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Neurotrophins and target interactions in the development and regulation of sympathetic neuron electrical and synaptic properties

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Abstract

The electrical and synaptic properties of neurons are essential for determining the function of the nervous system. Thus, understanding the mechanisms that control the appropriate developmental acquisition and maintenance of these properties is a critical problem in neuroscience. A great deal of our understanding of these developmental mechanisms comes from studies of soluble growth factor signaling between cells in the peripheral nervous system. The sympathetic nervous system has provided a model for studying the role of these factors both in early development and in the establishment of mature properties. In particular, neurotrophins produced by the targets of sympathetic innervation regulate the synaptic and electrophysiological properties of postnatal sympathetic neurons. In this review we examine the role of neurotrophin signaling in the regulation of synaptic strength, neurotransmitter phenotype, voltage-gated currents and repetitive firing properties of sympathetic neurons. Together, these properties determine the level of sympathetic drive to target organs such as the heart. Changes in this sympathetic drive, which may be linked to dysfunctions in neurotrophin signaling, are associated with devastating diseases such as high blood pressure, arrhythmias and heart attack. Neurotrophins appear to play similar roles in modulating the synaptic and electrical properties of other peripheral and central neuronal systems, suggesting that information provided from studies in the sympathetic nervous system will be widely applicable for understanding the neurotrophic regulation of neuronal function in other systems.

Keywords

Sympathetic neurons; autonomic; cholinergic; noradrenergic; co-transmission; firing properties; ion channels; M-current; potassium channels; neurotrophins; NGF; BDNF; TrkA; p75

1. Introduction

Different types of neurons are often defined based on the expression of distinct functional properties, such as neurotransmitter expression, connectivity and repetitive firing patterns. These phenotypic properties arise through developmental programs and provide the basis for the proper functioning of the nervous system. However, significant evidence suggests that these

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properties maintain some degree of plasticity in adults and may provide the substrate for adaptive changes in the nervous system, which are presumed to underlie processes such as learning and memory formation, for reviews see: (Maren and Baudry, 1995; Bruel-Jungerman et al., 2007). Thus, a critical question in neuroscience is how the nervous system maintains neuronal phenotypic properties and how adaptive changes in these properties are regulated.

This question has been studied extensively in the sympathetic nervous system, providing a model for understanding the proteins and processes that contribute to the development and plasticity of neural systems. In this review we will discuss evidence suggesting that neurotrophic growth factor signaling provides a regulatory mechanism for the rapid modulation of synaptic and repetitive firing properties in sympathetic neurons. This type of signaling appears to play similar roles in many central nervous system structures as well, suggesting that neurotrophic signaling provides a global regulatory mechanism for neuronal properties. Understanding the role of growth factor signaling in the sympathetic nervous system will contribute not only to our understanding of neural regulation of cardiac function and other sympathetically regulated processes, but to adaptive and maladaptive processes in neurons of the central nervous system as well.

2. Target regulation of neurotransmitter phenotype

Target-dependent regulation of postnatal sympathetic transmitter phenotype

The sympathetic nervous system is involved in the regulation of homeostatic physiological functions including the regulation of blood pressure via vasoconstriction, cardiac contractility, glandular secretions and digestive system motility. These processes are regulated by sympathetic neurons via synaptic contacts with somatic tissues, and are adjusted according to prevailing physiological conditions relayed from central and reflex centers (Elghozi and Julien, 2007; Grassi et al., 2009).

Neurotransmitter expression in postnatal sympathetic neurons is influenced by soluble growth factor signaling via innervated target tissues. Most adult sympathetic neurons are noradrenergic, utilizing norepinephrine (NE) as their primary neurotransmitter and begin to express this phenotype early in development via both cell intrinsic and extrinsic-dependent signaling mechanisms, discussed in detail elsewhere, see (Elfvin et al., 1993; Ernsberger and Rohrer, 1996; Ernsberger, 2000; Sarkar and Howard, 2006). However sympathetic neurons innervating sweat glands, periosteum and some vascular beds express the transmitter acetylcholine (ACh) (Schotzinger and Landis, 1988; Elfvin et al., 1993; Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Asmus et al., 2000). These neurons express NE early in development, but switch to a cholinergic phenotype during the postnatal period after forming connections with target tissue. This cholinergic switch is induced by retrograde signaling from the target (Rao and Landis, 1990; Rao et al., 1992; Rohrer, 1992). Whole protein extracts made from cholinergically innervated tissues induced expression of a cholinergic phenotype in cultured sympathetic neurons, suggesting that a soluble signaling protein was responsible for the phenotype shift (Rao and Landis, 1990; Habecker et al., 1995). The cholinergic differentiation factor seems likely to be a member of the ciliary neurotrophic/leukemia inhibitory factor cytokine family since it appears to act through the same gp130 receptor signaling system and multiple members of this family induce a cholinergic phenotype in cultured sympathetic neurons (Bazan, 1991; Habecker et al., 1997). Several members of gp130-signaling cytokines are expressed in the sweat gland during the period of cholinergic differentiation (Stanke et al., 2006), suggesting that multiple factors, acting via a common signaling pathway influence the cholinergic state of sympathetic neurons. Thus, postnatal expression patterns of particular transmitter phenotypes in sympathetic neurons appear to be regulated by signaling molecules released from target tissues.

Regulation of postnatal sympathetic transmitter phenotype by neurotrophins

Another class of target-derived molecules that regulates sympathetic transmitter expression is the neurotrophin family of growth factors, which include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neurotrophins play an important role in the development of the sympathetic nervous system, acting as trophic survival factors and as regulators of neuronal growth and arborization (Bibel and Barde, 2000; Huang and Reichardt, 2003; Glebova and Ginty, 2005; Ernsberger, 2009). In addition to their developmental roles in the embryo, neurotrophins may also play an important role in regulating transmitter phenotype in postnatal sympathetic neurons. Developmentally, the pattern of expression of the cholinergic phenotype in chick sympathetic ganglia matches the expression pattern of the NT-3 receptor, tropomyosin related kinase C (TrkC) (Brodski et al., 2000). Furthermore, culturing chick sympathetic ganglia explants in the presence of NT-3 induces cholinergic marker expression while reducing the expression of noradrenergic markers (Brodski et al., 2000), suggesting that neurotrophin signaling may act to modulate the neurotransmitter phenotype of sympathetic neurons. However it remains unclear what role this type of regulation plays *in vivo* since cholinergic sympathetic neurons innervating sweat glands develop normally in NT-3 knockout mice, whereas sympathetic neurons innervating other tissues fail to proliferate during development, apparently due to dependence on NT-3 as a developmental survival factor (ElShamy et al., 1996; Ernsberger, 2009).

In contrast to the effects of NT-3, culturing chick sympathetic explants in the presence of NGF promotes a noradrenergic phenotype and reduces the expression of cholinergic markers (Brodski et al., 2000). Expression of the NGF receptor tropomyosin related kinase A (TrkA) within the mammalian sympathetic ganglia chain initiates later than expression of TrkC (Birren et al., 1993; DiCicco-Bloom, 1993; Wyatt et al., 1997). TrkA signaling is therefore unlikely to mediate early expression of the noradrenergic phenotype, which can be detected as early as E10 in the mouse (Wyatt et al., 1997; Callahan et al., 2008) and which is regulated through a pathway that includes bone morphogenetic protein signaling and the Phox2 transcription factors (Sarkar and Howard, 2006).

However, NGF is clearly a modulator of the noradrenergic phenotype subsequent to the initial expression of TrkA. Application of NGF to cultured sympathetic and sensory neurons increases the expression of the NE biosynthetic enzyme tyrosine hydroxylase (TH) (Chun and Patterson, 1977; Max et al., 1978; Katz et al., 1986; Ma et al., 1992), which appears to occur via a post-transcriptional mechanism since this effect is not blocked by RNA synthesis inhibitors (Rohrer et al., 1978). Additionally, NGF rapidly upregulates TH expression in the sympathetically derived PC12 cell line (Greene et al., 1984). NGF can also influence NE marker expression *in vivo*, since injection into newborn or adult rats up-regulates the expression of TH and dopamine- β -hydroxylase, another biosynthetic enzyme involved in NE production (Thoenen et al., 1971; Otten et al., 1977). This increase takes place via an increase in protein synthesis as evidenced by incorporation of radio-tagged amino acids into protein and by sensitivity to block by a protein synthesis inhibitor. Furthermore, injection of animals with an anti-NGF antiserum causes opposite regulation, decreasing the activity and expression of noradrenergic markers (Angeletti et al., 1972; Hendry, 1973). These results suggest that target-derived NGF regulates and maintains the noradrenergic neurotransmitter phenotype expressed by sympathetic neurons both *in vitro* and *in vivo*.

These findings suggest that changes in neurotrophin signaling alter the levels and activity of the enzymatic machinery necessary for the production of neurotransmitter and hence could play a role in the modulation of neurotransmission, or even allow transmitter phenotype switches in postnatal neurons. Regulated neurotrophin signaling may thus provide a mechanism for adaptive changes in autonomic nervous system function such as those induced

by physical activity, which confers cardioprotection and reduces cardiovascular related morbidity (De Angelis et al., 2004; Raczak et al., 2005; Sugawara et al., 2009). Conversely, neurotrophic mechanisms could play a role in maladaptive changes that occur following heart attacks (Chen et al., 2001; Chaldakov et al., 2004). Indeed, following myocardial infarction synaptic reorganization and an increase in NE release occurs that are predictive for life threatening complications such as ventricular arrhythmias coincident with increased NGF production in cardiac tissue (Zhou et al., 2004; Jardine et al., 2005; Hasan et al., 2006).

