THE ROLE OF POSTSYNAPTIC ACTIVITY IN DEVELOPMENTAL INPUT ELIMINATION IN THE SYMPATHETIC NERVOUS SYSTEM

by

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ABSTRACT

Input elimination is a common developmental motif throughout the mammalian nervous system to establish precise innervation in mature circuits. Many studies show that this process is mediated by activity in developing circuits. It has been difficult to block postsynaptic activity in neuron-to-neuron systems because postsynaptic knockout phenotypes are lethal and kill the model animal before the period of input elimination. The sympathetic nervous system shows input elimination: neonatal ganglionic cells have many more pre-ganglionic inputs than adult ganglionic cells. A novel knockout mouse that survives for several months in good health and shows no fast synaptic transmission in sympathetic ganglia has provided a good opportunity to study the role of postsynaptic activity in developing neuronal circuitry. We investigated the number and strength of axonal inputs innervating sympathetic neurons in mouse superior cervical ganglia (SCG) by stimulating the pre-ganglionic nerve while recording form SCG neurons intracellularly. Inputs were quantified by the number of discrete EPSP amplitudes evoked at different stimulus thresholds. To isolate the role of postsynaptic activity, we used postsynaptically silent α3 knockout SCG neurons that lack the α3 sub-unit of the nAChR. We briefly restored sympathetic postsynaptic activity by infection of a adenovirus containing the α3 sub-unit gene to record from knockout SCGs. In wild-type SCG neurons there are about 3 inputs in maturity and 7 inputs at birth. The number of inputs to adult knockout SCG neurons remains elevated (at 7) and resembles the neonatal innervation pattern. We conclude the postsynaptic activity of sympathetic ganglionic neurons is necessary for developmental input elimination to achieve the adult innervation pattern.

RÉSUMÉ

L'élimination des branches axonales afférents est un motif commun dans le système nerveux des mammifères afin d'établir des réseaux neuronales précis. Beaucoup de travail démontre que cela est contrôlé par l'activité in les circuits en développement. C'était difficile de bloquer l'activité post-synaptique dans les circuits entre deux neurones par ce que les supprimations des recepteurs postsynaptiques sont léthales, et les animaux meurent avant le période normal d'élimination. L'élimination des contactes axonales fait partie du développement normal du système nerveux sympathique. Un modèle de souries knockouts survive pour plusieurs mois et qui n'a pas de transmission synaptique dans ses ganglions sympathiques nous présent une bonne oppotunité à enquêter sur le rôle d'activité dans le développement des circuits nerveux. On a regardé le nombre et la force des branches afférents qui innervent les cellules du ganglion supérieur cervicale (SCG) en stimulant les nerfs afférents pendant prenant des enregistrements intracellulaires. On a compter les contacts axonales par compté les réponses postsynaptiques discrètes à des niveaux discrets de stimulation. Pour isoler la rôle d'activité postsynaptique, on a employé les souries sans les sous-unités α3 de nAChR (α3 KO), ou les neurones de l'SCG sont silencieux. On a redonné l'activité aux souries α3 KO brièvement afin de compter les réponses discrètes dans leurs neurones d'SCG. Dans le type naturel, il y a environ 7 afférents à chaque neurone dans l'adulte et 3 à chaque neurone dans le jeune. Le nombre des contacts dans l'adulte α3 KO ressemble le jeune, à 3 contacts. On conclut que l'activité postsynaptique est nécessaire

pour l'élimination des contacts axonales développmentale au but d'atteindre un plan d'innervation mature.

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1. INTRODUCTION

1.1 GENERAL OVERVIEW AND QUESTION

The nervous system depends on highly specific connections to function. During embryogenesis, as developing populations of neurons are organized into nuclei and ganglia, they extend axons along stereotyped pathways to find their synaptic targets. Neurons grow axons that follow distal and proximal chemical cues, to grow towards their signaling partners. When the growing axons arrive at their destination, they form specific synapses with a specific subset of neurons to form specific neuronal circuits (Goodman and Shatz, 1993).

