2016.) (*E*) Illustration of a super soft, micro LED-based optoelectronic device for illumination of sensory nerve endings on the bladder in awake, freely moving mice. (Panel reprinted from Samineni et al. 2017a courtesy of Scientific Reports in conjunction with Creative Commons Licensing.)

DIVERGENT VAGAL NEURON POPULATIONS AND OPTOGENETIC CONTROL OF AUTONOMIC PHYSIOLOGY

The vagus nerve, the 10th cranial nerve, is a major conduit between body organs and brain that senses visceral changes and controls autonomic functions (Fig. 3). The vagus nerve innervates a variety of visceral organs, including the larynx, pharynx, trachea, lung, heart, aortic arch, stomach, intestine, liver, kidney, esophagus, intestine, and others (Berthoud and Neuhuber 2000; Yuan and Silberstein 2016b). Both ascending sensory afferent fibers and descending motor efferent fibers travel in the vagus nerve. Vagal sensory fibers, which comprise the majority

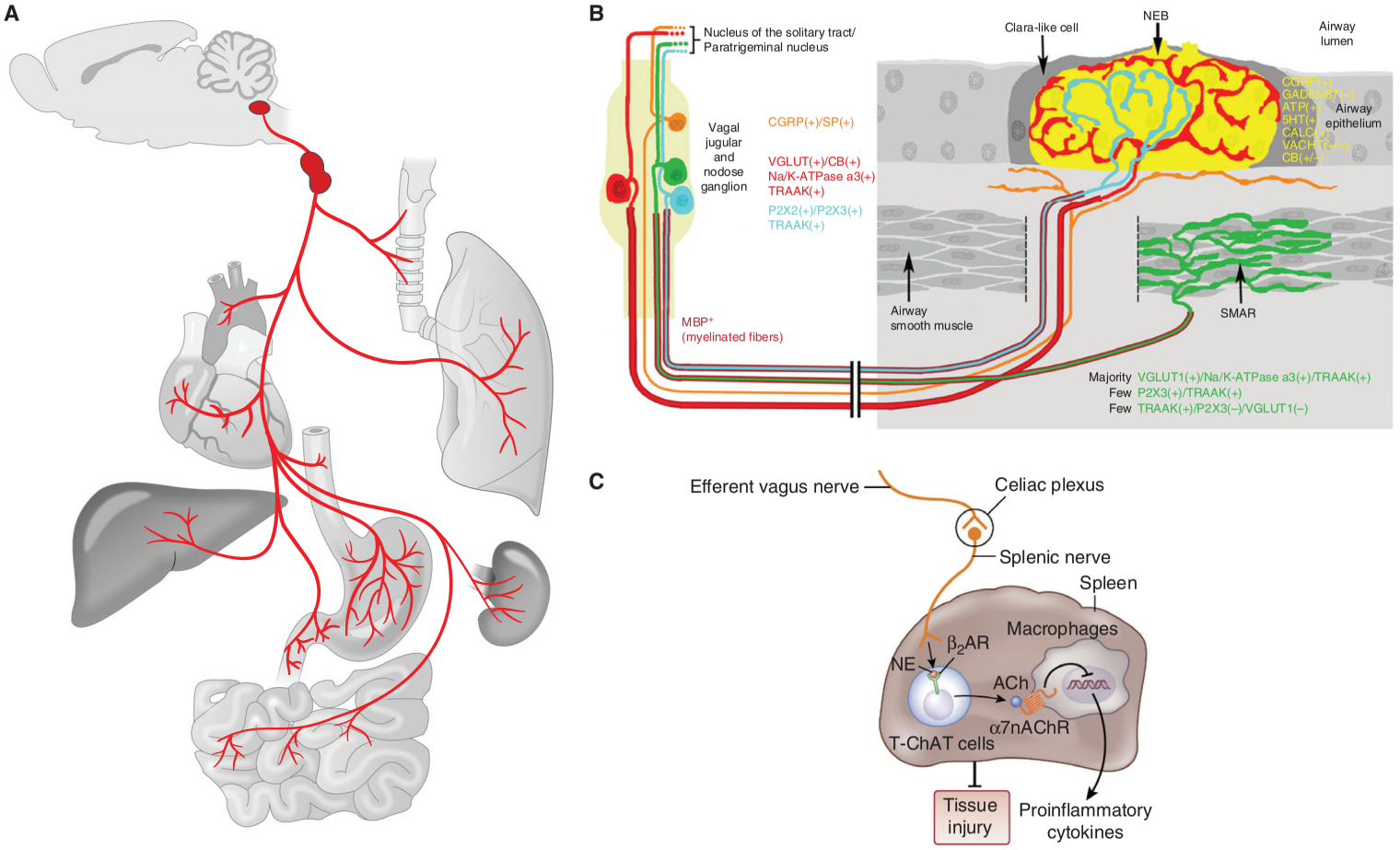

Figure 3. Anatomical and functional properties of the vagus nerve. (*A*) Anatomy of the vagus nerve. The vagus nerve serves as a major connection between a variety of body organs and the brain in both directions. (Panel based on data in Chang et al. 2015.) (*B*) Schematic illustration of diverse vagal sensory fiber types within the lung and airways. (Panel based on data in Mazzone and Undem 2016.) (*C*) Schematic representation of vagal efferent control of inflammation. (Panel based on data in Pavlov and Tracey 2017.)

(~80%) of the vagus nerve (Foley and DuBois 1937), carry a variety of environmental, physiological, and pathological information from the periphery, including airway pressure changes, inhaled air contents, ingested substances, blood pressure fluctuations, mechanical stretches along the gastrointestinal tract, inflammatory cues, and many others (Paintal 1973). On the other hand, vagal motor fibers participate in the control of diverse visceral functions such as bronchoconstriction, heart regulation, pancreas enzyme secretion, gastrointestinal motility, and inflammatory responses via acetylcholine release. Cell bodies of vagal sensory neurons reside in a pair of ganglia named nodose and jugular ganglion near the jugular foramen, while cell bodies of vagal motor neurons are located within the dorsal motor nucleus of the vagus (DMV) and the nucleus ambiguus in the brainstem. Gross anatomy, response properties, and physiological functions of vagal neurons have been extensively studied using histological, electrophysiological, pharmacological, and surgical approaches for over a century, yet our knowledge about the coding logic and some basic principles by which the vagus nerve regulates diverse autonomic physiology is still poor, largely a result of a lack of appropriate tools, presenting a major challenge for a deeper understanding of the underlying molecular mechanisms. A variety of cellular analyses regarding expression