BDNF does not appear to regulate the expression of cholinergic or noradrenergic phenotype genes in postnatal paravertebral sympathetic neurons (Slonimsky et al., 2003), as would be expected from the lack of expression of Trk B at this stage (Causing et al., 1997). However TrkB does appear to be expressed in at least some prevertebral ganglia innervating the gut suggesting a potential role for BDNF in those cells (Dixon and McKinnon, 1994). Despite the lack of TrkB in most sympathetic neurons, BDNF is able to modulate synaptic and electrical properties, a topic that is discussed further below.

3. Regulation of co-release of neurotransmitters in sympathetic neurons

Regulation of NE and ACh co-release in sympathetic neuronal cultures by soluble cell signaling factors

A great deal has been learned about the role of neurotrophins in regulating growth, survival and the neurotransmitter properties of developing sympathetic neurons from studies in which the neurons are grown in culture either alone or with target tissues such as cardiac myocytes (Levi-Montalcini and Angeletti, 1963; Bunge et al., 1974). Under these conditions, the sympathetic neurons extend processes and form active synapses onto neighboring neurons and myocytes, as well as forming autaptic synapses onto themselves (Bunge et al., 1974; O'Lague et al., 1974; Lockhart et al., 1997; Yang et al., 2002). Interestingly, co-culture of initially noradrenergic superior cervical sympathetic neurons with other tissues, such as cardiac myocytes, leads to a time-dependent increase in cholinergic and mixed noradrenergic/cholinergic neurotransmission at synapses between sympathetic neurons and myocytes (Furshpan et al., 1976; O'Lague et al., 1978c; O'Lague et al., 1978b, a; Furshpan et al., 1986b; Potter et al., 1986). Neurotransmission in younger cultures is initially almost exclusively noradrenergic, but release of ACh gradually increases with time. This switch appears to be dependent on soluble cell signals, since media conditioned by target cells also induces cholinergic transmission in sympathetic neurons cultured alone, which otherwise tend to remain noradrenergic (Patterson and Chun, 1977; O'Lague et al., 1978c; Slonimsky et al., 2003). The soluble cholinergic differentiation factor released from cardiac myocytes was identified as leukemia-inhibitory factor (Yamamori et al., 1989), which has been shown to activate LIFR β and gp130 receptors (Taga and Kishimoto, 1997), the same signaling pathway as the differentiation factors responsible for the noradrenergic to cholinergic shift in sympathetic neurons that innervate sweat glands and other tissues, as discussed above (Habecker et al., 1997; Stanke et al., 2006).

Electrophysiological evidence for NE and ACh co-transmission in individual sympathetic neurons

Evidence suggesting that individual cultured sympathetic neurons can release NE and/or ACh in cardiac myocyte co-cultures was determined using electrophysiological recordings to measure changes in membrane potential in response to synaptic transmission (O'Lague et al., 1974; O'Lague et al., 1978c; O'Lague et al., 1978b, a). Electrophysiological stimulation of neurons induces neurotransmitter release at the synapse that results in different postsynaptic responses depending on whether NE or ACh is released. The transmitter NE is excitatory and causes a depolarization of the myocyte membrane potential, while ACh is inhibitory for

myocytes and causes a hyperpolarization of the membrane. These changes in membrane potential can be monitored electrophysiologically and can be blocked with specific pharmacological compounds to determine relative contributions of each transmitter. These studies showed that direct neuronal stimulation leads to excitatory cholinergic transmission between neurons and at least two types of transmission from neuron to myocyte: excitatory NE signaling and inhibitory ACh signaling. These studies suggested that individual sympathetic neurons in myocyte co-cultures can release either NE or ACh. In addition to these classical transmitters sympathetic neurons are also known to co-release other transmitter substances including neuropeptides and adenosine (Furshpan et al., 1986a; Matsumoto et al., 1987).

While the previous experiments suggested that sympathetic neurons are plastic in respect to transmitter expression, it was unclear whether individual neurons co-release both transmitters or if different subpopulations of neurons are responsible for releasing NE or ACh individually. By culturing neurons and myocytes on collagen microisland cultures (300-500 μ m), individual synaptically connected pairs of neurons and myocytes can be isolated and neurotransmission can be investigated unequivocally in individual neurons (Furshpan et al., 1976; Furshpan et al., 1986b; Potter et al., 1986). These elegant studies demonstrate that individual postnatal sympathetic neurons in culture remain plastic in regard to transmitter expression, and in fact simultaneously co-release two classical transmitters (NE and ACh) in varying proportions, for review see (Luther and Birren, 2008). Some neurons were able to elicit an entirely excitatory response in connected myocytes upon stimulation. Antagonists for β -adrenergic receptors were found to block this response, indicating that NE was being synaptically released. Other neurons were found to inhibit connected myocytes when stimulated, an effect that was sensitive to block by muscarinic cholinergic antagonists, suggesting that they release only ACh. Still other neurons had combinatorial effects on myocytes, indicating that they release both NE and ACh.

Individual neurons showing the different synaptic responses in microisland cultures display ultrastructural differences consistent with the transmitter release observed in the electrophysiological experiments (Furshpan et al., 1976; Furshpan et al., 1986b; Potter et al., 1986). In electron microscopic examination of identified neurons previously subjected to electrophysiological analysis it was shown that synaptic terminals of neurons that display an excitatory, noradrenergic effect on myocytes contain predominately small granular vesicles that are associated with the packaging of monoamine transmitters. Neurons that display inhibitory, cholinergic neurotransmission predominately contain small, clear synaptic vesicles consistent with the release of ACh. Furthermore, neurons that display dual transmitter function at connected myocytes contain both types of vesicles. While these studies provide evidence that sympathetic neurons are able to release multiple neurotransmitters, they raise the important question of whether these properties are regulated by physiological conditions such as target-derived factor signaling. In the next few sections we will review the evidence suggesting that target-derived neurotrophin signaling plays a role in rapidly regulating transmitter release from sympathetic neurons.

Neurotrophin receptors and signaling pathways

As will be detailed below, neurotrophins rapidly influence neurotransmission in cultured sympathetic neurons in a manner that is differentially regulated through different neurotrophin receptors (Lockhart et al., 1997; Yang et al., 2002; Slonimsky et al., 2003; Slonimsky et al., 2006). Therefore it will be helpful to briefly review neurotrophin receptor expression in sympathetic neurons, for a more detailed treatment see (Chao and Hempstead, 1995; Chao, 2003; Reichardt, 2006). Neurotrophins act through two types of receptors: the pan-neurotrophin receptor (p75), a member of the tumor necrosis factor receptor family that binds all neurotrophins with similar affinity, and the tropomyosin related kinase receptors (Trk) of

which there are three family members that bind with high affinity to specific members of the neurotrophin family (Reichardt, 2006). Superior cervical sympathetic neurons express both TrkA and TrkC, which bind predominantly NGF and NT-3, respectively, and the p75 receptor but, except for a transient period during embryonic development (Straub et al., 2007), do not express the BDNF-specific TrkB receptor (Dixon and McKinnon, 1994; Wyatt and Davies, 1995; Bamji et al., 1998).

Trk and p75 receptors have complex, interacting signaling responses and, in some instances have opposing effects on a range of neuronal properties, including cell survival, process outgrowth, neurotransmission and firing properties (Barrett and Bartlett, 1994; Chao and Hempstead, 1995; Dechant and Barde, 2002; Yang et al., 2002; Lu et al., 2005; Woo et al., 2005; Luther and Birren, 2009). In cells that co-express both p75 and Trk receptors there is evidence for complex interactions between their respective signaling pathways. For example, activation of p75 induces axon pruning in mouse sympathetic neurons via inhibition of TrkA-mediated signaling pathways (Singh et al., 2008) and p75 and TrkA oppositely regulate process growth in cultured rat sympathetic neurons (Kohn et al., 1999). However, p75 has also been shown to facilitate Trk signaling (Hantzopoulos et al., 1994; Miller and Kaplan, 2001). TrkA autophosphorylation occurring after NGF binding is enhanced by p75 in a sympathetic progenitor cell line (Verdi et al., 1994) and PC12 cells (Barker and Shooter, 1994). Furthermore, p75 is found to directly interact with shc, a downstream target of activated TrkA, and to facilitate its phosphorylation by TrkA (Epa et al., 2004). One way to interpret these data is that in cells that express both receptors the physiological effect of neurotrophin signaling is dependent not only on the relative levels of p75 and Trk activation, but the cell context specific nature of interactions between the signaling pathways. Such a model would be consistent with studies showing that p75-dependent mediation of survival and death in sensory neurons is sensitive to developmental stage (Bartlett and Barrett 1994). The idea that cell contexts play a role in determining the nature of neurotrophins signaling has recently gained support by studies showing that p75 interacts with additional partners beyond Trk family members to mediate a variety of functions that including cell death and neurite retraction (Bronfman and Fainzilber, 2004; AlShawi et al., 2007).