Knockout studies have demonstrated that synaptic activity is not required for the initial formation of synapses. One prolific study is the knockout of Munc18, where animals have no synaptic activity and die at birth. Munc18 is a protein that binds with syntaxin and mediates membrane fusion of neurotransmitter-containing vesicles in presynaptic terminals. Researchers can, however, investigate knockout embryos, which survive until birth. Embryonic Munc18 knockout mice show no evoked synaptic transmission at any synapse. With global loss of presynaptic activity, however, researchers found functional synapses and brain development was normal up to embryonic day 12 (E12). Furthermore, at birth, the morphology of late-forming structures like the neocortex showed normal projections and synapse assembly, while earlier-forming brain areas like the brainstem, were severely degenerated (Verhage et al, 2000). Another study knocking out SNAP-25 (a vesicle-fusion mediator that is likely downstream of Munc18) shows some spontaneous synaptic transmission, but no evoked transmission. This lethal knockout does not show degeneration of any embryonic brain areas, indicating that low levels of activity are sufficient to maintain neural circuitry (Washbourne et al, 2001). These studies show

that the formation of neural circuits is activity-independent, though activity may be necessary in maintaining them. Specifically, synaptic activity does not mediate the developmental extension of axons or formation of synapses. What is the role, if any, of activity in the formation and development of functional neural circuits in early postnatal life?

A great deal of research in several different nervous circuits has investigated the role of activity in development. Work by Hubel and Wiesel showed that normal innervation patterns in the visual cortex are sculpted by experience-driven activity. When presynaptic activity is blocked through the occlusion of one eye, cortical innervation is greatly changed (Hubel and Wiesel, 1963; Hubel and Wiesel, 1977). When both eyes are occluded, and there is no experience-driven activity to the visual cortex, the researchers record no change in cortical innervation patterns (Hubel and Wiesel, 1965). These seminal experiments and follow-up investigations have elaborated Hebb's Law *cells that fire together, wire together* (Katz and Shatz,1996). When a presynaptic, axonal action potential elicits a postsynaptic action potential, the axonal connection is preferentially strengthened. When no postsynaptic action potential is elicited, there is no change in synaptic strength.

Further research has examined the role of the postsynaptic cell by pharmacologically silencing neurons in the visual cortex. Inhibiting postsynaptic activity in the visual cortex has yielded interesting results on the role of the postsynaptic cell: less active presynaptic inputs are actually *strengthened* when they synapse on postsynaptic cells (Reiter, et al, 1986; Reiter and Stryker, 1988). The explanation of this phenomenon is still obscure. The authors assert that the underlying mechanism is unclear partially due to the complex multi-synaptic architecture of the visual cortex (Reiter and Stryker. 1988). This experimental model could also be examined in a simpler circuit where the postsynaptic neuron population is silenced.

Apart from the visual system, the neuromuscular junction (NMJ) is another circuit commonly observed and manipulated to study input elimination. Initially, researchers observed that muscle fibers in young rats were innervated by many axonal inputs, but in adulthood received only a single axonal input (Sanes and Lichtman, 1999). When investigators block activity in the neuromuscular junction - pharmacologically or by genetically increasing the number of potassium rectifier channels - multiple innervation by motor axon inputs persists, where it is eliminated in wild-type animals. (Thompson et al., 1979; Favero et al., 2009). Studies like these have highlighted the importance of postsynaptic depolarization in input elimination. Although the NMJ is a classic model of input elimination, it is highly-specialized and quite distinct from neuron-to-neuron synapses. The differences are significant enough that conclusions from this synapse may not be applicable to neuron-to-neuron synapses (Sanes and Lichtman, 1999). Still the question remains, what is the role of postsynaptic activity in input elimination in neuron-to-neuron circuits?

This question is difficult to answer because blocking postsynaptic activity in the central nervous system would likely be lethal. Alternatively, to conditionally knockout postsynaptic receptors in one non-crucial brain area would require an extensive knockout design removing many postsynaptic receptors and all of their subtypes.

On the other hand, previous work has shown that the sympathetic nervous system is not necessary for survival. When NGF and TrkA are knocked out in mouse models, animals show a severe degeneration of the sympathetic nervous ganglia, yet animals survive from one week to almost two months (Crowley et al, 1994; Smeyne et al., 1994). Because mice can survive without sympathetic nervous systems, sympathetic circuits are an ideal model to knock out postsynaptic receptors without lethality. To examine the role of postsynaptic activity in this

circuit, the knockout mice must live long enough to study post-natal developmental changes. Recently, a novel knockout mouse model has been developed where sympathetic postsynaptic activity has been silenced, normal numbers of synapses persist and, importantly, the animal survives in good health for several months. Krishnaswamy and Cooper showed that this mouse, which lacks the α3 sub-unit of the nicotinic acetylcholine receptor, has no synaptic transmission in sympathetic ganglia. The knockout lacks a sub-unit of the nicotinic acetylcholine receptor (nAChR), which is necessary for sympathetic synaptic transmission (Krishnaswamy and Cooper, 2009). The α3 knockout provides an ideal preparation to explore the role of activity in the postsynaptic cell in input elimination in neuron-to-neuron circuits.