patterns of neuropeptides, receptors, ion channels, transporters, and transcription factors in vagal neurons were carried out using immunocytochemistry, RNA in situ hybridization, and single-cell-based gene analysis to uncover neuron heterogeneity and to understand potential cell signaling pathways (Helke and Hill 1988; Zhuo et al. 1997; Chang et al. 2015; Sajgo et al. 2016; Wang et al. 2017). However, the link between previous anatomical and functional findings and such molecularly defined neuron populations is still largely missing. Recent studies employing state-of-the-art mouse genetic tools were shown to be powerful in dissecting these essential body-to-brain circuits (Sisley et al. 2014; Trankner et al. 2014; Chang et al. 2015; Williams et al. 2016; Nonomura et al. 2017; Baral et al. 2018; Han et al. 2018). Here we will review some initial work with optogenetics in the vagus nerve (Fig. 4) and discuss the potential future applications.

Optogenetic Control of Breathing

Breathing is an essential physiological function for animal survival, providing appropriate oxygen into the body and taking away the metabolic wastes such as carbon dioxide (CO_2). Breathing is tightly controlled by the nervous system. In particular, groups of neurons located in the medulla oblongata in the brainstem form respiratory centers responsible for respiratory rhythm generation and motor pattern formation. Such neurons receive numerous regulatory inputs from both peripheral and other brain regions to ensure a precise regulation of respiration for appropriate tissue oxygenation at all conditions (Ikeda et al. 2017). Environmental, physiological, and psychological factors, such as inhaled air content, blood oxygen, CO_2, and pH levels, temperature, cardiovascular functions, circadian rhythm, mood, stress, and many others influence respiratory functions, including breathing frequency, depth, and pattern through a variety of mechanisms and neural pathways. Some essential respiratory reflexes involving mechanosensation along the respiratory tract and chemosensation from the lung, airways, and bloodstream are mediated by the vagal afferents (Paintal 1973; Widdicombe 2001; Mazzone and Undem 2016). Several classes of airway afferents were described (Fig. 3B; Widdicombe 2001; Carr and Undem 2003; Mazzone and Undem 2016). In general, airway mechanosensory fibers are myelinated fast-conducting A fibers that respond to airway inflation or deflation from diverse locations with different mechanosensitivities but not to capsaicin, a TRPV1 channel agonist commonly used to assess fiber chemosensitivity. Based on their response adaptation properties, airway mechanosensory fibers are further divided into rapidly adapting receptors (RARs) and slowly adapting receptors (SARs). Locations of RAR and SAR endings were determined using electrophysiology, yet their terminal structures were not precisely resolved. SARs are believed to mediate the

