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REVIEW

Cell-Type Identification in the Autonomic Nervous System

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Abstract The autonomic nervous system controls various internal organs and executes crucial functions through sophisticated neural connectivity and circuits. Its dysfunction causes an imbalance of homeostasis and numerous human disorders. In the past decades, great efforts have been made to study the structure and functions of this system, but so far, our understanding of the classification of autonomic neuronal subpopulations remains limited and a precise map of their connectivity has not been achieved. One of the major challenges that hinder rapid progress in these areas is the complexity and heterogeneity of autonomic neurons. To facilitate the identification of neuronal subgroups in the autonomic nervous system, here we review the well-established and cutting-edge technologies that are frequently used in peripheral neuronal tracing and profiling, and discuss their operating mechanisms, advantages, and targeted applications.

Keywords Autonomic nervous system · Neuronal tracing · Genetic marker · Molecular profiling · Cell-type diversity

Introduction

The autonomic nervous system (ANS) is one part of the peripheral nervous system and comprises sympathetic and parasympathetic divisions [1]. The ANS innervates a large number of internal organs tonically or phasically and controls many crucial physiological functions, such as blood circulation, digestion, respiration, urination, and the immune response [2]. The ANS also contributes to glucose and fat metabolism and water-sodium homeostasis (the enteric nervous system, which is also a large part of the peripheral nervous system, comprises a huge and complex network with a great number of neurons that operates more independently, thus many investigators prefer to classify the enteric system as a component separate from the ANS [3]). Dysplasia or dysfunction of the ANS causes a broad spectrum of human disorders, including dysautonomia [4] and neuroblastoma [5, 6]. The importance of the ANS has intrigued numerous investigators over the past decades – efforts have been made to delineate the neural anatomy and connectivity of the ANS and identify specific neuronal subsets with discrete functions. However, autonomic neurons are highly heterogeneous and complex, not only on the basis of retrograde and genetic tracing but also from functional electrophysiological recordings and immunohistochemical labeling [7]. To further elucidate the sophisticated structures and functions of ANS neurons, the first step is to mark, distinguish, and characterize the neuronal subtypes (Fig. 1). Nowadays, rapid progress is being made in profiling and clustering ANS neuronal subtypes, thanks to constantly-emerging new technologies with high performance and efficiency, which provide new opportunities to 'barcode' neurons in the ANS. Here, we summarize the well-established and novel techniques that have greatly improved our understanding of ANS functions.

Retrograde Labeling

One of the most classical and efficient methods of tracing autonomic nerves and identifying neurons in peripheral ganglia is retrograde labeling with fluorescent dyes.

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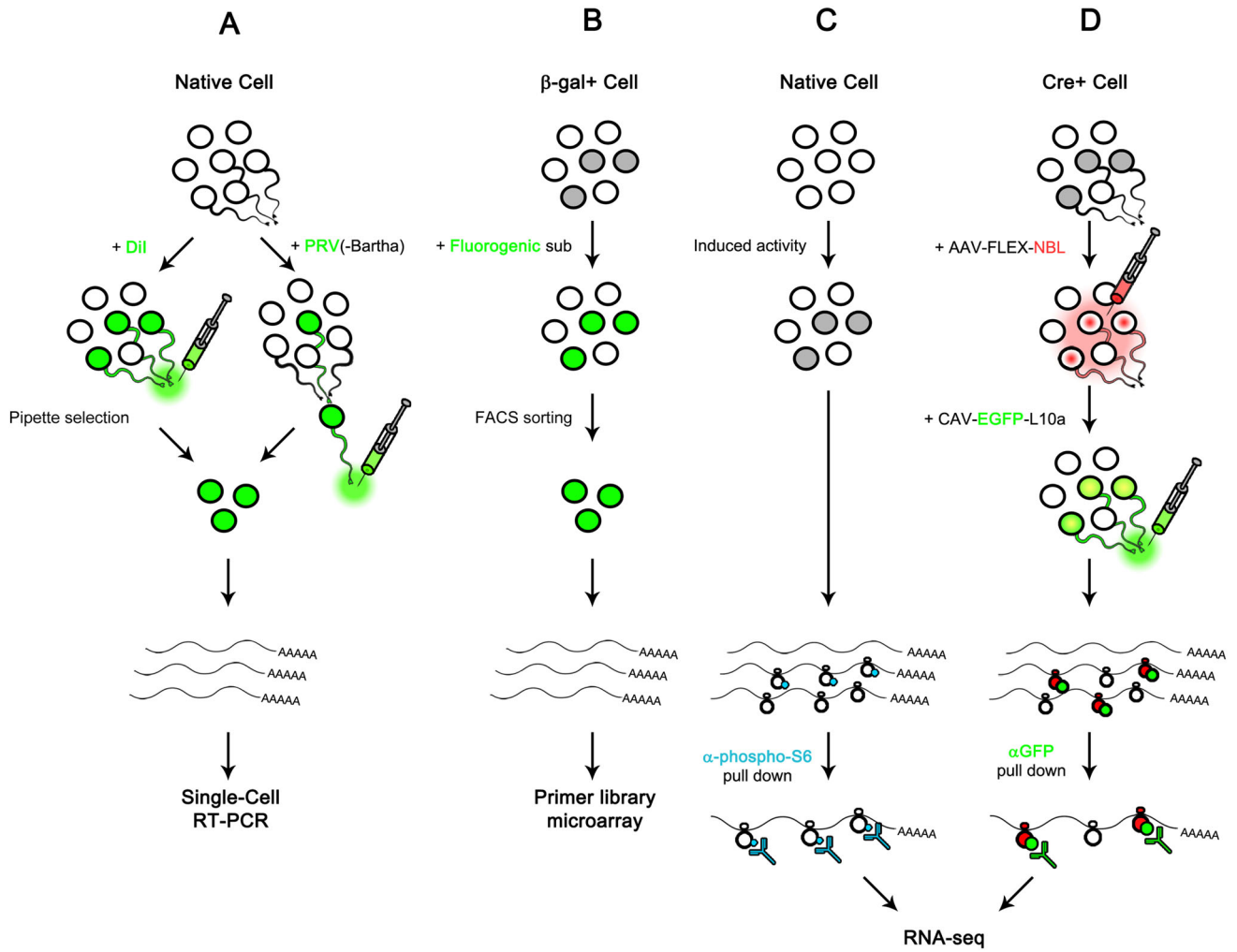