Rapid regulation of transmitter phenotype in sympathetic neurons by neurotrophins

The effect of neurotrophin signaling through TrkA and the p75 receptor on sympathetic neurotransmission has been assessed in co-cultures containing neurons grown with cardiac myocytes. Sympathetic neurons grown in this manner form functional synaptic contacts with myocytes that are similar to the anatomy seen *in vivo* (Smolen, 1988). Myocytes in these cultures beat spontaneously, and the beat rate is influenced by synaptic transmission from connected sympathetic neurons (Conforti et al., 1991; Lockhart et al., 1997; Yang et al., 2002). Using electrophysiological techniques the neurons can be stimulated to fire action potentials at controlled frequencies to induce transmitter release, the effect of which can be observed as a change in the beat rate of the myocyte (Conforti et al., 1991; Lockhart et al., 1997; Yang et al., 2002). Within the first few days in co-culture superior cervical sympathetic neurons express almost exclusively NE, which is an excitatory transmitter for myocytes. Stimulation of neurons in these young cultures leads to an increase in the beat rate of connected myocytes (Conforti et al., 1991; Lockhart et al., 1997). Application of NGF for as little as 10 minutes leads to a reversible facilitation of noradrenergic transmission as assessed by an increase in the effect of neuronal stimulation on beat rate acceleration (Lockhart et al., 1997).

The rapid effect of NGF on excitatory neurotransmission appears to be due to a TrkA-mediated presynaptic potentiation of NE release from sympathetic neurons. Application of the Trk antagonist K252a blocks the NGF-mediated potentiation (Lockhart et al., 1997) and TrkA is expressed by sympathetic neurons, but not myocytes, suggesting a presynaptic role for this

signaling. In addition, the effect of pressure ejection application of NE directly to myocytes is not influenced by NGF (Lockhart et al., 1997), suggesting that postsynaptic responses are not modulated by NGF. These results suggest that NGF promotes noradrenergic synaptic transmission in sympathetic neurons via a TrkA mediated mechanism.

In contrast to the potentiating effect of NGF on release of NE in neonatal neuron/myocyte co-cultures grown for three days, application of BDNF leads to the release of the inhibitory neurotransmitter ACh (Yang et al., 2002). This is surprising since young neurons tend to be noradrenergic, express low levels of cholinergic markers, and show no electrophysiological evidence of cholinergic transmission until many days in co-culture with myocytes (O'Laigue et al., 1978c; O'Laigue et al., 1978b, a; Yang et al., 2002). However, a fifteen minute application of BDNF causes a reversible switch in the effect of neuronal stimulation from excitatory to inhibitory (Yang et al., 2002). This switch is blocked by the muscarinic cholinergic antagonist, atropine, suggesting that BDNF promotes synaptic release of the inhibitory transmitter ACh (Yang et al., 2002). Similar to the results found for NGF, the effect of BDNF appears to be presynaptic since the effect of direct application of muscarine, a muscarinic cholinergic agonist, or NE is unaltered by BDNF. Application of BDNF does not induce expression of cholinergic markers over the time frame of the experiment and unlike other cholinergic differentiation factors, such as ciliary neurotrophic factor, does not induce cholinergic markers even after three days in culture (Slonimsky et al., 2003). These data suggest that BDNF leads to a rapid induction of ACh release from sympathetic neurons through a mechanism distinct from the cholinergic differentiation factors generated by cholinergically innervated tissues such as sweat glands. Further, while increasing cholinergic transmission, BDNF does not appear to affect the release of NE, thus BDNF treatment results in the rapid onset of co-transmission in sympathetic neurons.

The induction of ACh release from sympathetic neurons is dependent on p75 signaling. Sympathetic neurons express TrkA and TrkC, which are not activated by BDNF, and express p75, but not the BDNF-specific TrkB (Dixon and McKinnon, 1994; Wyatt and Davies, 1995; Bamji et al., 1998; Yang et al., 2002; Luther and Birren, 2009). Therefore, BDNF acts as a specific p75 agonist in sympathetic neurons. Several lines of evidence support the action of p75 activation as an acute modulator of cholinergic transmission. BDNF-dependent induction of ACh release is not altered by the Trk inhibitor K252a, suggesting that Trk involvement is unlikely (Yang et al., 2002). Furthermore, application of C₂-Ceramide, an analog for the ceramide second messenger generated following p75 activation (Dobrowsky et al., 1994; Dobrowsky et al., 1995), mimics the effect of BDNF on myocyte responses to neuronal stimulation. Overexpression of human p75 in cultured rat sympathetic neurons leads to activity-dependent ACh release even without BDNF application (Yang et al., 2002). Finally, application of BDNF to sympathetic/myocyte co-cultures made from mutant mice lacking p75 does not lead to ACh release (Lee et al., 1992; Yang et al., 2002). Together, these data suggest that neurotrophin-dependent activation of p75 rapidly and reversibly promotes cholinergic transmission in noradrenergic sympathetic neurons.

p75 regulation of segregation and release of separate vesicle pools containing ACh or NE

Studies using pharmacological blockers suggest that the relative levels of ACh and NE released from sympathetic neurons are determined by the extent of Trk and p75 signaling. When neonatal neurons and myocytes were cultured for three days in 5-50 ng/ml NGF, stimulation of neurons resulted in only potentiation of beat rate increases (Yang et al., 2002). Application of the β -adrenergic antagonist, propranolol, blocked the potentiation and revealed a small inhibitory response (Yang et al., 2002). In contrast, culturing in the presence of BDNF led to only inhibition of beat rate when neurons were stimulated. This inhibition was blocked by atropine revealing a small noradrenergic response (Yang et al., 2002). Thus, sympathetic

neurons release both NE and ACh, albeit to different extents depending upon the neurotrophin conditions. In contrast to the effects of known cholinergic differentiation factors such as CNTF, however, culture in the presence BDNF for three days does not induce the expression of cholinergic markers (Yang et al., 2002; Slonimsky et al., 2003). These data suggest that even apparently noradrenergically committed sympathetic neurons can co-release NE and ACh in the absence of an up-regulation of cholinergic biosynthetic and packaging enzymes. One model that could account for this regulation is an action of p75 signaling in the preferential release of a segregated pool of ACh containing vesicle. A true understanding of these synaptic effects of neurotrophins will require more detailed analysis of neurotransmitter storage and release properties.

While the intracellular processes that contribute to p75-dependent cholinergic transmission remain to be fully defined, recent studies have shown effects of p75 signaling on segregation of distinct pools of transmitter vesicles in sympathetic neurons. Neurotrophic signaling influences the trafficking and segregation of specific ACh and NE vesicular proteins in sympathetic neurons, processes that are likely to be important for regulated co-transmission (Felder and Dechant, 2007). The authors studied segregation and co-expression of neurotransmitter machinery by transfecting co-cultured sympathetic neurons with fluorescently tagged vesicular acetylcholine transporter, a protein responsible for transporting ACh into cholinergic vesicles, and fluorescently tagged vesicular monoamine transporter 2, which transports NE into vesicles. The authors showed that the two types of transporters, tagged with different chromophores, demonstrate increased segregation following a 30 minute application of NGF or BDNF. The effect of the neurotrophins appears to be mediated via p75 activation since application of a p75 function blocking antibody, but not the Trk inhibitor K252a, blocks the effects of neurotrophins. This study demonstrates a rapid effect of p75 on the sorting of different transmitter pools and provides a potential mechanism for the acute modulation of sympathetic synapses onto cardiac myocytes by BDNF.

The rapid modulation of ACh release in neonatal neurons after three days of culture suggests the presence of a pre-existing pool of cholinergic vesicles in these predominantly noradrenergic neurons. As mentioned in previous sections, contact with particular targets such as sweat gland induces the expression of cholinergic markers in sympathetic neurons. However, earlier developmental mechanisms exist that lead to expression of cholinergic markers independent of target interactions in a subset of sympathetic neurons (Schafer et al., 1997; Stanke et al., 2000; Schutz et al., 2008). This regulation occurs through a mechanism distinct from target-dependent cholinergic induction since it is not influenced by genetic disruption of LIFRb and gp130 signaling, the signaling system used by the sweat-gland differentiation factor (Stanke et al., 2000; Stanke et al., 2006). This raises the question of whether all postnatal neurons express low levels of ACh, which can be regulated via neurotrophin signaling, or if this only occurs in a subset of neurons. It is also unclear to what extent co-culture of postnatal sympathetic neurons with myocytes influences the expression of cholinergic neurotransmission markers over the first three days in culture. Future experiments will be necessary to determine if all postnatal sympathetic neurons possess small numbers of cholinergic vesicles that have not been detected through conventional means and whether three day co-culture times result in the induction of a physiologically relevant cholinergic phenotype.

The question of whether or to what degree adult sympathetic neurons *in vivo* remain plastic with respect to transmitter phenotype remains open. Adult sympathetic neurons co-cultured with cardiac myocytes retain the capacity to switch to a cholinergic phenotype, although they appear to be less plastic than neurons cultured from neonatal animals (Potter et al., 1986). The ability to co-release multiple neurotransmitters may also be maintained. Cholinergic sympathetic neurons contacting sweat glands continue to express noradrenergic markers in the adult, suggesting that they may retain the ability to release both transmitters (Habecker et al.,

2000). Furthermore, human sympathetic neurons innervating vascular specializations called Hoyer-Grosser organs in the hands and feet and parasympathetic neurons innervating the heart express markers for both NE and ACh (Weihe et al., 2005), suggesting that human adult neurons maintain some plasticity with regard to neurotransmitter release properties. More work will be needed to confirm the extent of adult plasticity *in vivo* and to determine the potential physiological role of NE/ACh cotransmission in the sympathetic system.