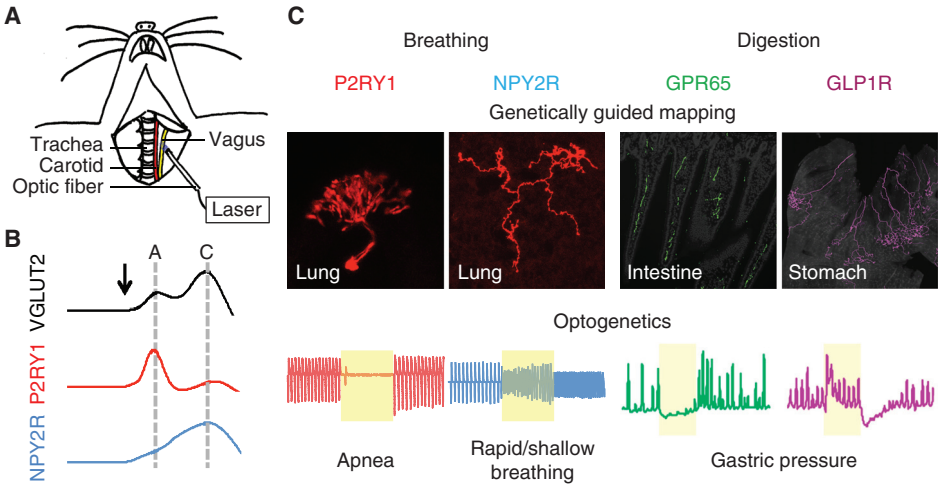

Figure 4. Optogenetic control of the vagus nerve in anesthetized mice. (*A*) Cartoon depiction of optogenetic stimulation of surgically exposed vagus nerve in anesthetized mice. (Panel from Chang et al. 2015; reprinted, with permission, from Elsevier © 2015.) (*B*) Measurements of conduction velocity in genetically defined sensory neuron populations with optogenetics. Compound action potentials evoked by brief optogenetic stimulation indicated that a majority of vagal P2RY1 fibers are fast-conducting A fibers and NPY2R fibers are largely slow-conducting C fibers. (Panel based on data in Chang et al. 2015.) (*C*) Summary of a few genetically defined vagal sensory neuron populations involved in control of respiration and digestion. (Panel based on data in Chang et al. 2015 and Williams et al. 2016.)

pulmonary stretch response, also named the Hering–Breuer reflex, which is an inhibitory respiratory reflex to lung inflation or deflation while RARs may mediate cough and other irritant responses, although their roles are still under debate. In contrast, airway chemosensory fibers are nonmyelinated, capsaicin-responsive, slow-conducting C fibers that mediate defensive respiratory responses such as rapid and shallow breathing. Although extensively investigated, a major challenge in understanding the respiratory reflexes is the lack of tools to selectively target particular fiber populations in intact or awake animals. It has been difficult to associate fiber types defined by electrophysiology with anatomical findings and physiological studies.

Recent application of genetic tools to the vagus nerve may help eventually overcome this problem (Chang et al. 2015; Nonomura et al. 2017). Using transgenic mouse models based on *Cre-LoxP* technology, diverse vagal subpopulations were genetically targeted and their projection patterns, neuronal response properties, and physiological roles were assessed. In particular, the feasibility of applying optogenetics in the vagus nerve to control autonomic physiology, such as breathing, was demonstrated in these studies. ChR2 (H134R) was introduced to genetically targeted vagal sensory neuron populations by crossing Cre mice with reporter mice carrying a Cre-dependent ChR2 H134R allele (*lox-ChR2* mice, Jackson Lab Stock #024109) to obtain *driver-Cre; lox-ChR2* mice. For example, to express ChR2 (H134R) in all vagal sensory but not vagal moter neurons, *Vglut2-ires-Cre*, which selectively target vagal sensory neurons, were used to obtain *Vglut2-ires-Cre; lox-ChR2* mice. Blue light generated from a 473-nm DPSS laser was delivered to the vagus nerve through a 200- to 400-μm optic fiber precisely placed on top of the carefully dissected vagus nerve or vagal ganglia using a micromanipulator in anesthetized animals (Fig. 4). Light intensity was adjusted to 75–125 mW/mm^2 to achieve optimized activation without causing thermal side effects. Stimulation frequency and duration was controlled using an optical shutter that physically blocks the light beam. Robust