Fig. 1 Strategies to trace and identify distinct neuronal subgroups in the autonomic nervous system. **A** Native ANS neurons are labeled with vital fluorescent dyes, such as DiI (left) or neurotropic viruses (right), and then manually selected and analyzed by single-cell RT-PCR. **B** Cells expressing endogenous or exogenous β-galactosidase activity are labeled with the fluorescent substrate fluorescein di-β-D-galactopyranoside (FDG) by fluorogenic reactions, followed by fluorescence-activated cell sorting (FACS) and microarray analysis using a pre-designed primer library. **C** Active ribosomes and associated translating mRNAs are pulled down with anti-phospho-S6 and subsequently analyzed by high-throughput RNA sequencing. **D** Combination of Cre mouse line, flanked-LoxP-AAV virus, retrograde CAV virus, and anti-GFP pull-down approaches. Retro-TRAP (translating ribosome affinity purification) technology can effectively target specific neuronal subpopulations and facilitate downstream molecular profiling.

Retrograde transport of these dyes from the peripheral target organ/area to the neuronal cell bodies can label a subpopulation of ganglionic neurons based on their anatomical projections. Once injected into internal organs, the tracer diffuses from axon terminals back along the sympathetic/parasympathetic nerves and eventually reaches the ganglionic cell bodies, where it effectively marks the nerve bundles that innervate the target areas without significantly influencing neuronal development, function, and viability (Fig. 1A, left). Lipophilic and polar dyes are two families of the most commonly-used vital fluorescent tracers (also including biotin derivatives, dextran conjugates, fluorescent microspheres, and protein conjugates [8]). Long-chain dialkylcarbocyanines and dialkylaminostyryl dyes, such as DiI [9, 10] and DiO [11], are lipophilic dyes which trace neurons by passive lateral diffusion. These tracers not only move rapidly and efficiently in live neurons, both *in vitro* and *in vivo*, but also function in fixed samples (with a lower diffusion speed). DiI and DiO have excitation and emission spectra similar to fluorescein and rhodamine, respectively, and are suitable for long-term and long-distance neuronal tracing. Fast Blue and True Blue [12, 13] are polar fluorescent dyes frequently used in retrograde neuronal tracing. To perform dual-color labeling, Fast Blue and True Blue may be conveniently combined with Diamidino Yellow, Nuclear Yellow, or other dyes. Aminostilbamidine and hydroxystillbamidine (Fluoro-Gold) [14] are also cationic dyes

normally used in ANS tracing studies. These polar dyes are usually excited by UV light and stain neuronal cells blue. Because of their distinct mechanisms, these fluorescent dyes label different components and organelles of neurons: lipophilic dyes mainly stain plasma membranes and lipid compartments, while polar dyes prefer to mark the cytoplasm (such as True Blue) or the nucleus (such as Diamidino Yellow and Nuclear Yellow) [8]. In addition, lipophilic dyes are less toxic and also compatible with fixed tissue samples. Comparison studies have also shown that both lipophilic and polar dyes have high labeling efficacy for the retrograde labeling of peripheral neurons [15]. However, it is difficult to discriminate the ascending and descending pathways and more specific neuronal subgroups which connect the same target organ using these approaches.