Involvement of CamKII in p75 induction of ACh release

The downstream signaling pathways required for cholinergic modulation have only partially been worked out. The effects of BDNF are mimicked by a ceramide analog (Yang et al., 2002), suggesting a role for this second messenger in p75-dependent effects on synaptic properties. In addition, activation of calcium/calmodulin-dependent protein kinase II (CamKII) appears to play a role in the BDNF-dependent induction of ACh release from cultured sympathetic neurons (Slonimsky et al., 2006). The shift to cholinergic transmission is blocked by presynaptic inhibition of CamKII, whereas transfection of neurons with constitutively active CamKII leads to cholinergic induction in the absence of exogenous BDNF (Slonimsky et al., 2006). However, a p75-CamKII interaction appears critical since transfection of constitutively active CamKII into neurons cultured from p75 knockout mice does not lead to a cholinergic shift (Slonimsky et al., 2006). These effects of CaMKII in the regulation of synaptic transmission are consistent with previously described actions of this kinase in the central nervous system (Wang, 2008) and suggest a complex role for CaMKII signaling in the modulation of sympathetic synaptic transmission, the details of which are still unknown.

Physiological role of neurotrophin-dependent regulation of co-transmission

Together, these studies suggest that the physiological effect of sympathetic transmission at the heart and other target tissues could be dynamically regulated by neurotrophin-dependent activation of p75 and TrkA. The relative signaling through these pathways could determine the level of excitation by modifying the level of excitatory (NE), and possibly inhibitory (ACh), transmitters released. Different types of sympathetically innervated tissues release distinct profiles of neurotrophin family members (Bierl et al., 2005; Randolph et al., 2007), which suggests a mechanism whereby targets could dynamically regulate the influence of sympathetic tone based on the prevailing physiological conditions. Adaptive changes in autonomic nervous system function are implicated in health benefits conferred by physical activity (De Angelis et al., 2004; Raczak et al., 2005; Sugawara et al., 2009) and pathological changes in sympathetic function are implicated in cardiovascular diseases (Chen et al., 2001; Watson et al., 2006). Neurotrophin-dependent regulation of transmitter release may provide a mechanism for the modulation of sympathetic nervous system function in disease and health.

Interestingly, recent evidence suggests that neurotrophin regulation of co-transmission may also take place in the CNS. Neurotrophin signaling regulates neurotransmitter release in cultured basal forebrain neurons expressing ACh (Auld et al., 2001b; Auld et al., 2001a). Basal forebrain neurons express ACh, γ -amino butyric acid (GABA) or glutamate (glut), and recently have been shown to co-express mixtures of the three transmitters (Sotty et al., 2003; Huh et al., 2008). In order to unequivocally examine neurotransmission in single cells, individual neurons were grown on glial microislands and autaptic synaptic currents were monitored (Huh et al., 2008). Neurotransmitter phenotype was determined using pharmacological dissection and cells expressing either one or a combination of two or all three transmitters were identified. The authors found that chronic NGF exposure (16 to 30 days) increased the amplitude and frequency of both ACh and glut components of neurotransmission in autaptic microislands. This effect was dependent on p75 since it was blocked by a p75 function blocking antibody but not by the Trk inhibitor K252a. These results suggest that neurotrophin signaling may

represent a global mechanism controlling transmitter strength and phenotype on multiple time scales.

4. Regulation of repetitive firing properties of sympathetic neurons

Excitability and firing properties of sympathetic neurons

We have discussed how neurotransmitter phenotype is regulated in sympathetic neurons by target-derived factors and how this regulation may impact adaptive changes in sympathetic neuronal functioning. We will next discuss the electrophysiological properties of sympathetic neurons that control action potential discharge and hence neurotransmission. We will review data suggesting that, in addition to regulating transmitter phenotype, neurotrophin signaling also regulates intrinsic excitability and repetitive firing properties of sympathetic neurons and discuss how these mechanisms could act together to influence the functional output of sympathetic innervation.

Characteristic repetitive firing patterns of sympathetic neurons

Intrinsic excitability and the repetitive firing properties of neurons control transmitter release and are therefore essential determinants of nervous system function. Mammalian sympathetic neurons have long been used to study the relationship between the electrical action potential and release of neurotransmitter (Eccles, 1944; Eccles, 1955), and it was recognized that there are distinct classes of sympathetic neurons that can be differentiated based on electrophysiological properties and firing patterns (Weems and Szurszewski, 1978; Kreulen and Szurszewski, 1979; Jule and Szurszewski, 1983).

The firing patterns of sympathetic neurons have been typically described as being either phasic, that is firing a burst of one or more spikes early after excitation and then becoming silent, or tonic, firing throughout an excitation. The relative proportion of cells firing in each of these patterns varies with the ganglion from which the neurons are isolated (Erulkar and Woodward, 1968; Blackman and Purves, 1969; Decktor and Weems, 1981, 1983; Cassell et al., 1986; Wang and McKinnon, 1995; Jobling and Gibbins, 1999).

The different firing patterns are associated with physiologically distinct classes of sympathetic neurons. Anatomic tracer studies show that most sympathetic neurons in the caudal lumbar ganglia chain project to the vasculature of the hind limbs, whereas another group of neurons in the inferior mesenteric ganglion project to visceral organs, controlling secretion and contractility (McLachlan and Janig, 1983; Baron et al., 1985). It is found that neurons projecting to vascular targets fired in the phasic pattern while neurons projecting to visceral organs fire tonically, suggesting discrete physiological roles for different firing patterns (Cassell et al., 1986). In addition to projecting to different targets these two classes of neurons are also found to receive different types of synaptic input (Cassell and McLachlan, 1986). When afferent nerves are stimulated, phasic vasomotor neurons typically receive at least one suprathreshold synaptic input whereas tonic, visceral neurons typically receive multiple subthreshold synaptic inputs (Cassell and McLachlan, 1986). Sympathetic neurons of the celiac ganglion can also be divided into tonic and phasic firing groups, and project either to vascular, muscular or glandular targets within the intestine (Meckler and McLachlan, 1988; McLachlan and Meckler, 1989). Interestingly, stimulation of the afferent nerve leads to suprathreshold synaptic inputs to phasic neurons and multiple subthreshold inputs to tonic neurons, similar to the situation in the lumbar chain and inferior mesenteric ganglion (Meckler and McLachlan, 1988; McLachlan and Meckler, 1989). This suggests that the repetitive firing properties of the neurons may be matched to both the specific target of innervation and to the types of inputs they receive.

While tonic and phasic firing phenotypes of sympathetic neurons that project to the lower body and gut appear to be correlated to the identity of the innervated tissue, neurons in the intact adult superior cervical ganglion (SCG) appear to be predominately phasic (Erulkar and Woodward, 1968; Wang and McKinnon, 1995; Jobling and Gibbins, 1999). Cultured SCG neurons however, display both phasic and tonic phenotypes (O'Laigue et al., 1978b; Malin and Nerbonne, 2000, 2002; Jia et al., 2008; Luther and Birren, 2009), suggesting that properties of the intact ganglion influence sympathetic firing patterns. While all SCG neurons in intact ganglia fire phasically regardless of target innervation, they do express target-specific patterns of functional properties. For example, stimulation of the afferent nerve providing input to the SCG elicits action potentials in ganglionic neurons that can be recorded electrophysiologically and whose threshold for activation correlates to the identity of tissue innervated, suggesting that different classes of SCG neurons are electrophysiologically distinct in some ways (Li and Horn, 2006). Furthermore, retrograde tracer studies show that SCG neurons that innervate vascular targets express both the peptide transmitter neuropeptide Y and tyrosine hydroxylase, while neurons that project to piloerector or salivary gland targets express only tyrosine hydroxylase (Gibbins, 1991). Additionally, the size and position of the SCG neuronal somata varies within the rostrocaudal extent of the ganglion according to the identity of innervated target (Gibbins, 1991; Headley et al., 2005). Neurons that project to salivary glands are the largest and are located caudally, those projecting to vascular targets are the smallest and located throughout the rostrocaudal axis and secretomotor neurons are intermediate in size and located rostrally. Thus, at least for SCG neurons, establishment of firing properties is likely to depend upon multiple regulatory factors, but target interactions appear to play a role in determining other functional properties.

Firing patterns also vary among other ganglia in the sympathetic system. Neurons of thoracic paravertebral sympathetic ganglia have been described as maintaining a predominately tonic firing pattern (Blackman and Purves, 1969), while renal and splenic sympathetic neurons in the celiac and renal ganglia have been shown to fire either phasically or tonically (Decktor and Weems, 1981, 1983). These findings suggest that, in general, physiologically distinct classes of sympathetic neurons projecting to different targets express distinct constellations of functional properties, including electrophysiological properties.