1.2 THE SYMPATHETIC NERVOUS SYSTEM

1.2.1 Overview of the sympathetic nervous system

The sympathetic nervous system is a branch of the autonomic nervous system (ANS), which is constitutively active to maintain homeostasis in an organism. All synaptic transmission to sympathetic ganglion neurons is mediated by acetylcholine (ACh) release onto acetylcholine receptors (AChRs). Sympathetic pre-ganglionic neurons in the spinal cord extend axons and form synapses with post-ganglionic cells in the paravertebral chain ganglia, located bilateral and ventral to the spinal cord, or the prevertebral ganglia. Post-ganglionic neurons innervate endocrine and exocrine glands, smooth muscle or cardiac muscle using noradrenaline or, in a few ganglia, ACh as their neurotransmitter (Purves, et al., 1980; Purves, et al., 2004; Glebova and Ginty, 2005; McLeod and Tuck,1987). Activity in the sympathetic nervous system is driven by internal or external cues, such as stress and changes in posture. The sympathetic nervous system

regulates body temperature, blood pressure, heart rate and contractility, blood glucose levels, gastrointestinal peristalsis and ejaculation. Dysfunction of the sympathetic nervous system can cause congestive heart failure, hypertension or hypotension and other neuropathies (Purves, et al., 2004; Goldstein et al., 2002; Glebova and Ginty, 2005).

1.2.2 The superior cervical ganglion

The superior cervical ganglion (SCG) is an attractive model because of the accessibility of the ganglion and its pre-ganglionic and post-ganglionic nerves. The accessibility of the SCG has made it a classical model of developmental and mature sympathetic circuitry. The SCG is the rostral-most sympathetic ganglion in the paravertebral chain, found at the bifurcation of the common carotid artery. The ganglion innervates its targets (smooth muscle of blood vasculature and sweat glands) on the face, ears, and outside of the head through the external carotid nerve. Axons for targets in the skull, such as the pupils and pineal gland are located in the internal carotid nerve. (Glebova and Ginty, 2005; Nja and Purves, 1977; Moller and Baeres, 2002). Descending pathways of the hypothalamus and various nuclei in the brainstem innervate pre-ganglionic cells in the intermediolateral (IML) horn in the thoracic spinal cord. Pre-ganglionic axons emerge from the ventral roots of the spinal cord to innervate the sympathetic ganglia (Rosenberg, 2002).

Innervation to the SCG originates in segments T1-T5 of the spinal cord, with pre-ganglionic neurons in T2 and T3 innervating most SCG cells. Each SCG neuron is dominantly innervated by pre-ganglionic cells of one segment. The spinal location of a pre-ganglionic neuron predicts the target of a SCG neuron. SCG cells with pre-ganglionic inputs from segments T1 and T2 typically innervate the pupil and the eyelid, while those with inputs from T3 and T4

innervate the ear and neck. (Nja and Purves, 1977). When SCG neurons are wholly or partially denervated, the same innervation is reestablished within two months, indicating specificity in the pre-ganglionic-to-target pathway (Nja and Purves, 1978; Maehlen and Nja, 1980). The strength of a pre-ganglionic input to a SCG cell decreases respective to the distance of the input's segmental origin from the dominant input's segmental origin (Nja and Purves, 1977). Because adult SCG neurons are innervated by pre-ganglionic cells from one segment, input elimination could create input specificity by eliminating pre-ganglionic inputs from distant segments to inappropriate SCG neurons (Purves and Lichman, 1983).

Morphological studies show there are approximately 10 000 neurons in the adult mouse SCG. Pre-ganglionic cells can be stained through retrograde transport of horse radish peroxidase injected in the cervical trunk nerve bundle; In the IML nuclei of the spinal cord, there are about 700 neurons that innervate the SCG (Purves et al., 1986). According to Purves et al., adult mouse SCG cells each receive 4 or 5 inputs. Because each of the 10 000 cells receives 4 or 5 inputs, and there are 700 pre-ganglionic cells, each pre-ganglionic cell extends inputs to approximately 65 neurons in the adult ([10 000/700] x 4.5 ≈ 65). Between the 4.5 inputs on each SCG neurons, there are about 250 synapses formed on each cell (Purves et al., 1986).