Neurotropic virus is another choice for efficient neuronal tracing [16]. Virus infection occurs on the skin surface or peripheral mucosae, like the gastrointestinal and respiratory tracts. The life cycles of the viruses rely on trans-synaptic spread from the peripheral to the central nervous system. As major controllers of internal organs, ANS nerves are easily and abundantly infected by neurotropic viruses, making these viruses natural and ideal tracing agents for ANS neurons. In the late 1980s, pseudorabies virus (PRV) was first used to trace sympathetic ANS nerves (Fig. 1A, right) [17]. The original PRV has both retrograde and anterograde trans-synaptic abilities [18]. PRV-Bartha, an attenuated strain, was later discovered; it can only spread from post- to pre-synaptic neurons and has greatly facilitated ANS circuitry mapping [19]. PRV has a broad host range and capacity for retrograde trans-synaptic spread, allowing its extensive use in studies that are intended to delineate the multi-synaptic architecture of neuronal chains. Like PRV, herpes simplex virus (HSV) is also a member of the alpha-herpesvirus family and is able to spread along ANS nerves [20]. Interestingly, the HSV-1 H129 strain only spreads from pre- to post-synaptic neurons, and thus has become the most commonly-used virus for anterograde trans-synaptic tracing [21]. The prominent advantages of these neurotropic viruses are directional trans-synaptic activity, self-amplification, and carrying capacity. They can display the multi-level hierarchical structure of ANS circuitry in a designated direction, and the number of trans-synaptic events can also be controlled *via* the transduction period. During viral amplification and spread, the signal intensity and labeling efficiency do not decrease. Furthermore, these virus tools can also carry and introduce genes into the host neurons, making them suitable and convenient for functional studies, or even giving them translational and therapeutic potential.

After labeling with vital dyes or trans-synaptic viruses, fluorescent ANS neurons can be separated by laser capture microdissection, or collected using a glass pipette or fluorescence-activated cell sorting (FACS) following digestion [9]. As the functional units are usually mixed together into one nerve bundle, the labeled neurons may be further analyzed for categorization into discrete neuronal subgroups based on their cell body sizes, electrophysiological features, and immunochemical labeling with various markers [10]. Neuronal tracing techniques provide information on the projection and connection relationships between neurons and their target organs, and help to identify neuronal distributions from the anatomical and topological viewpoints. These methods are effective and convenient, and are easily combined with other downstream analyses. These methods are still frequently used in a wide range of neuronal tracing studies in ANS research.

Neurodevelopment and Cell Lineage

Cell lineage describes the developmental history of a nervous system from a fertilized embryo [22]. By providing lineage and differentiation information, neurodevelopment studies help distinguish different subtypes of ANS neurons from another perspective [23]. Generally, ANS divisions originate from neural crest stem cells, which are initially located at the dorsal margin of the neural tube [24]. These cells undergo the epithelial-mesenchymal transition, begin to migrate at embryonic day 8 (E8, cranial neural crest cells, *Mus musculus*) (LifeMap Discovery® Embryonic Development & Stem Cell Compendium v1.9.3. 2018), and then form the sympathetic primordia chain. Bone morphogenic proteins, which are secreted by ectoderm, the neural tube roof plate [25], and the dorsal aorta [26, 27], play important roles in activating downstream key transcription factors, such as Ascl1 and Phox2b, that induce the noradrenergic features of sympathetic neuronal cells [28]. On the other hand, the parasympathetic division arises from cranial and sacral neural crest cells, and recently, remarkable discoveries have further revealed that parasympathetic ganglia form close to visceral organs and their precursors at distal peripheral sites, suggesting that the progenitor lineages of sympathetic and parasympathetic divisions diverge at an earlier stage than previously thought [29]. By combing multicolor Cre-reporter lineage tracing with immunostaining, two back-to-back studies independently demonstrated that parasympathetic neurons develop largely from nerve-associated Schwann cell precursors within the preganglionic peripheral nerves and are dependent on the activation of Ascl1 [30, 31] (Fig. 2). Interestingly, although the two research groups had distinct genetic resources and research logic – Espinosa-Medina *et al.* used Neurog2-Cre, Phox2a/Pgk-Cre, Wnt1-Cre, and Phox2b-Cre mouse lines [23], while Dyachuk *et al.* used Sox10-