Growth factor signaling within sympathetic ganglia

In addition to signals from the targets of innervation, it is also possible that neurotrophins and other factors released by cells within sympathetic ganglia provide important regulation of functional neuronal properties for sympathetic neurons. It has been shown that BDNF released by sympathetic neurons in the SCG regulates the density of innervation by spinal preganglionic neurons (Causing et al., 1997). Little is known about the production of neurotrophic factors within different sympathetic ganglia and how responses to those factors might interact with target-derived neurotrophic signaling to regulate the functional properties of neurons. However, recent studies demonstrating an effect of BDNF on sympathetic firing properties (see below) further support the idea that local ganglionic signaling could play an important role in determining neuronal properties. Future experiments will be necessary to further define the role of factors produced within individual ganglia in regulating sympathetic neuronal function.

Differential regulation of sympathetic firing patterns via p75 and TrkA signaling

The repetitive firing pattern of cultured SCG sympathetic neurons is influenced by neurotrophin signaling, with differential effects of NGF, a target-derived neurotrophin, and BDNF, a factor produced within the SCG (Luther and Birren, 2009). Specific patterns of firing in these neurons are regulated via signaling through the p75 and TrkA receptors. Under control conditions cultured SCG neurons predominately fire tonically, but after a fifteen minute

application of NGF electrophysiological recordings demonstrate a shift in some cells to a phasic firing pattern. Sympathetic neurons express p75 and TrkA, both of which are activated by NGF. When NGF is co-applied with the kinase inhibitor K252a to inhibit Trk, but not p75 signaling, cells fired predominantly in a phasic pattern. Conversely, selective activation of TrkA following co-application of NGF with a p75 function blocking antibody led to an increase in tonic firing cells. This suggests that activation of p75 promotes phasic and TrkA activation promotes tonic firing in SCG neurons. This model is further supported by the finding that BDNF, a p75 selective ligand in SCG sympathetic neurons causes phasic firing, an effect blocked by a p75 function blocking antibody. Furthermore, application of the p75 second messenger analog C₂-ceramide also causes SCG neurons to fire phasically and application of NGF or BDNF to SCG cultures established from p75 mutant mice does not promote phasic firing. Together these results provide strong evidence for a role for p75 in promoting phasic firing patterns and suggest that phasic and tonic firing phenotypes in sympathetic neurons can be rapidly regulated by differential neurotrophin signaling via p75 and Trk.

5. Repetitive firing and voltage-gated currents

Determination of sympathetic repetitive firing patterns: voltage-gated currents

Since the pioneering work of Hodgkin and Huxley it has been recognized that the electrical behavior of neurons is determined by the exquisitely regulated movement of ions across the cell membrane (Hodgkin and Huxley, 1952b, a). Through their work and the work of many others we know that different firing patterns are generated by the expression of particular compliments of ion channel proteins that respond to changes in membrane potential by transiently opening to allow the flow of specific ions across the membrane (Llinas, 1988).

M-type potassium current in tonic and phasic sympathetic neurons

Extensive evidence suggests that tonic and phasic firing patterns in sympathetic neurons are, in part, determined by differences in potassium currents. Sympathetic neurons express the M-type potassium current, which causes a slowly activating, prolonged hyperpolarization of the membrane that inhibits repetitive firing (Brown and Adams, 1980; Constanti and Brown, 1981). The M-current is larger in amplitude in phasic compared to tonic neurons and is likely to be responsible for the cessation of firing following the initial discharge (Cassell et al., 1986; Wang and McKinnon, 1995; Jia et al., 2008; Luther and Birren, 2009). Pharmacologically blocking or reducing the M-current can induce tonic firing in previously phasic neurons (Wang and McKinnon, 1995; Luther and Birren, 2009), providing further evidence for a key role of the current in setting up a phasic pattern.

Delayed Rectifier type potassium currents in tonic and phasic sympathetic neurons

Other types of potassium currents also play a role in shaping specific sympathetic firing patterns. Besides the M-current, sympathetic neurons express multiple voltage-gated potassium currents that can be differentiated in voltage-clamp recordings based on channel kinetic properties in response to depolarization (McFarlane and Cooper, 1992; Malin and Nerbonne, 2000; Luther and Birren, 2006). Broadly speaking, channels that remain open or close slowly during a maintained depolarization are categorized as delayed rectifier type channels, while those that close rapidly during depolarization (inactivation) are termed A-type channels (Rudy, 1988). Voltage clamp recordings have shown that the amplitude of the delayed rectifier current is larger in tonic compared to phasic neurons (Luther and Birren, 2009). Perturbation of the expression of Kv2 potassium channel subunits, which form a delayed rectifier type, has implicated these channels in the establishment of a tonic pattern of firing. Transfection of cultured SCG neurons with dominant negative gene constructs for Kv2 reduces the number of tonic cells from 25 % to 8 % of the population, and conversely, transfecting with the wild-type construct increases the number of tonic cells to 50 % of the population (Malin

and Nerbonne, 2002). Delayed rectifier currents are implicated in regulating membrane repolarization following an action potential, relieving sodium channel inactivation and resetting the cell to prepare for the next action potential (Rudy, 1988). Indeed some other neurons, such as fast-spiking hippocampal interneurons that fire at rapid frequencies, are found to express large, fast delayed rectifier type currents to facilitate rapid action potential repolarization (Lien and Jonas, 2003).

A-type potassium current and tonic and phasic sympathetic neurons

Rapidly inactivating, A-type currents have also been suggested to play a role in determining tonic and phasic firing patterns in sympathetic neurons, however their exact role appears to be complicated. Some studies have detected A-current in only tonic neurons (Cassell et al., 1986) while other studies have detected it in both tonic and phasic cells and found that it is unlikely to play a major role in determining firing patterns (Wang and McKinnon, 1995). However, species differences may account for the discrepancies as the work by Cassell et al. was performed in the guinea pig while the studies by Wang and McKinnon used rat sympathetic neurons.

Nevertheless, in cultures derived from rat SCGs, both phasic and tonic neurons express A-currents, and they appear to play a complex role in regulating firing patterns (McFarlane and Cooper, 1991, 1992; Malin and Nerbonne, 2000, 2001; Luther and Birren, 2006). Transfection of SCG neurons with gene constructs encoding dominant negative Kv4 potassium channels selectively blocked the expression of the A-current in 70 % of cells and led to a change in the proportion of tonic and phasic neurons (Malin and Nerbonne, 2000). The authors describe phasic and two classes of repetitively firing neurons in their cultures: adapting and tonic. Adapting neurons are differentiated from tonic neurons by firing repetitively, but with increasing interspike intervals throughout a prolonged depolarization. Tonic neurons fire with consistent intervals throughout the stimuli. Phasic, adapting and tonic cells were seen following block of the Kv4 A-current, similar to untransfected cultures, but the percentage of phasic neurons was reduced from 43 % to 24 %, while the percentage of adapting neurons increased from 32 % to 52 % and the number of tonic neurons remained the same. This suggests that reduction of Kv4 A-current converts phasically firing cells to adapting cells, consistent with the idea that the A-current plays a role in generating phasic firing of SCG neurons.

The role of the A-current in promoting phasic firing is further supported by experiments in which the activity of Kv1, another potassium channel subunit family that can contribute to A-currents, was manipulated. Similar to the results with the Kv4 dominant negative, transfection of SCG neurons with Kv1 dominant negative constructs blocks A-current in a percentage of cells and transfection of both mutant Kv4 and Kv1 blocks A-current in all cells (Malin and Nerbonne, 2000, 2001). Similar to blocking Kv4 A-currents, blocking Kv1 decreased the percentage of phasic cells, while increasing both tonic and adapting cells (Malin and Nerbonne, 2001). Co-transfection of both dominant negative constructs dramatically reduced phasic cells (43 % in control to 13 %) while increasing both adapting and tonic neurons (Malin and Nerbonne, 2001). Thus, experiments in which the A-currents are reduced support a role for these currents in the establishment of phasic firing.

In contrast, overexpression experiments suggest a more nuanced role for A-currents in setting firing patterns. Consistent with the results of the A-channel blocking experiments, overexpression of the wild-type Kv1.4 causes an increase in the A-current and a large increase in phasic cells (43 % in control to 67 %), a decrease in adapting cells (32 % in control to 11 %) and no change in tonic neurons (Malin and Nerbonne, 2001). A different response was seen, however, following overexpression of the wild-type Kv4.2 construct. Transfection with Kv4.2 also increases A-current and leads to a reduction of adapting neurons, but increases tonic as well as phasic neurons (Malin and Nerbonne, 2000). These results suggest that generally low

A-current expression is associated with repetitive firing, particularly the adapting phenotype, and high A-current expression contributes to phasic firing. However, it appears that under some conditions at least Kv4.2 may contribute to tonic firing in sympathetic neurons since overexpression increased that firing phenotype. Clearly the interaction of different channels subtypes and how they contribute to distinct firing patterns is complex and more work needs to be done to elucidate these relationships.