neuronal activation was observed in the vagus nerve in response to blue light stimulation in these animals, as revealed by whole nerve electrophysiology, suggesting in vivo optogenetic stimulation was successful. High-frequency stimulation (5 msec, 50 Hz) of all vagal sensory neurons using *Vglut2-ires-Cre; lox-ChR2* mice caused an acute apnea, followed by a second phase of rapid and shallow breathing over a 10-sec trial. This effect was not observed in control animals that either lack the Cre driver or the ChR2 reporter, or in *Chat-ires-Cre; lox-ChR2* mice in which ChR2 is expressed in vagal motor neurons, suggesting that the light-evoked response requires functional ChR2 in the sensory arm of the vagus nerve. Interestingly, this effect was not obvious when using lower frequency stimulation (5 msec, 5 Hz), consistent with previous findings that physiological responses triggered by VNS is frequency dependent.

Optogenetic stimulation of two distinct vagal sensory neuron populations elicited powerful but opposite respiratory responses. Activating vagal P2RY1 neurons using *P2ry1-ires-Cre; lox-ChR2* mice resulted in a similar respiratory pause without the second phase observed when all sensory neurons were stimulated, while activating NPY2R neurons in *Npy2r-ires-Cre; lox-ChR2 mice* elicited rapid and shallow breathing. Not all vagal neuron types contribute to breathing, as activating vagal GPR65 neurons had no effect on respiratory patterns. Anatomical studies further confirmed that both vagal P2RY1 and NPY2R but not GPR65 neurons project extensively to the lung. Intriguingly, vagal P2RY1 and NPY2R neurons have different pulmonary arborization patterns and terminal morphologies. P2RY1 neurons account for the majority of vagal afferent fibers innervating the neuroepithelial bodies, while NPY2R fibers are enriched near the alveoli in the lung respiratory zone. This morphological difference suggests that they may be involved in distinct respiratory reflexes, and may provide an essential anatomical basis for understanding the underlying sensory mechanisms.

Conduction velocity is a widely used parameter to assess the electrophysiological property of peripheral nerve fibers, including vagal afferents. To determine electrical properties of genetically defined vagal neuron types, an optogenetics-assisted approach was developed. Instead of using a pair of wires for electrical stimulation, an optic fiber was used for ultrafast (0.8 msec) optogenetic stimulation (Fig. 4), and propagation speed was calculated by varying the distance between the optic fiber and recording electrodes. Both characteristics of A and C fiber responses were observed when all vagal sensory neurons are stimulated in *Vglut2-ires-Cre; lox-ChR2* mice, with an A/C ratio around 0.6, consistent with previous findings that the majority of vagal afferents are C fibers. Similar studies further demonstrated that the majority of vagal P2RY1 neurons are fast-conducting A fibers, while most NPY2R neurons are slow-conducting C fibers.

Intriguingly, a subset of vagal P2RY1 neurons coexpresses Piezo2, the mechanoreceptor essential for gentle touch and proprioception. Optogenetic stimulation of vagal Piezo2 neurons similarly caused apnea, suggesting that they may detect mechanical signals from the lung (Nonomura et al. 2017). Indeed, when Piezo2 was conditionally deleted from vagal sensory neurons using *Phox2b-Cre*, lung inflation-induced vagus nerve activation was abolished and the Hering–Breuer reflex was diminished, providing for the first time a molecular mechanism for pulmonary stretch detection.

Optogenetic Control of Gastrointestinal Functions

Organs along the gastrointestinal tract, including esophagus, stomach, and intestine are highly coordinated to achieve efficient food digestion and nutrient absorption (Cummings and Overduin 2007). Gastrointestinal organs also play a defensive role in preventing absorption of noxious or toxic substances by inducing vomiting or diarrhea (Horn 2014). Ingested food contents and mechanical stretch along the gastrointestinal tract are important sensory cues for appropriate regulation of digestive functions and feeding behaviors. Gastrointestinal organs are densely innervated by vagal sensory nerves (Paintal 1973; Fox et al. 2000; Berthoud et al.