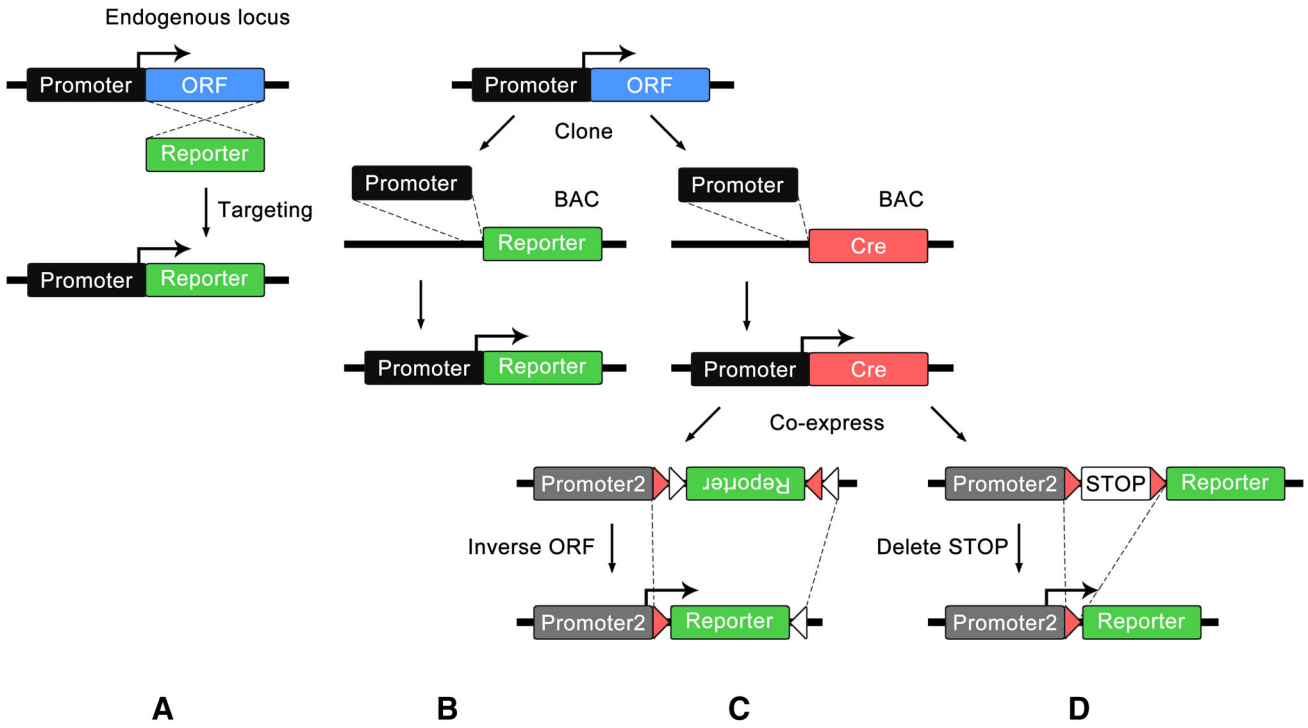

Fig. 2 Genetic tracing strategies for the autonomic nervous system. **A** Targeting an endogenous gene locus to generate reporter mice: in both alleles, the open reading frames of a marker gene are substituted by fluorescent reporter genes. Driven by the endogenous promoter of the marker gene, the fluorescent reporter protein is only expressed in the subpopulation of neurons in which the promoter has been activated. **B** In an alternative approach, the endogenous promoter of the marker gene is cloned into a bacterial artificial chromosome (BAC) to make a reporter line, leaving the endogenous gene intact. **C–D** To further narrow down the labeling range and increase the spatio-temporal resolution, a Cre-LoxP system controlled by two promoters has been introduced: Cre recombinase is driven by the promoter of the marker gene, while the reporter gene is driven by another promoter (specific or nonspecific). The expression of Cre protein leads to the recombination of the FLEX switch (**C**), or the excision of the LoxP-flanked stop cassette (STOP) (**D**), thus the fluorescent reporter protein is only expressed in the neurons in which both of the promoters have been activated.

Cre, Ascl1-Cre, and Plp-Cre lines [24] – they arrived at very similar conclusions.

Developmental studies provide critical information about cell lineage, which helps to trace cells that share a common origin. A large number of genetic tracing studies have been successfully accomplished by applying this knowledge. Using promoters of transcriptional factors previously defined to be crucial for ANS neurodevelopment, researchers have been able to temporally and spatially drive the expression of Cre recombinase in BAC (bacterial artificial chromosome) mice and sequentially perform genetic tracing by crossing these Cre mouse lines with LoxP-dependent reporter mice (Fig. 2). For instance, Wnt1 and Phox2b are commonly used markers to distinguish parasympathetic neurons in the vagal complex (Fig. 3) [11]. Phox2b-positive neurons are primarily localized in the nodose ganglion, which is derived from epibranchial placodes [32], while Wnt1-positive neurons are preferentially enriched in the jugular, trigeminal, and dorsal root ganglia, which are derived from the neural crest [14, 33]. Combining the Wnt1-Cre and Phox2b-Cre mouse lines with LoxP-dependent gene-knockout mice, Nonomura *et al*. successfully targeted different neuronal subpopulations in the nodose-jugular complex and found that knockout of the *piezo2* gene in these two ganglionic types caused distinct respiratory defects at different developmental stages. So far, many key transcription factors during ANS development have been identified, such as Phox2a, Phox2b, Gata2/3, Ascl1 (Mash), Hand2, Foxd3, Hmx1, and Sox family members [34, 35], and some of their promoters have been cloned and constructed into Cre mouse lines [36, 37].