Other channel types and tonic and phasic sympathetic neurons

Other types of voltage-gated currents have been implicated in controlling firing patterns in sympathetic neurons as well. Voltage-clamp recordings suggest that calcium-dependent potassium currents are larger in tonic compared to phasic cultured SCG neurons (Luther and Birren, 2009). Similar to delayed rectifier currents, some calcium-dependent potassium currents are implicated in action potential repolarization and appear to be important for generating tonic firing patterns (Muller and Yool, 1998; Muller et al., 2000; Gu et al., 2007). Voltage-dependent sodium current also has a larger amplitude in tonic compared to phasic neurons (Luther and Birren, 2009). This may contribute to the phasic firing pattern by reducing sodium channel availability during progressive channel inactivation caused by repetitive firing (Khaliq and Raman, 2006). Additionally, the inwardly rectifying potassium current is larger in tonic compared to phasic rat sympathetic neurons (Wang and McKinnon, 1996). Blockade of voltage-gated calcium currents in guinea pig sympathetic neurons reduces action potential amplitude and increases half width in phasic, but not tonic neurons, suggesting that calcium does not prominently contribute to the action potential in tonic neurons (Davies et al., 1999). In contrast, calcium current blockade reduces the spike afterhyperpolarization mediated by calcium-dependent potassium currents in both types of neurons, suggesting that voltage-gated calcium currents play different roles in the two types of cells (Davies et al., 1999).

The identification and function of multiple ionic currents in phasic and tonic neurons suggests that different firing patterns in sympathetic neurons are determined through the expression of distinct sets of properties for multiple voltage-gated ionic conductances. Because these channels are regulated by membrane potential changes, differences in the properties of any one channel type are likely to influence the behavior of the other expressed channels in a complex manner. Understanding the contribution of each type of current to the global firing pattern will likely require more detailed pharmacological and molecular biological manipulations of currents combined with electrophysiological recordings, as well as computer model simulations.

6. Target-dependent regulation of excitability and neuronal firing properties

Regulation of voltage-gated currents by neurotrophins

Many studies have implicated neurotrophins in modulating the function of synapses and ion channels in multiple types of neurons in the central and peripheral nervous systems, for reviews see, (Black, 1999; McAllister et al., 1999; Poo, 2001; Davis, 2003; Rose et al., 2004; Blum and Konnerth, 2005; Dulon et al., 2006; Amaral et al., 2007; Nicol and Vasko, 2007; Carvalho et al., 2008; Nicol, 2008). Here we will focus primarily on neurotrophin-dependent ion channel modulation occurring postnatally in neurons of the peripheral nervous system and the relationship of this regulation to the functional properties of the neurons.

Target-derived NGF regulates sympathetic neuronal electrophysiology and ion channel properties

Early studies suggesting that target-derived regulatory signals are important for controlling neuronal electrophysiological properties were carried out using adult bullfrog paravertebral sympathetic neurons. It was recognized that disruption of connections to target tissues results

in changes in sympathetic electrophysiological properties. These changes include broadening of the action potential and a reduction in the spike afterhyperpolarization as a result of a decrease in calcium-dependent potassium currents, which act to repolarize the membrane following the action potential (Kelly et al., 1986; Gordon et al., 1987). This decrease in calcium-dependent potassium current was due to a reduction of calcium entry through voltage-gated calcium channels (Jassar et al., 1993). Additionally, axotomy increased the amplitude of both the M-current and sodium current and decreased the amplitude of the delayed rectifier current (Jassar et al., 1993, 1994). This effect was not likely to be due simply to damage to the neurons since crushing the nerve, which allows regeneration of connections to target, resulted in partial recovery of the electrical properties, while cutting the nerve, which does not allow regeneration, does not result in recovery of electrical properties (Kelly et al., 1988). These studies suggest that interaction with target tissues influences the electrophysiological behavior of sympathetic neurons.

The electrophysiological changes that occur following axotomy appear to be due, at least in part to a loss of target-derived NGF signaling. Incubating explanted adult paravertebral bullfrog sympathetic ganglia in the absence of NGF *in vitro* results in changes in cellular electrophysiology that are consistent with those seen in axotomized neurons (Traynor et al., 1992). Addition of NGF to these explants is able to partially rescue the electrophysiology while addition of NGF antibodies exacerbated the loss of calcium-dependent potassium current and spike broadening (Traynor et al., 1992). Similarly, NGF prevents the decrease over time of calcium currents in cultured frog sympathetic neurons (Lei et al., 1997). While target-derived NGF appears to play a role in regulating sodium and calcium channels (Traynor et al., 1992; Jassar et al., 1993; Lei et al., 1997; Petrov et al., 2001), it does not appear to be essential for potassium channel regulation in the frog, suggesting that additional target-derived factors mediate these effects (Lei et al., 2001).

Interestingly, in contrast to the effects of axotomy and removal of NGF, which increase sodium current, long-term culture of frog sympathetic neurons with exogenous NGF has been shown to also increase sodium current (Lei et al., 2001; Ford et al., 2008). The explanation for these results remains to be determined, but it could be due to differential time-dependent effects of NGF signaling or to multiple interacting signaling factors lost through axotomy. Such factors would not be present in long-term culture conditions and could alter NGF-dependent current modulation (Jassar et al., 1993; Lei et al., 2001).

Target-dependent regulation of sympathetic electrophysiology *in vivo*

The regulation of ionic currents and firing properties by neurotrophins, and the effects of axotomy described above, implies that these factors modulate electrophysiological properties in the animal. In fact, such regulation of sympathetic properties by target-derived factors has been demonstrated. NGF increases calcium currents in paravertebral sympathetic neurons in the adult bullfrog *in vivo*, as shown in experiments in which sympathetic nerve endings were exposed to exogenous NGF via subcutaneous injections of NGF into the thigh followed by culture and electrophysiological analysis. Calcium currents were larger when frogs received prior NGF injections and smaller when they received injections of a blocking NGF antiserum (Lei et al., 1997). These results are consistent with observations described above that sympathetic axotomy causes a reduction in calcium current (Kelly et al., 1986; Gordon et al., 1987; Jassar et al., 1993), and together provide strong evidence supporting a role for NGF in the regulation of these channels in the animal.

In addition to perturbing NGF levels *in vivo*, it is possible to reversibly lesion noradrenergic sympathetic neuronal axon terminals without damaging cell bodies, allowing for the reestablishment of damaged target connections (Petrov et al., 2001). The reversible loss of sympathetic innervation after 6-hydroxydopamine treatment was evident from a loss and

resumption of the sympathetically regulated pupillary reflex in living frogs, as well as through analysis of staining for the noradrenergic marker, tyrosine hydroxylase (Petrov et al., 2001). In explant cultures prepared at times before, during and after 6-hydroxydopamine induced loss of sympathetic connectivity, calcium channel amplitude was decreased coincident with loss of connectivity and increased when connections were reestablished. These findings suggest that interactions between sympathetic neurons and target tissue influenced calcium current properties (Petrov et al., 2001). Together these data strongly suggest that the electrophysiology of sympathetic neurons is regulated *in vivo* via target-derived growth factor signals, including NGF. While the studies described above implicate target interactions in regulating neuronal electrical properties, it also appears that neurotrophin sources in the ganglia can be important regulators of electrophysiology. This idea is suggested by the shift in firing properties from predominately phasic to a tonic and phasic mixture in cultured mammalian SCG neurons (McFarlane and Cooper, 1991, 1992; Malin and Nerbonne, 2000, 2001; Luther and Birren, 2009). Thus, the final pattern of conductances, and the firing properties of sympathetic neurons are likely to be influenced by complex interactions between local and target-generated neurotrophic signals.

Target-dependent regulation of the development of electrophysiological properties

In addition to regulating mature electrophysiological properties, target-derived cell signaling plays a role in setting these properties during autonomic neuron development (Dryer, 1998; Dryer et al., 2003). An example of this is seen in the neurons of the chick ciliary ganglion that express a prominent calcium-dependent potassium current (K_{Ca}) that is important for determining action potential waveform and repetitive discharge patterns (Cameron and Dryer, 2000; Dryer et al., 2003). Expression of K_{Ca} begins after embryonic day (E)8-9 and peaks around E13, coincident with the period of target contact with in the eye (Landmesser and Pilar, 1972; Dourado and Dryer, 1992). The developmental expression of K_{Ca} in parasympathetic neurons is blocked by removal of either target tissue or preganglionic innervation from the Edinger-Westphal nucleus, suggesting that multiple interactions regulate development of the current (Dourado et al., 1994).

The target-dependent developmental regulation of K_{Ca} is dependent on transforming growth factor (TGF β) signaling (Cameron et al., 1998; Cameron et al., 1999). Expression of TGF β transcripts are detected in E9 to E13 chick iris, coincident with target-neuron contact. Treatment of cultured E9 ciliary neurons, which express little to no K_{Ca} , with exogenous TGF β promotes expression of K_{Ca} currents. Furthermore, injection of TGF β into the eye of E8 chicks causes a robust, early increase in K_{Ca} expression by the following day, and injection of TGF β antiserum blocks K_{Ca} expression. These data suggest that target-derived TGF β signaling is an important developmental regulator for potassium currents in chick parasympathetic neurons.

While TGF β acts as a target-derived regulatory factor, the factor responsible for preganglionic regulation of K_{Ca} in chick ciliary neurons appears to be β -neuregulin (β -NEU) derived from the Edinger-Westphal nucleus. The expression of β -NEU in the preganglionic neurons coincides with the onset of K_{Ca} expression (Cameron et al., 2001). Additionally, β -NEU induces expression of K_{Ca} in cultured E9 ciliary neurons and when injected into the eye of E9 chicks (Subramony and Dryer, 1997; Cameron et al., 2001). In contrast injection of saline or a β -NEU neutralizing antibody did not induce K_{Ca} expression (Cameron et al., 2001). These data suggest that both pre- and postganglionic cell-cell interactions provide growth factor signaling that plays an important developmental role in setting the electrophysiological properties of parasympathetic neurons.