There is a relationship between the size of a sympathetic neuron, measured by diameter, and a feedback signal it receives from its target. In mice, pre-ganglionic neurons in the spinal cord are roughly 10 μm in diameter, whereas post-ganglionic SCG cells are between 20 and 30 μm (Purves, et al., 1986; Smolen, 1988). Interestingly, the size of the pre-ganglionic cells depends on how many SCG cells it contacts in rodents and the size of the SCG nerve cells vary depending on their targets (Purves, et al., 1986; Gibbins, 1991).

The number and size of SCG neurons and the number of boutons on SCG neurons increases modestly as the size of the model animal increases from mouse to rabbit. Dendritic growth, however, is significantly greater in larger animals. Dendrites influence the innervation of SCG cells; specifically, dendritic complexity affects the distribution of synapses on the SCG. In the mouse SCG, about 10% of synapses are on the cell body (axosomatic), where only about 1% of synapses in rabbit SCG are axosomatic. When a SCG cell has extensive a dendritic arbor, it receives less axosomatic innervation. From this data, Purves and his colleagues assert that dendrites are the preferred target for synapses (Purves, et al., 1986). Dendritic complexity in SCG cells, therefore, may be an important factor that influences developmental input stability in the sympathetic nervous system.

1.3 SYNAPTIC TRANSMISSION IN THE SCG

1.3.1 The sympathetic cholinergic synapse

Theories of input elimination in the sympathetic nervous system have been outlined by other groups (Hume and Purves, 1981; Purves and Hume, 1981; Forehand and Purves, 1985; Forehand, et al., 1985). More recent work has focused on how elimination is mediated based on the morphology of the postsynaptic cell and ganglionic synapses. But to directly investigate the role of activity on input elimination, I have used a mouse model with sympathetic synapses that lack synaptic transmission and that survives beyond the normal period of sympathetic input elimination. To describe the knockout model, I will first describe the normal features of the sympathetic cholinergic synapse.

Synaptic transmission between pre-ganglionic cells and post-ganglion cells is mediated by acetylcholine (ACh). In a simple summary, vesicles loaded with ACh fuse with the active zone on the inner membrane of the presynaptic terminal. Once fused, the vesicles release ACh into the synaptic cleft. In fast synaptic transmission, ACh binds to nAChRs anchored on the postsynaptic density on the post-ganglionic neuron. When ACh binds, the nAChRs open to allow the inward flow of cations into the cell, which initiates the depolarization of the neuron.

1.3.2 The presynaptic terminal

The presynaptic terminal has a few distinctive markers commonly used to identify cholinergic synapses. These molecules have been used to observe changes in innervation and can also serve as indicators of the maturity of a cholinergic circuit.

ChAT: ACh is synthesized by choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A (acetyl-CoA). ChAT is globular protein and exists in a soluble form and a membrane-bound form. ChAT and synaptic vesicle protein (SV) co-immunoprecipitate, indicating that ChAT may synthesize ACh onsite at the vesicle itself. ChAT is an indicator of functional status in cholinergic neurons in the nervous system, and deficiencies are apparent in cholinergic pathologies like Alzheimer's and schizophrenia (Oda, 1999).

VAChT: ACh is packed into synaptic vesicles by vesicular acetylcholine transferase (VAChT). Electron microscopy shows the c-terminus in the cytosol and the n-terminus in the vesicular lumen. VAChT has a strangely low affinity for ACh, which implies high concentrations of ACh in the presynaptic terminal. VAChT uses the energy of protons moving down the concentration gradient out of the vesicle to pump ACh into the vesicle (Eiden, 1998).

Expression of ChAT and VAChT are coordinated and the open-reading frame for VAChT is contained in the first intron for ChAT. In *Drosophila*, ChAT and VAChT have the same first intron, and likely share a promoter. In vertebrates, however, ChAT and VAChT mRNA are transcribed from different promoters so expression is likely coordinated, but not directly paired (Oda, 1999; Eiden, 1998). These two molecules are commonly stained to mark presynaptic terminals, but cannot identify distinct inputs, only synapses.