In general, by combing specific Cre lines with LoxP-dependent reporter lines or gene-knockout/knockin lines, the genetic tracing or neuronal ablation approaches from the neurodevelopmental point of view are useful for labeling a specific cell lineage or neural pathway, or for finding the developmental role of a group of precursor cells, thus helping researchers narrow the search range. However, these genetic strategies also have their own limitations, because cells that share a common progenitor type may still have distinct molecular profiles and be responsible for distinct functions after differentiation [38]. Sometimes, controversial results have been obtained from

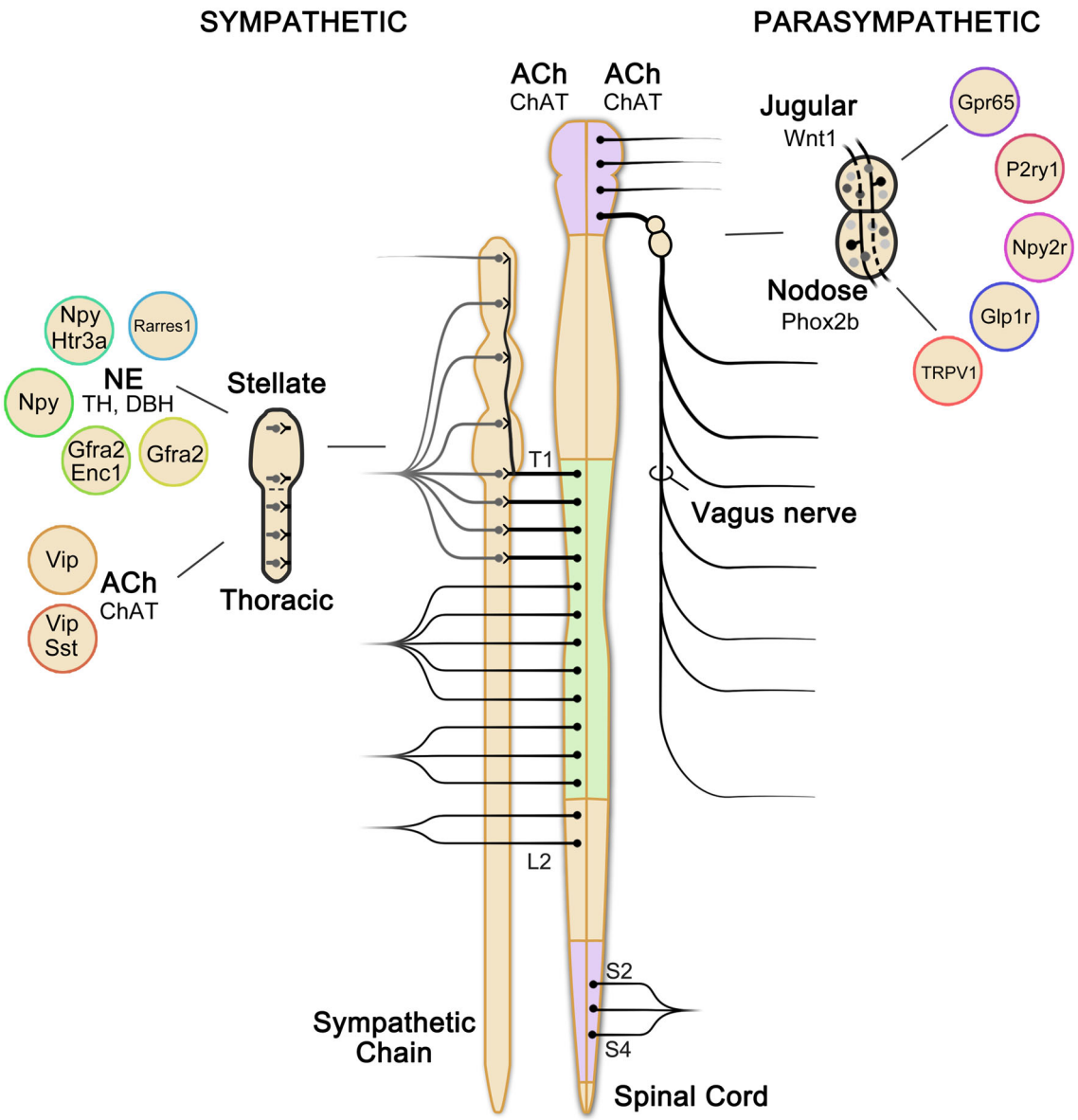

Fig. 3 Identified neuronal subpopulations in the autonomic nervous system. ACh is the main neurotransmitter of preganglionic neurons for both the sympathetic and parasympathetic divisions. ChAT is a commonly-used marker enzyme for these neurons. NE (synthesized from TH and DBH) and ACh are the major neurotransmitters of postganglionic neurons for the sympathetic and parasympathetic divisions, respectively. In the sympathetic division, neuronal somata in the stellate and thoracic ganglia have been clustered into seven subgroups, which contain five NE subtypes and two ACh subtypes [13]. In the parasympathetic division, Phox2b mainly targets the nodose ganglion, while Wnt1 targets the jugular ganglion [11]. The vagal ganglia also comprise multiple neuronal subsets, such as TRPV1$^+$, Grp65$^+$, Glp1r$^+$, P2ry1$^+$, and Npy2r$^+$ cells, which mediate distinct functions [40, 50, 51].