Less is known about the specific factors that control ion channel expression in the sympathetic nervous system, but target interactions are clearly important for the expression of A-type (I_A)

and K_{Ca} potassium currents in chick sympathetic neurons (Raucher and Dryer, 1994, 1995). Expression of I_A in chick sympathetic neurons increases between E7 and E20 and neurons isolated and maintained in culture at E9 fail to develop I_A normally (Raucher and Dryer, 1994). Neurons that contacted other neurons or were grown on lysed neuronal fragments, cardiac myocytes, spinal cord explants or aortic smooth muscle developed normal I_A densities, but with altered gating kinetics, suggesting target interactions are important for regulating current density (Raucher and Dryer, 1994). The signaling factor responsible for this regulation is unknown, and is not NGF since exogenous application of NGF to cultured E9 sympathetic neurons does not rescue I_A amplitude (Raucher and Dryer, 1994).

Target derived signaling also regulates the development and expression of K_{Ca} in chick sympathetic neurons. Expression of K_{Ca} is low during days E9 to E16 and increases from E17 to E19, but expression of voltage-gated calcium currents remain constant over this time period (Raucher and Dryer, 1995). Embryonic sympathetic neurons isolated at E13 and cultured for five days showed a much lower amplitude K_{Ca} than acutely isolated neurons at E18, although calcium currents were not different between the two conditions (Raucher and Dryer, 1995). Co-culture of E13 neurons with cardiac myocytes or application of NGF or media conditioned by myocytes rescued K_{Ca} expression, but co-culture with spinal cord explants did not (Raucher and Dryer, 1995). However, NGF treatment resulted in K_{Ca} expression in only half of the neurons, suggesting the actions of additional target factors in this regulation. Together, these data provide strong evidence that target interactions are important determinants in the development of appropriate electrophysiological properties in chick sympathetic neurons. Further, these studies define both long-term developmental and acute modulatory actions for target-derived signals in the autonomic nervous system.

Neurotrophin regulation of electrophysiology in cultured SCG sympathetic neurons

Neurotrophin signaling also regulates voltage-gated currents and firing properties in cultured rat SCG neurons, suggesting that target-derived regulation of sympathetic neurons occurs in mammals as well as frogs and birds (Jia et al., 2008; Luther and Birren, 2009; Raucher and Dryer, 1995). Relatively short duration exposure to neurotrophins (15-120 minutes) shifts the proportions of cultured neurons firing in phasic and tonic patterns and causes systematic changes in several voltage-gated currents that are dependent on the type of neurotrophin receptor activated (Luther and Birren, 2009). Specific pharmacological manipulations determined that activation of p75 promotes phasic firing by increasing the amplitude of the M-type current and decreasing the delayed rectifier, calcium-dependent potassium and sodium currents. Activation of TrkA promotes tonic firing and causes opposite regulation of the four ionic conductances. Single channel recordings suggest that neurotrophins modulate the M-current channel open probability in SCG neurons, which leads to a regulation of the macroscopic current (Jia et al., 2008), while no evidence was found for a shift in the voltage-dependence of activation for the M-current (Jia et al., 2008; Luther and Birren, 2009). While the regulation of the M-current has been investigated, neurotrophin-dependent changes in sympathetic firing properties involve changes in multiple currents and the mechanisms of neurotrophin regulation of these other currents remain to be determined.

Recently, a direct link between neurotrophin regulation of ionic currents and firing patterns in sympathetic neurons was provided by experiments showing that the p75-mediated shift to phasic firing was occluded by using the M-current antagonist linopirdine to partially block the M-current (Luther and Birren, 2009). These results demonstrate that neurotrophin-dependent regulation of an ionic current contributes to the effects of p75 activation on sympathetic excitability and repetitive firing and suggest that further analysis of the roles of p75 and Trk signaling activity on setting patterns of ionic conductances will provide new insight into the mechanisms that control sympathetic neuron functional properties.

Neurotrophin-dependent regulation of electrophysiological properties of dorsal root ganglia sensory neurons

Another example of neurotrophin regulation of voltage-gated currents and firing properties is seen in cultured rat dorsal root ganglia (DRG) sensory neurons (Zhang et al., 2002; Zhang and Nicol, 2004; Zhang et al., 2008). Application of NGF to cultured DRG neurons leads to an increase in excitability, occurring within minutes, as evidenced by a decrease in latency and an increase in spike output in response to depolarizing current ramp injections made using electrophysiological recording techniques. This enhanced excitability is due to an increase in the amplitude of a voltage-gated sodium current and a shift in its voltage dependence, which causes it to activate at more hyperpolarized potentials. NGF further reduces the amplitude of a delayed rectifier type potassium current. These effects appear to be due to NGF activation of the p75 receptor since they were mimicked by application of C₂-ceramide, and blocked by application of a p75 function blocking antibody, and when sphingomyelinase, an enzyme responsible for the generation of ceramide, was inhibited with intracellularly applied glutathione.

Modulation of firing patterns and voltage-gated currents by neurotrophins in auditory sensory neurons

It seems likely that the modulation of channel activity by neurotrophins has functional consequences for the function of neural systems. An example of this is seen in the cochlea where neurotrophins regulate the electrical properties of auditory sensory neurons in the spiral ganglion, which are arranged tonotopically (Adamson et al., 2002a; Adamson et al., 2002b). These neurons are responsible for transduction of specific auditory tones detected in cochlear hair cells and express distinct firing properties that are related to their tonal sensitivities (Adamson et al., 2002a; Adamson et al., 2002b). In this system, the regulation of ion channels and neuronal firing properties by neurotrophins has functional implications for discriminatory sensory detection. When examined electrophysiologically with a maintained depolarization, neurons in the apex, corresponding to low frequency responsive neurons, show a slowly adapting repetitive firing pattern with relatively broad action potentials. Neurons of the base, corresponding to high frequency coding, display a short latency phasic response with brief action potentials. Culturing spiral ganglion neurons for three days in the presence of NT-3 caused all cells to fire adaptively, while culturing with BDNF resulted only in cells that fired phasically regardless of their original position within the cochlea (Adamson et al., 2002a; Zhou et al., 2005). The neurotrophins NT-3 and BDNF are expressed in opposing gradients along the length of the cochlea *in vivo*, consistent with a role of neurotrophins in determining the tonotopic organization of neuronal firing patterns (Fritzsche et al., 1997; Schimmang et al., 2003). The *in vitro* shift in firing phenotype is unlikely to be due to a differential neurotrophin-mediated survival effect on cell subpopulations since culturing cells for three days in the absence of neurotrophins followed by NT-3 treatment yielded similar results to including NT-3 from the initial isolation of the neurons (Zhou et al., 2005). This suggests that an endogenous gradient of NT-3 and BDNF in the cochlea is important for regulating the electrophysiological phenotypes of postnatal auditory sensory neurons.

The differences in spiral ganglion firing patterns correspond to differences in the expression of several voltage-gated potassium currents (Adamson et al., 2002a; Adamson et al., 2002b). Immunocytochemistry using antibodies directed against potassium channel subunits show that apical, adapting neurons have higher expression of Kv4.2 channel subunits, while the phasic basilar neurons show relatively higher expression of large-conductance calcium-activated potassium current (K_{Ca}) and Kv1.1 and Kv3.1 type potassium channel subunits (Adamson et al., 2002a; Adamson et al., 2002b). Culturing neurons in the presence of NT-3, which leads to adaptive firing, increased Kv4.2 expression, while BDNF exposure, resulting in phasic firing, leads to increased expression of K_{Ca}, Kv1.1 and Kv3.1 (Adamson et al., 2002a). These studies

demonstrate neurotrophin-dependent regulation of channels implicated in the establishment of phasic and tonic firing patterns, and suggest a direct link between neurotrophin signaling and regulation of ionic currents.

Role of cellular context in determining neurotrophin effects

These results taken together suggest that the exact effect of neurotrophin signaling on firing patterns and voltage-gated currents is dependent on cell context. In auditory sensory neurons phasic cells have a larger K_{Ca} and delayed rectifier and adapting cells show a larger A-current, which is not consistent with findings for sympathetic neurons described above, which showed that phasic neurons had smaller K_{Ca} and delayed rectifier than tonic neurons (Luther and Birren, 2009). Additionally, the increase of sodium current and excitability in DRG neurons via p75 signaling is at odds to the finding that in SCG sympathetic neurons p75 signaling causes a decrease of sodium current and firing (Luther and Birren, 2009). These findings suggest that a range of voltage-gated currents, intracellular signaling pathways and neurotrophins interact in complex cell-specific ways to regulate firing properties.

7. Downstream signaling pathways in the regulation of electrophysiological properties

Intracellular signaling cascades linking TrkA and p75 to ion channel modulation

While it is clear that neurotrophin signaling regulates ionic conductances, repetitive firing and synaptic properties in many types of neurons, much is still left to be learned about the signaling pathways that link neurotrophin receptors to ion channels. The complex and interacting intracellular pathways that are activated downstream of the Trk and p75 receptors have been extensively studied (Chao and Hempstead, 1995; Chao, 2003; Gentry et al., 2004; Reichardt, 2006), but the specific components of these pathways involved in the regulation of neuronal functional properties remain to be fully identified. In this section we will review experiments that have begun to shed light on intracellular pathways that are involved in this linkage, concentrating mostly on experiments involving the peripheral nervous system.

