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Wiring Sympathetic Neuron Subpopulations into Target-Selective Circuits for Homeostasis

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Abstract

The molecular characterization of postganglionic sympathetic neurons by RNA sequencing has allowed the full assessment of gene expression in individual cells and classification of neurons into subpopulations defined by their gene expression profile and developmental history. The identification of growth factor receptor subunits specifically expressed by select neuron subpopulations enabled the demonstration of GDNF family ligands and the respective receptor subunits as instrumental in the innervation of certain targets. These are first critical steps in the attempt to characterize the molecular processes leading to the establishment and maintenance of target-specific sympathetic efferent pathways.

Introduction

The knowledge on the cellular elements of the autonomic nervous system has made enormous progress since the description of preganglionic and postganglionic elements by John Newport Langley at the turn to the 20th century[1]. Molecular analysis focusing on key functional properties was successively refined from physiological[2,3] and histological[4] detection of the transmitter in noradrenergic sympathetic neurons to the detection of immunoreactivity as well as enzyme activity for the transmitter-synthesizing enzymes in the case of both noradrenergic as well as cholinergic autonomic neurons[5-7]. Histochemical detection of the mRNAs coding for these enzymes as well as the vesicular neurotransmitter transporters coexpressed from synexpression gene groups enabled, with highly enhanced specificity and sensitivity, the detection and developmental surveillance of neurons with a given transmitter phenotype[8,9]. With the dramatic advances in RNA sequencing and bioinformatical processing, data sets for the transcriptomes of individual cells are available that have been dreamed of two decades ago[10,11].

Noradrenergic and Cholinergic Sympathetic Neuron Classes in the Era of Single Cell RNA Sequencing

With the advent of high throughput sequencing technologies and the massif reduction in the amount of starting RNA required for analysis, the generation of single cell transcriptomes displaced microarrays for gene expression profiling[12,13]. Data acquisition and analysis set complex demands on quality control and data normalization, read mapping, dimensionality reduction, feature selection and cluster analysis[14]. The method then allows classification of neural subtypes and characterization of marker genes in addition to the quantitative interrogation of many features of the gene expression profile. For postganglionic sympathetic neurons, this approach has enabled the

characterization of several noradrenergic and cholinergic subpopulations together with the partial assignment to anatomically, histologically and developmentally defined neuronal features[10]. In addition, analysis of the same data set allowed the characterization of noradrenergic and pan-neuronal synexpression groups of genes[15]. Yet the full correlation with the multitude of anatomically and physiologically defined sympathetic pathways is still open[16].

With the availability of transcriptomes for sufficiently large numbers of neurons derived from mouse stellate and thoracic sympathetic ganglia, a classification into different classes of noradrenergic (NA 1-5) and cholinergic (ACH 1 and 2) sympathetic neurons was resolved[10]. Interestingly, the transmitter phenotype of the ACH 1 and 2 populations is in the meantime categorized as cholinergic and noradrenergic [11, www.mousebrain.org]. indeed, cells in the ACH 1 and 2 populations may not only express the cholinergic marker gene vesicular acetylcholine transporter (VAChT, Slc18a3) but also the noradrenergic marker vesicular monoamine transporter 2 (VMAT2, Slc18a2) in addition to the transmitter synthesizing enzymes[10,15]. This speaks to the question raised already 40 years ago whether sympathetic neurons can release acetylcholine in addition to noradrenaline[17]. The very large majority of the characterized neurons, however, in particular nearly all neurons classified as noradrenergic (NA) are devoid of cholinergic markers such as the vesicular acetylcholine transporter.

Some neurons first classified as cholinergic (ACH 1 and 2) as a result of the general gene expression profile lack expression of detectable transcripts for the cholinergic marker genes choline acetyltransferase (ChAT) and VAChT altogether[10]. The significance of this peculiar observation is currently not fully understood and entails the question for the detection limit of the RNA sequencing procedures. An explanation may be the low levels for the transcripts from the cholinergic locus genes, ranging roughly tenfold below those for the genes coding for the enzymes of the noradrenaline biosynthesis pathway[10,15]. In addition, their transport and distribution within the cell compartments may be responsible for the lack of reliable detection in RNA extraction procedures from the isolated soma. Another explanation may be the plasticity of transmitter phenotype in a subpopulation of sympathetic neurons[18,19]. These cells are distinguished by expression of cholinergic as well as noradrenergic marker genes with transcript levels depending on neuronal activity or environmental growth factor supply. However, it needs to be emphasized that the postganglionic sympathetic neurons innervating rodent sweat glands are functionally cholinergic by several physiological criteria[6].