functional studies using these approaches, indicating that the functional neuronal subgroups which arise from the same cell lineage still need to be separated and discriminated more precisely [39, 40]. In addition, recent studies have shown that a subgroup of sympathetic neurons (Ret$^+$ and TrkA$^+$) remain unspecialized until target organogenesis occurs postnatally. The neuronal differentiation appears to be coordinated with organ innervation and the unique interactions between growth factors and their receptors on the cell membrane [13]. This means that a neuronal cell's fate is determined not only by its intrinsic genetic program, but also by intercellular recognition and co-development, suggesting a highly sophisticated developmental process and organization of the ANS. Furthermore, ligand-receptor recognition (during cell-cell interactions) still mediates specific transmembrane signaling cascades and is indispensable for normal neuronal functions even after cell differentiation and maturation,

making these molecules potentially useful as markers that can be used to distinguish various neuronal subpopulations in the ANS.

Neurotransmitters, Hormones, and Neuropeptides

Autonomic neurons communicate with each other and their target organs by means of neurotransmission [41]. Once autonomic neurons are mature and functioning, they begin to secrete unique combinations of neurotransmitters, hormones, and neuropeptides [42]. It is therefore possible to use these specific signaling molecules or the enzymes that synthesize them as tracing markers. In the ANS, acetylcholine (ACh) is the major preganglionic neurotransmitter for both divisions. In the postganglionic divisions, sympathetic nerves mainly release noradrenaline (norepinephrine, NE) and its interacting partners on recipient cells are adrenergic receptors α1, α2, β1, β2, and β3 (some sympathetic nerve terminals also release ACh, which activates postsynaptic nicotinic ACh receptors (Fig. 3)). The synthesis of NE requires tyrosine hydroxylase (TH) [43] and dopamine beta-hydroxylase (DBH) [44]; hence TH has become one of the most commonly-used genetic markers of sympathetic neurons in adult mice. Parasympathetic neurons mainly release ACh, which activates postsynaptic muscarinic M1, M2, and M3 receptors (Fig. 3) [45, 46]. These cholinergic neurons were first identified by immunostaining with ACh esterase, and later verified with antisera for choline acetyltransferase (ChAT), the main synthetase of ACh. Nowadays, both ChAT antibodies and ChAT-Cre transgenic lines are frequently used to mark cholinergic neurons in the parasympathetic division. In the upstream central neural circuits from hypothalamus to brain stem, which control ANS neurons, α-melanocyte-stimulating hormone (α-MSH) and agouti-related peptide (AgRP) are an agonist and antagonist of melanocortin receptor signaling, respectively. Interestingly, AgRP and pro-opiomelanocortin, the precursor of α-MSH, are expressed in distinct small neuronal populations, making these neurons the most studied in appetite, obesity, and metabolic control [47, 48].

In addition, to control different organs and execute various functions, peripheral ANS neurons are also enriched with and secrete many neuropeptides and neuromodulators, such as enkephalin, substance P, neuropeptide Y, calcitonin gene-related peptide, gastrin-releasing peptide, neurotensin, dynorphin, vasoactive intestinal polypeptide, somatostatin, galanin, and cholecystokinin [12, 33, 49]. The diversity of these neurotransmitters and neuropeptides reflects the functional differentiation of ANS neurons in adult animals. Coupling with specific receptors expressed on postsynaptic cells, these messenger molecules effectively execute distinct intercellular signals, and consequently define the neuronal identities and functions to a large extent. Depending on the specificity and reliability of ligand-receptor interactions, one or a combination of these neurotransmitters/neuropeptides and their synthetases with their receptors is now frequently used to barcode or verify a subgroup of ANS neurons.

Membrane Receptors and Ion Channels

An important way to study familial dysautonomia is to identify natural disease-causing genetic mutations in humans. Because of the greater length and higher mutation tolerance of the coding sequences, harmful mutations are much more likely to act on receptor genes than neuropeptide genes. For example, multiple pathogenic heterozygous mutations have been found in the coding sequence of melanocortin 4 receptor (MC4R), which underlies severe obesity in $>$ 10 families [52, 53], making this receptor the most common monogenic cause of human obesity. The loss of MC4R function in mice also causes dysfunction of the ANS and obesity, suggesting that it contributes to the regulation of thermogenesis and glycaemia [54, 55]. Recently, by using ChAT-Cre and Phox2b-Cre mouse lines to target different ANS neuronal subpopulations, Berglund *et al*. further found that MC4R in sympathetic, but not parasympathetic, cholinergic neurons is essential for the control of energy expenditure and body weight [52]. Another example is leptin receptor (LepR) deficiency, which is caused by mutations in the *lepr* gene and dysfunction of LepR. In the hypothalamus, loss of LepR function prevents leptin signaling and leads to excessive hunger and obesity [56]. In the central neurocircuits from hypothalamus to brain stem which control the ANS, there are also many neuropeptide-receptor pairs like α-MSH-MC4R and leptin-LepR that contribute crucially to homeostasis [57]. Making use of the functional specificity of these membrane receptors, researchers have successfully targeted them to study the actions of neuropeptide signaling on ANS modulation and homeostasis. For example, Ryan *et al*. recently found that neurons expressing the oxytocin receptor in the parabrachial nucleus of the brain stem form a small neuronal subgroup that regulates water intake [58].