2004). Similar to vagal airway afferents, sensory fibers that project to the gastrointestinal tract are largely mechanosensitive or chemosensitive. Mechanosensory afferents form specialized terminal structures such as intraganglionic laminar endings and intramuscular arrays on the muscular wall of the esophagus, stomach, and small intestine to detect stretch along the gastrointestinal tract, whereas mucosal endings likely sense ingested or digested substances from the digestive tract indirectly. Neural circuits between vagal sensory and motor fibers, so-called vago-vagal reflexes, are important for coordinating gastrointestinal motility and regulation of enzyme secretion after food ingestion (Rogers et al. 1995). Yet, the underlying neural architectures and molecular mechanisms are not well understood.

Vagal afferents likely detect nutrients from multiple locations along the gastrointestinal tract (Maljaars et al. 2008). Within the proximal small intestine, food contents do not contact vagal sensory endings directly but instead are detected by enteroendocrine cells or enterochromaffin cells, specialized epithelial cells on the intestinal villi that release a myriad of gut-derived signals, including glucagon-like peptide 1 (GLP1), peptide YY, cholecystokinin, and serotonin to regulate digestive processes like acid secretion and stomach contractility, as well as feeding behaviors (Chambers et al. 2013; Kim et al. 2018). The sensory vagus nerve is involved in a variety of gastrointestinal reflexes; however, the underlying sensory mechanisms are under debate. As vagal afferents innervating small intestinal villi terminate close to enteroendocrine and enterochromaffin cells and receptors for many gut-derived peptides are expressed in vagal sensory neurons, it is commonly assumed that gut signals released from enteroendocrine and enterochromaffin cells bind to their receptors at peripheral afferent terminals to initiate signal transduction. GLP-1 is one gut hormone proposed to mediate aspects of nutrient detection by the vagus nerve, such as food intake, gastric emptying, and glycemia (Krieger et al. 2016). However, genetic deletion of its receptor GLP1R from vagal afferents did not impact GLP-1 agonist-induced changes in body weight and glucose homeostasis (Sisley et al. 2014), challenging the role of vagal GLP1R in digestion and feeding behavior. Moreover, vagal GLP1R neurons displayed minimum projection to intestinal villi but densely innervated the muscular layer of stomach and small intestine, as revealed by AAV-guided anatomical tracing in *Glp1r-ires-cre* mice (Williams et al. 2016). Consistently, vagal GLP1R neurons accounted for the vast majority of stretch sensations from stomach and small intestine, yet vagal neurons that responded to intestinal nutrients were largely GLP1R negative. Through an optogenetics-based screening performed to identify vagal sensory neuron populations that regulate gastric contractility, vagal GPR65 neurons were discovered as a potent inhibitor of gastric contractions (Williams et al. 2016). In this study, ChR2 was similarly expressed in selective vagal neuron populations using diverse Cre lines harboring a Cre-dependent ChR2 allele, and light delivery was achieved through an optic fiber positioned next to the vagus nerve or vagal ganglia. Gastric contractility was measured as intragastric pressure changes using a pressure transducer cannulated into the stomach. Optogenetic stimulation was similarly performed as aforementioned, using a low stimulation frequency (5 msec, 5 Hz), which was efficient for gastric responses without causing drastic respiratory or heart rate changes. Optogenetic activation of vagal GPR65 neurons potently reduced stomach contractions, as revealed by intraluminal gastric pressure measurements. Moreover, anatomical and calcium imaging data indicated that vagal GPR65 neurons account for the majority of vagal afferents that detect food from the intestinal villi. Such results provided a new and proper neural target for understanding the communication between intestinal epithelial cells and the sensory vagus nerve regarding nutrient sensation. The exact role of GLP1R in the sensory vagus nerve still needs to be elucidated, and such genetic data supports an alternative model that at least some gut hormones released in response to ingested food may regulate gastrointestinal functions through modulating gastrointestinal mechanoreceptors.