Taken together, single cell RNA sequencing has strongly promoted the characterization and classification of sympathetic neurons. For the further analysis of cell types in the autonomic nervous system, it provides a platform for the comparison of sympathetic neurons in the different paravertebral and prevertebral ganglia and for the comparison with parasympathetic neurons.

In addition to the transmitter phenotype-related and neuropeptide markers, electrophysiological and morphological features are key determinants of sympathetic neuron diversity[20]. The degree to which this diversity as described in mice[21,22] can be correlated and approximated by RNA sequencing approaches is still open. For the appreciation of the physiological role of neurons in sympathetic efferent channels it poses an important consideration.

Assigning Postganglionic Neurons to Target-Specific Sympathetic Pathways

With increasing understanding of gene expression patterns and biochemical properties of individual autonomic neurons, the question gains momentum, how these neurons are recruited to distinct sympathetic outflow channels conveying appropriate regulation to diverse target tissues. With the analysis of reflex activation patterns in sympathetic neurons innervating different target structures, it became apparent that the SNS possesses diverse functional pathways distinguished at molecular, cellular and integrative levels[23,24]. Specific central autonomic networks allow for selective control of the sympathetic outflow to individual tissues and thus for the realization of patterned autonomic responses[25,26]. The questions whether these precisely organized pathways in the neuraxis[27-29] possess specific molecular signatures and how these arise during development are now becoming addressed.

Apart from noradrenergic and cholinergic neurotransmitter phenotype-related markers and neuropeptides, neurotrophin receptor (Ntrk) and GDNF family ligand receptor (Gfr alpha and Ret) subunits appear as preferentially expressed in selected sympathetic neuron subpopulations[10]. The noradrenergic NTRK1/TRKA-expressing neuronal subpopulations NA2, NA4 and NA5 reexpress Ret during postnatal development concomitant with target innervation. By analyzing expression with immunohistochemistry for the receptor subunit proteins and neuropeptide NPY in sympathetic fiber tracts adjacent to the target structures in combination with retrograde labeling from target areas, selected neuron subpopulations were further characterized. NA2 neurons represent the Gfra3- and NPY-positive sympathetic subpopulation innervating nipple erector muscle. NA5 neurons constitute the Gfra2-positive but NPY-negative subpopulation innervating piloerector muscle. Importantly, with a combination of conditional gene inactivation at advanced

developmental stages and immunohistochemical detection of sympathetic fibers in the mutant target tissues, it was demonstrated that, via developmentally regulated expression of the Gfra2 and 3 ligands artemin and neurturin in combination with postnatal induction of Ret expression in a subpopulation of sympathetic neurons, the innervation of the respective target tissues is regulated[10].

The study demonstrates how the knowledge on subpopulation-specific gene expression provides tools not only to identify the diverse neuronal populations but also to characterize the mechanisms of integration of these populations into autonomic neuron circuits. These data extend earlier knowledge on the role of neurotrophin[30-32] and GDNF family ligand[10,33,34] signaling in sympathetic neuron target innervation. Additional growth factor families are involved in this process such as endothelins and semaphorins[35,36] or the vascular endothelial growth factor and Eph/ephrin family members[37,38]. The full complement of signaling decisions on the route from the developing sympathetic ganglion through the appropriate nerve branches to the proper target still have to be worked out.

Uncovering Aspects of the Neurochemical Code of Sympathetic Pathways by Immunohistochemistry and Retrograde Labeling

Neurochemical and retrograde labeling approaches have provided early insight into the molecular code of selected sympathetic target-directed pathways. Immunohistochemistry for neuropeptide Y (NPY), expressed in noradrenergic cardiovascular sympathetic neurons[39,40] as well as vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) expressed in cholinergic sudomotor neurons[41] has been used for the characterization of the respective preganglionic neurons.