CHT: The high-affinity choline transporter (CHT) recovers choline from the synaptic cleft back into the presynaptic terminal to synthesize ACh. The CHT protein was proposed in 1961 by Birks and McIntosh as the recovery of choline is the rate-limiting step in ACh synthesis. The CHT gene was only recently identified, characterized and cloned from *c. elegans*, human and mouse genomes (Okuda, et al., 2000; Apparsundaram, et al., 2000; Apparsundaram, et al., 2001) . CHT is located on a subpopulation of synaptic vesicles, called the reserve pool, that fuse with the presynaptic membrane in high-frequency or sustained firing. High levels of activity delivers CHT to the membrane only after a certain extent of usage, when the reserve pool is recruited in signaling. The lack of CHT-mediated choline recovery at the neuromuscular junction may explain why CHT knockouts die soon after birth. Without the ability to sustain prolonged activity at the diaphragm, the animals cannot breath. (Birks and McIntosh, 1961; Blakely and Ferguson, 2004).

Recent work in our lab shows that presynaptic CHT is regulated by postsynaptic activity. Following up work indicating that the presynaptic output of ACh was impaired in α3 knockout mice (Rassadi et al, 2005), Krishnaswamy and Cooper investigated CHT expression in the silent SCG. CHT is expressed in presynaptic terminals in the wild-type SCG, but not in knockout. When α3 expression was restored to the knockouts by adenovirus, synaptic transmission was

restored in sympathetic ganglion. CHT was found in the pre-ganglionic varicosities 7 days after viral rescue. These findings indicate that there is a retrograde signal in cholinergic synapses: the expression of presynaptic CHT depends on postsynaptic activity (Krishnaswamy and Cooper, 2009). In respect to my study, the important implication is the role of a retrograde signal. To better outline how the α3 knockout silences postsynaptic cells in the sympathetic nervous system, I will describe the postsynaptic receptors of the sympathetic cholinergic synapse.

1.3.3 The postsynaptic density

Nicotinic acetylcholine receptors: nAChRs mediate fast synaptic activity in sympathetic nervous ganglia (Sargent, 1993). In SCG neurons, nAChRs are pentamers composed of α and β sub-units. These hetero- and homopentamers are different combinations of five sub-units, α3, α5, α7, β2 and β4 (Mandelzys, et al., 1994). This differs from nAChRs of the neuromuscular junction, where α, β, γ and δ sub-units are expressed at birth and α, β, δ and ε sub-units are expressed post-natally (Hall and Sanes, 1993). The extracellular domain and three transmembrane domains of these sub-units are highly conserved. An intracellular linking loop, a fourth transmembrane domain and the intracellular domain differ between subtypes. The pore of the nAChR opens upon ACh binding and selectively allows the flow of cations into the cell. Haghighi and Cooper have shown in earlier studies in our lab that neuronal nAChRs are inwardly rectifying because of their interaction with intracellular polyamines. At positive membrane potentials, polyamines are electrostatically attracted to the receptor pore and occlude current flow. At negative potentials the polyamines are repulsed by the membrane and allow current flow (Haghighi and Cooper, 1998). Haghighi and Cooper eliminated inward rectification by removing the negative charges from amino acids between the first and second transmembrane

domains. This experimental mutation also reduced the calcium permeability of the channel (Haghighi and Cooper, 2000).

In SCG neurons, the following pentamers were quantified by immunoprecipitation. The subunits combine in the following proportions in the SCG α3β4 at 55%, α3β4α5 at 24% and α3β4β2 at 21% (David, et al, 2010). In neonatal rat SCG cells, mRNA transcripts for all of these subunits are present, with the mRNA levels for α3 and α7 both tripling within the first two postnatal weeks (Mendelzys, et al., 1994). Our lab investigated the homopentameric nAChRs composed of α7, found in the neonatal SCG. The α7-nAChRs, like muscle AChRs, can be labelled with α-bgt. In culture, α7 mRNA levels drop dramatically. When cultured neurons are depolarized, transcript levels and α7-pentamers labeled with α-bgt increase with activity in cultured SCG neurons. This change can be blocked by inhibiting CaM kinase (De Konick and Cooper, 1995). Interestingly, there is no change in SCG current density correlated to α7 mRNA or αbgt-nAChR increases and it is unlikely that α7 contributes to synaptic transmission in the SCG (De Koninck and Cooper, 1995).

Because the α7 does not contribute to synaptic transmission, the α3 knockout will not have residual activity through normal homopentameric α7 receptors. In the α3 knockout it is possible the expression of another α sub-unit may be upregulated to compensate. The α5 subunit is also expressed in sympathetic nAChRs (Mandelzys, et al., 1994). The α3 knockout will be further discussed on page 13.