Ion channels are a large group of membrane receptors that confer distinct characteristics on neuronal subsets. Recently, TRPM2 (transient receptor potential channel, subfamily M, member 2) was reported to be a warm temperature sensor that displays very restricted expression in the hypothalamus. TRPM2-positive neurons control body temperature, limit fever, and contribute to hypothermia [59–61]. Similarly, TRPV1/TRPA1 channels have

been shown to be expressed in one neuronal subset in the vagal ganglia and contribute to airway hyper-reactivity, the pathology of asthma [51], and gastrointestinal mechanosensation (Fig. 3) [62], while TRPV4 is selectively expressed in a colonic sensory population of visceral afferents which regulate mechanically-induced visceral pain [63]. Acid-sensing ion channels have also been reported to be differentially expressed in ANS neurons and are important for the autonomic control of cardiovascular [64–66] and gastrointestinal mechanosensory functions [67, 68]. In addition, genetic tracing studies have shown that Nav1.8 channels are enriched in a subset of nodose and dorsal root ganglia neurons where they contribute to acute-phase responses to dietary fat [69, 70].

G-protein-coupled receptors (GPCRs) make up another huge protein superfamily responsible for various signal transduction processes between the extracellular space and the cytoplasm. Nearly 800 GPCR members have been identified and about half of them mediate olfactory function. The extreme diversity in their distribution and function makes GPCRs ideal for genetic marker screening in the ANS [71, 72]. In a series of studies, Liberles *et al*. used a high-throughput approach to screen GPCRs expressed in specific neuronal subgroups. These investigators used fluorescein di-β-D-galactopyranoside (FDG), a fluorescent substrate of β-galactosidase, to label cells that possess either endogenous [73, 74] or exogenous (LacZ-positive) [75, 76] β-galactosidase activity. They then either picked fluorescent cells with a glass pipette and performed single-cell PCR analysis or used FACS to enrich the fluorescently-labeled subpopulations [77] and screened by microarray of GPCR mRNAs with a GPCR-specific primer library (Fig. 1B). After discovering two novel families of olfactory receptors, they used this method to screen for GPCR mRNAs in vagal ganglia and identified several useful genetic markers for neuronal subpopulations in these ganglia, which were then successfully validated by *in situ* hybridization. In 2015, Chang *et al*. reported that P2ry1 (11.6% of neurons), Npy2r (29.2%), and Gpr65 (10.2%) are specifically expressed in different neuronal subgroups of the vagal ganglia and differentially innervate lung, heart, and stomach, as well as the upstream brainstem (Fig. 3) [40, 78]. Optogenetic activation of P2ry1 neurons largely decreases the respiration rate and minute volume, while the activation of Npy2r neurons causes rapid and shallow breathing. In the digestive system, Williams *et al*. further discovered that Gpr65 and Glp1r differentially target intestinal villi and stomach/intestinal muscle, respectively, and also contribute to distinct functions – Gpr65-positive neurons are responsible for nutrient sensation in the intestine, while Glp1r-positive neurons mainly detect stretch in the stomach and intestine [50].

On the other hand, GPCRs have not only been used as cellular markers to trace specific subgroups of neurons, but have also been engineered to interact with drug-like small molecules which had been unrecognized previously and were specifically used as functional manipulators of neuronal excitability. This popular method in neuroscience is termed "Chemogenetics" [79], which is as well-known as the "Optogenetics" that uses opsins and light to control neuronal activity [80]. In this technology, several human muscarinic receptors have been engineered by site-directed mutagenesis to bind non-natural ligands like clozapine-N-oxide (CNO), and eventually achieved nanomolecular potency without harmful constitutive activity [81]. The engineered M3 muscarinic receptor (termed hM3Dq) couples with $G_{\alpha q}$ and can activate neurons when CNO is presented, while the engineered M4 muscarinic receptor (termed hM4Di) acts on $G_{\alpha i}$ and can inhibit neuronal activity under the same conditions [81]. These receptors were also named DREADDs (designer receptors exclusively activated by designer drugs); they have limited off-target effects and are unresponsive to the native ligand ACh [82, 83]. Once introduced into a subgroup of neurons using the genetic approach (or viral transduction) and expressed under the control of a specific promoter and CNO, these DREADDs can activate or inhibit neuronal firing both temporally and spatially, thus helping to identify the functional role of the neuronal subpopulation from the physiological outcomes.