Vagus nerve controls a variety of essential autonomic functions. In addition, VNS is an

FDA-approved treatment for patients with epilepsy, recurrent depression, clustered headache, and migraine pain (Schachter and Saper 1998; Rush et al. 2000; Uthman 2000; Groves and Brown 2005; O'Reardon et al. 2006; Krahl 2012; Yuan and Silberstein 2016a; Siefring 2017; Brauser 2018). However, deeper mechanistic understanding of autonomic regulation and better therapeutic specificity and efficacy are hindered because of the lack of appropriate cell-type-specific neuromodulation tools, calling for the development of new approaches. Optogenetics provides a powerful cell-specific modulatory approach for precise dissection of the physically intermingled but functionally distinct vagal fiber types. So far, most studies regarding optogenetic control of autonomic physiology via the vagus nerve has been limited to anesthetized animals. Because of technical challenges for light delivery, optogenetic control of vagal fibers in awake, freely moving animals has been difficult, which prevents a variety of essential physiological and behavioral studies. Many fascinating questions remain to be answered. Are vagal P2RY1 or NPY2R neurons involved in cough or asthma? Do vagal GPR65 neurons also regulate food intake? What is the designated neuron population for inflammation control? Which neurons are responsible for the effects of VNS on epilepsy and depression? In general, there are two ways to deliver light to awake, freely behaving animals: internal or transdermal light illumination. Both approaches have been explored in the PNS and could be potentially applied to the vagus nerve. Surgically implanted optical cuffs and LED arrays were designed to illuminate peripheral nerves deep inside the body and transdermal light stimulation has been widely used to illuminate nerve terminals on the skin in pain studies. A few examples will be summarized in the following sections.

OPTOGENETIC CONTROL OF THE PERIPHERAL NERVOUS SYSTEM IN AWAKE, FREELY BEHAVING ANIMALS

One of the best applications for transdermal light illumination is optogenetic modulation of sensory fibers that terminate on the skin. In transgenic animals in which ChR2 is expressed in DRG neuron populations either under specific promotors or delivered by viral infections, transdermal blue light–induced changes, including paw withdrawal and other pain-like behaviors and long-term sensitization to mechanical and thermal stimuli, were observed (Ji et al. 2012; Daou et al. 2013; Boada et al. 2014; Draxler et al. 2014; Iyer et al. 2014). Conversely, when inhibitory opsins were expressed in skin-innervating DRG neurons, transdermal illumination with green or yellow light reduced pain behaviors (Boada et al. 2014; Iyer et al. 2014; Daou et al. 2016). When ChR2 was expressed in intrinsic and extrinsic whisker pad muscles, blue light stimulation on the whisker pad caused whisker movements (Park et al. 2016). On the other hand, for nerve fibers located deeper inside the body, noninvasive neuromodulation with transdermal light illumination becomes much less efficient. One possible solution is to increase the opsin expression level to compensate for the light loss. As revealed in a recent study (Maimon et al. 2017), when motor neurons are retrogradely infected with an ultraconcentrated ChR2 virus, transdermal blue light stimulation were sufficient to elicit muscle movements. Moreover, ankle positions can be accurately controlled by light stimulation. However, it is unlikely to adapt this approach to nerves deeper in visceral organs as the penetration depth of blue light in skin tissue is around 2 mm (Avci et al. 2013; Ash et al. 2017). Longer wavelength light can reach deeper tissues. For example, red light can penetrate 5 mm into the skin. Other factors that will influence light penetration in tissues are scattering and absorption. Therefore, another strategy extensively explored is to use longer wavelength light and red-shifted opsins to achieve neuromodulation in nerves deeper in the body. For example, a flexible red OLED device was developed for transdermal optogenetic vagal stimulation (Smith et al. 2016), yet its in vivo application needs to be further determined.

Unlike light illumination onto neural targets on the skin or inside the brain, precise illumination of peripheral nerves deep inside the body with an implanted light source is quite challeng-

ing because of tremendous displacement during animal movement. The main issue comes from nerve damage when using conventional laser-coupled fiberoptic nerve cuffs. Super flexible cuffs constructed using biocompatible organic polymer and small diameter optic fiber at a well-designed angle were developed to control large peripheral nerves deep inside the body (Towne et al. 2013). Sciatic nerve, one of the largest nerves in an animal, has been successfully targeted using such optical cuffs in rats. When ChR2 was expressed in sciatic motor neurons using retrograde AAV infection, light illumination through an optical cuff surgically placed around the sciatic nerve resulted in fine control of muscle movement. Successful control of hindlimb muscle contraction using similar soft optical cuffs placed on the sciatic nerve has also been demonstrated in mice (Michoud et al. 2018). However, application of optical cuffs is so far still limited to large peripheral nerves like the sciatic nerve in rodents. The development of smaller and softer optical cuffs in the future may facilitate the application to other smaller peripheral nerves including the vagus nerve.