As discussed in previous sections, NGF positively regulates calcium currents in bullfrog sympathetic neurons. This effect is mimicked by a receptor-activating TrkA antibody and is unaffected by a p75 blocking antibody, demonstrating a role for TrkA signaling (Lei et al., 1998). Pharmacological manipulations have implicated the Ras-MAP kinase pathway downstream of TrkA activation in this regulation. This was shown using the Ras inhibitor α -hydroxyfarnesylphosphonic acid, the tyrosine kinase inhibitors genistein and lavendustin A, and the MAP kinase inhibitor PD98059, all of which inhibit NGF-mediated regulation of calcium currents (Lei et al., 1998). Treatment with transcriptional inhibitors also showed a requirement for RNA transcription for the calcium current increase, suggesting that transcriptional regulation via a MAP kinase pathway underlies regulatory effects of neurotrophins on a current important for setting the firing properties of peripheral neurons (Lei et al., 1998).

Studies of neurotrophin regulation of other currents suggest that multiple signaling pathways contribute to the final electrical state of sympathetic neurons. Long-term culture of frog sympathetic neurons in NGF leads to a dramatic increase in voltage-dependent sodium currents (Lei et al., 2001; Ford et al., 2008). This increase is blocked by the inhibitors wortmannin and LY294002, suggesting that NGF acts via TrkA activation of phosphatidylinositol 3-kinase signaling to mediate sodium channel up-regulation (Ford et al., 2008). Regulation of these currents is also blocked by the transcriptional inhibitors cordycepin and actinomycin-D, demonstrating a requirement for RNA transcription (Lei et al., 2001). Taken together these data suggests that effects of NGF on the electrophysiology of frog sympathetic neurons is

mediated through multiple TrkA signaling pathways that influence expression of ion channel message.

While less is known about the signaling pathways underlying p75-mediated regulation of neuronal excitability, some studies have suggested a role for the ceramide second messenger pathway. As discussed above, the excitability of DRG sensory neurons is increased by NGF and BDNF acting through a p75-mediated mechanism that involves upregulation of a sodium current and downregulation of a potassium current (Zhang et al., 2002; Zhang and Nicol, 2004; Zhang et al., 2008). The role of p75 in this regulation is supported by the observations that the effect is blocked by a p75 function blocking antibody but not by Trk inhibitors (Zhang and Nicol, 2004). Furthermore, the effects on excitability are mimicked by application of downstream ceramide derivatives generated by p75 activation and are blocked by an intracellular inhibitor of sphingomyelinase, the enzyme that produces ceramide (Zhang et al., 2002; Zhang and Nicol, 2004; Zhang et al., 2008). While the biochemical link between p75 activation and ion channel modulation is not fully understood, recent evidence suggests the involvement of sphingosine-1-phosphate, a downstream component of the ceramide signaling pathway, and a requirement for signaling through G protein coupled receptors in the regulation of sensory neuron ion channels and firing properties (Zhang et al., 2006b; Zhang et al., 2006a).

Neurotrophin modulation of channel function via regulation of channel phosphorylation

While progress has been made on identifying pathways involved in the long-term regulation of ion channel expression via Trk-mediated mechanisms, a critical question that remains to be answered in the sympathetic system is what mediates the rapid effects of neurotrophins on electrophysiological properties. While little is known about the pathways that lead to ion channel modification and a shift in firing pattern in the sympathetic system, studies in the olfactory bulb have provided some detailed information that can inform future studies.

In the olfactory bulb (OB), neurotrophins influence channel activity by regulating the phosphorylation state of channel subunits. The potassium current in OB neurons is predominately carried via channels composed of Kv1.3 channel subunits (Fadool and Levitan, 1998), which appear to play an important role in olfactory sensation (Fadool et al., 2004). The functional properties of these Kv1.3 channels are modulated by BDNF via ion channel phosphorylation (Tucker and Fadool, 2002; Colley et al., 2004; Colley et al., 2007; Colley et al., 2009). Acute application of BDNF to cultured rat OB neurons causes a decrease in potassium-mediated whole-cell current measured in voltage clamp without altering channel kinetics, an effect that is associated with increased tyrosine phosphorylation of Kv1.3 subunits (Tucker and Fadool, 2002; Colley et al., 2004). This effect was found to depend on the previous sensory experience of the animal since unilateral naris occlusion, which blocks odorant entry to the nasal epithelium, causes an increased tyrosine phosphorylation of Kv1.3 in response to acute BDNF exposure compared to the control non-occluded side (Tucker and Fadool, 2002). While the role of neurotrophin-dependent phosphorylation of Kv1.3 in functional regulation of olfaction is not clear, these data do suggest that neurotrophin signaling can modulate nervous system function through ion channel phosphorylation.

Interestingly, long-term exposure of BDNF (days) to cultured OB neurons results in an increase in whole-cell current and a speeding of channel kinetics, in contrast to the decrease seen with acute exposure (Tucker and Fadool, 2002). Co-expression of TrkB and Kv1.3 in HEK 293 cells leads to an increase in surface expression and a decreased rate of channel turnover as determined using pulse-chase experiments (Colley et al., 2007). This suggests the long-term increase in Kv1.3 current seen in OB neurons is due to an increased level of functional channel protein in the cell membrane. It was also found that Kv1.3 and TrkB were co-localized in the mouse olfactory bulb and both proteins co-immunoprecipitated, suggesting that TrkB directly

interacts with Kv1.3 to increase specific tyrosine phosphorylation leading to an decreased rate of channel turnover in OB neurons (Colley et al., 2007). These studies are similar to recent findings in peripheral sensory neurons where increased surface expression of TRPV1 channels in DRG neurons is induced via NGF-mediated activation of TrkA and subsequent channel phosphorylation by Src kinase (Zhang et al., 2005). Together, these data suggest that ion channels can be modulated by neurotrophin-induced phosphorylation that can differentially affect channel gating or surface expression.

These studies provide evidence suggesting that neurotrophins may represent an important regulatory mechanism that controls the properties of expressed ion channels. This regulation can occur through direct Trk dependent phosphorylation or through second messenger systems that alter the biochemical state of channels. Neurotrophins can also regulate channel function through alterations of the channel expression or cycling time in the membrane. Yet another mechanism whereby activated Trk receptors have been shown to modulate channel function is via rapid (within milliseconds) direct activation. BDNF has been shown to directly activate Nav1.9 voltage-gated sodium channels via a TrkB interaction in cortical, hippocampal and cerebellar neurons causing membrane depolarization and eliciting action potential discharge (Kafitz et al., 1999; Blum et al., 2002). While such a mechanism has not been reported in peripheral neurons, they suggest an additional mechanism by which rapid changes in ion channel activity could be achieved. Together, these studies show that the ways in which neurotrophins act to regulate ion channels and influence firing are complex and highly varied. An understanding of how these interactions influence the functional properties of the sympathetic nervous system will require an in depth analysis of the signals and interactions that control the expression and activity of the ion channels that control sympathetic electrophysiological properties.

8. Conclusions

Many lines of evidence suggest that neurotrophin signaling represent an important mechanism controlling the synaptic and electrical properties of multiple neuronal types. As discussed in this review, much is known about this type of regulation in the sympathetic nervous system, which notably regulates important physiological functions including cardiovascular and metabolic processes. It seems clear that neurotrophic regulation of the functional properties of sympathetic neurons spans a number of different processes that include ion channel expression and activity, intrinsic electrical and firing properties, and the regulated release and co-release of neurotransmitters. These patterns of regulation are intimately linked; changes in the activity of groups of ion channels underlie changes in firing properties in sympathetic and other neuron types, and firing rates and patterns are known to determine presynaptic neurotransmitter release properties in several systems including in sympathetic neurons (Bradley et al., 2003). As we understand more about the individual regulatory processes mediated by neurotrophins that control different aspects of sympathetic function it will be possible to build new, integrated models of how ganglionic and target-derived factors control the activity and output of sympathetic circuits.

The regulation of sympathetic function by neurotrophic signaling is likely to have significant physiological importance in both normal function and disease. The types and amounts of neurotrophins expressed are likely to vary between different tissue types. For example, sympathetic neurons form synaptic connections onto both NGF-expressing cardiac myocytes and onto BDNF-expressing sympathetic neurons (Ostberg et al., 1976; Bamji et al., 1998). Further, neurotrophin expression is modulated by disease and physiological state. For instance, both NGF and BDNF are up-regulated in the rat heart following ischemia and reperfusion, even though BDNF is not present in cardiac myocytes in a normal physiological state (Hiltunen et al., 2001). Neurotrophin levels change with age in tissues innervated by the sympathetic

nervous system (Cai et al., 2006) and NGF levels are reduced in human congestive heart failure (Kaye et al., 2000). These changes in neurotrophin levels are associated with altered sympathetic function in cardiac disorders, although the functional consequences of changes in neurotrophin levels are not understood. A more complete picture of the effects of neurotrophins on the electrical and synaptic properties of postnatal sympathetic neurons and the mechanisms underlying that regulation will drive a greater understanding of how sympathetic output is controlled in health and disease.

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