Molecular Profiling

Unlike microarray analysis, sequencing-based technologies have no requirement for the predesigned transcript-specific probes of candidate gene(s) and therefore are ideal for discovery-based studies. With next-generation sequencing technology, the dynamic range of detection is broader, the sensitivity has been greatly improved, and the cost of large-scale tests has become affordable for most academic laboratories, providing a new and unbiased means of discovering novel markers for tracing. Using this high-throughput technology, a number of large-scale and detailed single-cell analyses have been implemented [13, 84]. In a recent study, Furlan *et al*. carried out single-cell RNA sequencing of ~ 300 neuronal cells isolated from mouse sympathetic ganglia and were able to cluster them into 7 subpopulations: 5 noradrenergic and 2 cholinergic subtypes (Fig. 3).

New technologies now allow us to correlate gene profiles and neuronal functions more closely. To capture the active translational changes in specific neuronal subpopulations, multiple affinity-based approaches have been further developed to purify representative or

immediately-responsive mRNAs. For example, Knight *et al*. first used the antibody to phosphorylated ribosome protein S6 to enrich activated endogenous ribosomes and mRNAs from discrete neuronal subpopulations in the mouse hypothalamus and brain stem [85] (Fig. 1C). These investigators successfully identified galanin-positive neurons that are active during fasting, and prodynorphin neurons that have an inhibitory effect on feeding [85]. In another example, Sanz *et al*. created a mouse line called RiboTag, which expresses 3 × HA-tagged RPL22 under the control of flanked LoxP sites (FLEX). This allows pull-down of RPL22-containing ribosomes and active mRNAs in Cre-expressing cells using anti-HA [86]. Notably, Doyle *et al*. also developed a similar method that uses anti-GFP to pull down EGFP-L10a-labeled ribosomes and active mRNAs; this is referred to as translating ribosome affinity purification (TRAP) [87, 88] (Fig. 1D). Combining the visibility of a fluorescent protein and the selectivity of the Cre-LoxP system, the TRAP methodology was subsequently widely applied and has been significantly expanded. For example, Allison *et al*. collected LepRb-positive cells among mouse hypothalamic and brain stem neurons and performed TRAP and RNA-seq tests to successfully validate prodynorphin, which plays important roles in LepR function and energy expenditure, as a usable marker for a subgroup of LepRb neurons [85, 89]. In another case, Li *et al*. used the similar technology to detect highly-enriched neuropeptides in the prodynorphin-positive cells, and further found islet amyloid polypeptide, the precursor of amylin, to be enriched in a neuronal subset in the lateral hypothalamus that regulates food intake [90]. Recently, the TRAP technique has been expanded into a toolbox, including phosphoTRAP [85], bacTRAP (combined with the BAC Cre line) [91], vTRAP (with Cre-dependent virus) [92], and RetroTRAP (with retrograde-tracing GFP-expressing viruses and anti-GFP camelid nanobody-fused RPL10A) [93] (Fig. 1D) [94, 95]. Integrating the information on circuit connectivity and neuronal activity, these techniques impressively offer effective ways to capture activity-dependent and cell-type-specific transcripts and thereby illuminate more precise biological details of ANS activity.

Concluding Remarks and Perspectives

Various reliable tools and techniques with high performance, including vital dyes and trans-synaptic viruses, cell-lineage genetic tracing, neurotransmitter synthetase targeting, membrane receptor recognition, microarrays, and active ribosome enrichment, have been developed to allow the effective distinction and separation of neuronal subsets in the ANS. These methodologies are based on quite different mechanisms and perspectives, such as neural anatomy and connectivity, neurodevelopment, neurotransmitter or receptor specificity, and molecular machinery recruitment. High-throughput sequencing approaches further provide an unbiased means of discovering unpredictable changes or distinct expression patterns in small but critical neuronal subpopulations [96]. By applying these technologies, many neuronal subpopulations in the ANS have been identified and have subsequently undergone specific manipulation and functional exploration. Moreover, new technologies with improved efficiency are continuously emerging. For instance, by integrating high-throughput sequencing with Cas9 genomic editing, a whole-organism cell barcoding system [97] offers a novel systematic means of tracing developmental processes and revealing cell fates from the global viewpoint, which may provide an unprecedentedly robust engine for lineage studies and genetic marker discovery. The next generation of monosynaptic retrograde and anterograde viruses, which already have many applications in studies of the central nervous system, such as recombinant rabies virus, vesicular stomatitis virus, and type 2 adeno-associated virus, may have the potential to boost neural circuit tracing studies of the ANS [98]. It is highly likely that these new approaches will reveal further monosynaptic neural circuits between preganglionic and postganglionic neurons and more details of the hierarchical structure of ANS divisions, providing us with cell identity information in a network context [99].

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Compliance with Ethical Standards

Conflict of interest All authors claim that there are no conflicts of interest.

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