Recent advances in LED technologies provided a powerful alternative for light delivery. Compared to lasers, miniature high-powered LEDs have several advantages that make them ideal for in vivo light delivery in awake, freely moving animals. First, to ensure enough light illumination, optic fibers with a certain diameter and stiffness have to be used while LED arrays could be more flexible. Second, it is hard to illuminate a large surface area with implanted optic fibers while LED arrays can be designed to wrap around a whole organ. Third, animals have to be tethered with an optic fiber when using a laser, while LED arrays can be wirelessly controlled (Wentz et al. 2011; Kim et al. 2013; Yeh et al. 2013; Rossi et al. 2015; Samineni et al. 2017b), which enables truly freely moving studies. On the other hand, as light emitted from LEDs are not coherent, tissue penetration may not be as good, and thermal- induced side effects should always be considered and examined. An LED array–based optical cuff was demonstrated in 2010 to be effective in precise control of muscle movement when placed around the sciatic nerve in anesthetized *Thy1-ChR2* mice (Llewellyn et al. 2010). This effect was similarly achieved using a wireless LED-based optical cuff a few years later (Kang-Il et al. 2015). A variety of LED devices were thereafter designed and applied for optogenetic modulation of peripheral nerves in awake, freely moving animals. For example, in mice in which ChR2 was expressed in sciatic nociceptive neurons, nociceptive behaviors were elicited using a wireless LED stimulation device implanted subcutaneously near the sciatic nerve endings in the hind paw (Montgomery et al. 2015). Using a miniaturized stretchable μ-LED-based wireless optoelectronic system placed around the sciatic nerve, nociceptive behaviors were also successfully elicited in *Advillin-ChR2* and *TRPV1-ChR2* mice (Park et al. 2015). Such wireless μ-LED arrays can be designed to cover a large surface area. For example, using a green LED mesh placed around the bladder in SNS-Arch-GFP mice, genetically targeted nociceptive afferents that innervate the bladder were silenced in vivo, and induced bladder pain was reduced in awake, freely behaving animals (Samineni et al. 2017a).

OTHER GENETIC NEUROMODULATORY APPROACHES AND POTENTIAL THERAPEUTIC APPLICATIONS

Some of the recent neuromodulatory applications based on optogenetics are summarized here. While there are numerous advantages of using microbial opsins and optogenetics to modulate neural activity over conventional methods, including genetic access and precise spatial and temporal control, light delivery can be difficult to achieve especially for many peripheral nerves and surgical implantation is usually required. Moreover, although wireless LED devices are gaining increasing attention, laser or LED-coupled optic fiber implants are still used in most cases, meaning that animals are tethered and not completely free-moving. Chemical-based chemogenetic approaches involving designer receptors exclusively activated by designer drugs (so-called DREADDs) provide a great alternative for cell-type-specific

neuromodulation (Urban and Roth 2015). Compared to optogenetic methods, DREADDs render the capability of simple chronic neuromodulation in untethered animals. With i.p. injection or oral administration of DREADD-specific ligands, long-lasting neuronal activation/silencing can be easily achieved for 6–8 h. In the PNS, DREADDs-based chemogenetic approaches were widely used to control pain and nociceptive behaviors.

So far, cell-type-specific neuromodulatory approaches have greatly advanced our understanding of the PNS in animal studies, and the potential of using optogenetics and chemogenetics to treat or alleviate chronic pain and motor disorders has been demonstrated using rodent disease models. More attentions have been drawn to their potential therapeutic applications in human patients. One major challenge in human therapy is to safely, effectively, and stably express opsins or DREADDs in target cell populations. Recent advances in virus development have enabled such genetic neuromodulatory approaches in nonhuman primates (Gerits and Vanduffel 2013). As the first AAV-based gene therapy, Luxturna, was approved by the FDA in December 2017, it is reasonable to believe that this challenge will be overcome in the near future.

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