Applying immunohistochemistry for corticotrophin releasing factor-like immunoreactivity (CRF-LI) in cat stellate and lumbar sympathetic ganglia resulted in the staining of terminal baskets derived from innervating preganglionic neurons surrounding 96 to 99% TH-negative, but CGRP-positive postganglionic neuronal cell bodies[42]. Conversely, retrograde labeling from the paw innervated by sudomotor and vasoconstrictor neurons with fluorogold labeled postganglionic sympathetic ganglion cells including approximately 30% somata surrounded by CRF-LI terminal baskets.

Retrograde labeling with different dyes applied to the heart ventricle or the cut end of the cardiac nerve in the rat disclosed NPY-positive and negative postganglionic sympathetic neuron populations[43]. Association of NPY-positive cell bodies in rodent stellate and superior cervical ganglia with synaptic baskets positive for cocaine and amphetamine-regulated transcript peptide (CART) allowed the characterization of innervating preganglionic neurons in the cardiovascular pathways as CART-positive cells[44]. The finding was confirmed by transneuronal tracing using pseudorabies virus from skeletal muscle demonstrating the innervation of NPY-positive vasoconstrictor neurons by CART-positive preganglionic neurons.

By combination of immunohistochemistry for neuropeptides and calcium binding proteins with retrograde labeling and nerve transection, initial characterization of sympathetic pathways to additional targets such as the iris and submandibular glands were performed[45,46].

Thus, the combination of peptide immunohistochemistry and retrograde labeling was able to characterize several features of sympathetic pathways innervating the heart and the vasculature as well as sweat glands in two classical model systems, the cat and the rat. Transneuronal tracing with neurotropic virus expressing fluorescent marker proteins promises to allow selection of neurons in given sympathetic pathways for RNA sequencing. In this manner, the full transcriptome in these pathways may become unveiled and provide information on their equipment with gene products involved in contact formation and information propagation between neurons and targets.

The establishment of Specific Synaptic Connections from Preganglionic to Postganglionic Neurons

The formation of the proper synaptic connections between the pre- and postganglionic sympathetic neurons is key to the establishment of the diverse functional pathways supplying appropriately patterned sympathetic activity to the target organs[23,25].

Distinct regulation is demonstrated for sympathetic outflow channels subserving different functional contexts. This can be observed for sympathetic innervation to vasoregulatory and thermoregulatory effectors[47,48]. It is detected for the innervation of a range of target organs such as the heart as compared to kidney[49,50] or to spleen[51-53]. Also, differential regulation of the blood circulation in distinct somatic and intestinal vascular beds is accomplished by distinct patterns of efferent sympathetic activity[54,55]. Skin and muscle blood flow are distinctly regulated[56-59].

The molecular logic that enables the development and sustains the maintenance of this diversity in sympathetic efferent pathways is currently not fully understood. During development it is a multistep process including among others synapse formation of preganglionic onto postganglionic sympathetic neurons and formation of appropriate synaptic contacts between central pre-motor neurons and the appropriate preganglionic neurons.

A critical set of events in this multistep process is the final choice of the appropriate postganglionic targets by

preganglionic neurons, the initial synapse formation and the regulation of synapse number during maturation. In different populations of autonomic neurons the postnatal refinement of synapse numbers has been documented by electrophysiological recording[60]. Neurotrophin signaling depending on growth factors derived from the target[61,31] as well as from the preganglionic neurons[62,63], and their activity dependence has been characterized. However, the processes responsible for establishment of specificity in development of the diverse target-specific sympathetic pathways are unknown.

Lessons from Spinal Motor Neuron Specification

For spinal motor neurons, developmental specification and integration into circuits required for coordinated muscle movement has been analyzed using genetic approaches in mice[64,65]. Several features important for specific innervation of skeletal muscle targets by spinal motoneurons are also relevant for the development of target-specific sympathetic pathways: migration and coalescence of the differentiating motoneurons to their final dorsoventral positions in spinal cord columns[66,67], the innervation of the appropriate target[68], and target-dependent maturation processes that affect connectivity within the spinal cord[69].

The processes entail transcriptional specification involving Hox proteins and regulatory ret signaling[70-72]. The ret ligand GDNF and its receptor subunit GFRa1 are required for correct positioning of motor neuron cell bodies in the spinal cord, invasion of embryonic target tissue, and induction of the ETS transcription factor Pea3 in a motoneuron subpopulation[73]. Pea3 is prerequisite for correct positioning of the motoneurons within the spinal cord and normal axon branching within the target[74]. The observations demonstrate a signaling sequence from the target-derived growth factor resulting in proper maturation, positioning and target innervation of a motoneuron subpopulation. Hox proteins coordinate motoneuron subtype specification, determine ret expression levels and define GFRa subunit profiles[72].

Interestingly, the ETS transcription factor Er81, expressed in a nonoverlapping motor and a sensory neuron subpopulation, is required for establishment of the appropriate synaptic contacts between the motoneurons and their sensory input[75]. The neurotrophin NT3 is required for Er81 expression in the sensory neurons[76]. These observations illustrate the involvement of GDNF family ligand and neurotrophin signaling in the differentiation of spinal cord motor circuits. Thus, both growth factor and receptor families are involved in motoneuron and sympathetic neuron differentiation.

In a comparable manner, such an analysis is expected to not only provide understanding of the molecular mechanisms underlying target-selective outgrowth of sympathetic nerve fibers. In addition, it may shed light on the positioning of preganglionic cell bodies in target-specific cell clusters in the intermediolateral cell column of the spinal cord[77]. Moreover, an analysis of the principles underlying the synaptic organization of distinct functional pathways in sympathetic ganglia[78] appears to become experimentally accessible.

Progressing from Axonal Pathfinding to Selective Synapse Formation

A question of particular interest is the association and interaction of these signaling systems with proteins acting as guidance cues and in particular membrane-associated proteins suitable to mediate cell-adhesion and cell-type identification[79,80]. In the case of spinal motoneurons this has been shown for ret and Eph/ephrin signaling[81] by cooperative interaction of the two signaling systems[82]. Also, protocadherins expressed in motoneurons[83] are engaged with ret in mutual regulation and stabilization as shown in stem cell derived motoneurons and primary postganglionic sympathetic neurons[84].

in addition, synaptic organizer hubs of the neurexin and neuroligin[85,86] as well as the receptor - protein tyrosine phosphatase (LAR-RPTP)[87,88] families will attract interest. In postganglionic sympathetic neurons neurexin induction and splice variant expression is regulated during maturation and target innervation[89]. However, developmental regulation of their binding partners, in particular neuroligins, and the expression of both protein families in preganglionic sympathetic neurons is not analyzed. The study of the role of the above-mentioned signaling systems in interaction with these synaptic organizers in the establishment of target-specific sympathetic pathways can be expected to be of outstanding relevance.

Conclusions

The molecular characterization of the cellular elements in the sympathetic nervous system was greatly refined during the last three decades. From the demonstration of functional signature genes such as tyrosine hydroxylase and choline acetyltransferase it progressed to the characterization of the full transcriptome detected in cell bodies of postganglionic neurons in selected mouse sympathetic ganglia. With functional characterization of an ever-increasing selection of gene products, the interrogation of neuronal circuits moves to a new level. On the one hand, the characterization of transcriptomes of large numbers of individual neurons allows the classification of sympathetic neuron subpopulations and, to a certain extent, their functional characterization. On the other hand, the knowledge on the subpopulation-specific expression of a range of marker genes opens, comparable to the study of the spinal motoneuron circuits, the analysis

of target-specific sympathetic pathways. This involves not only the study of the projection of preganglionic neurons to their postganglionic partners and to the final targets but also the analysis of the innervation from the distinct autonomic CNS centers by premotor to preganglionic neurons. This compilation of data promises to unravel the sympathetic autonomic neural circuits required for the synchronization of circulation and temperature regulation, balancing of the fluid matrix, regulation of bowel function and sexual organs among others.

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