Muscarinic acetylcholine receptors: There is a second type of postynaptic receptor at the cholinergic synapse. When ACh is released into the synaptic cleft, there is some overflow into the perisynaptic area. This spillover activates muscarinic acetylcholine receptors (mAChRs) that

are widely distributed over sympathetic neurons. There are 5 different genes that encode these receptors (Hogger, et al., 1996). The muscarinic response works through Gq protein signaling (for types M1, M3 and M5) or Gi/o pathway (M2 and M4). Activation of mAChRs in sympathetic neurons closes rectifying potassium channels to depolarize the cell. The increase in excitability of the cell produces the cholinergic 'slow EPSP' (Brown, et al., 1997). Muscarinic responses do not evoke action potentials in the postsynaptic cell, so it is unlikely that they are directly involved in input elimination. Evidence from the neuromuscular junction of tonic muscles, which are depolarized but do not fire action potentials, shows multiple innervation in maturity, indicating that postsynaptic action potentials are required in input elimination (Ridge, 1971; Lichtman et al., 1985). If input elimination in sympathetic circuits depends on postsynaptic activity, muscarinic receptors might play a role by increasing excitability in the postsynaptic cells (Brown, et al., 1997).

1.4 THE α3 KNOCKOUT: model of investigation of activity-dependent signaling in input elimination

Our lab used a knockout mouse lacking the α3 sub-unit of the nicotinic acetyl choline receptor (α3 KO) to investigate the sympathetic cholinergic circuit in culture and *in vivo*. Investigators from our lab showed that low presynaptic ACh-output in the knockout is caused by the absence of CHT in the presynaptic cell, essentially a delay in the maturation of the cholinergic circuit when activity is eliminated.

It was known mice could survive without any sympathetic function, as NGF and TrkA knockout mice have no sympathetic nervous system and survived past birth. These animals, however, fail to thrive and rarely survived to one month (Crowley et al., 1994; Smeyne et al., 1994). Investigators assumed knocking out fast synaptic transmission in the sympathetic nervous

system would also yield a mouse that survived, at least, past birth. The α3 knockout mouse was generated by Xu and colleagues by removing exon 5 from the α3 gene, located on chromosome 9. Heterozygous α3 +/- mice show no notable phenotype or reduced lifespan. Homozygous α3 -/- mice, however, have notable characteristics associated with reduced sympathetic activity. Furthermore, Xu et al.'s knockout homozygotes failed to thrive and typically did not survive past 3-6 weeks (Xu et al, 1999). Knockout animals cannot fully open their eyelids and have over-dilated pupils, functions of the eye that are normally controlled by sympathetic tone (Bremner, 2009). Homozygous knockouts also have enlarged abdomens and dribbling urine caused by a lack of nAChR-mediated parasympathetic tone in the bladder or reduced sympathetic tone to the internal urethral sphincter (Xu et al., 1999; Purves et al., 2004). In our lab we have also identified that knockouts' heart rate is reduced by about 40% and knockouts' ability to thermoregulate is impaired, as their body temperature drops quickly in cold environments. When the knockout was generated by Xu and colleagues, it was not known whether the animals lacked all sympathetic activity. Another α sub-unit might be expressed to assemble sympathetic nAChRs in the α3 knockout mice to compensate for reduced synaptic transmission (Mandelzys et al., 1994).

Importantly, our lab observed no fast nicotinic EPSP in SCG neurons when stimulating the pre-ganglionic nerve in α3 KO mice. This confirmed that there was no compensatory nAChR assembly with an α3-substitute sub-unit and the circuit was indeed silent. Finding that preganglionic ACh output in the SCG was diminished, our lab investigated whether presynaptic varicosities in the knockout SCG were altered. The group visualized synapses by immunostaining presynaptic terminals (with VAChT) and postsynaptic densities (with PSD93) in neonatal α3 KO mice. Immunostaining showed that morphological synapses were present in P7

knockout SCGs, with no significant difference in number compared to wild-type SCGs (Rassadi, et al., 2005).

Further, electron microscopy showed neonatal knockout SCGs show morphological synapses with characteristic concentration of presynaptic vesicles, a density of proteins in the postsynaptic zone, a parallel thickened organization of pre- and postsynaptic membranes and a thickened synaptic cleft. The group found no significant morphological differences between the synapses of P7 KO and wild-type littermates. Immunolabeling and electron microscopy show synapses in the neonatal, P7 α3 knockout in comparable numbers and morphology to wild-type, showing synaptic activity is not necessary for synapse assembly (Rassadi et al., 2005).

In the mouse SCG, synapses are typically formed around E13-14 . Therefore, silent synapses observed in P7 knockout had persisted without activity for 2 weeks. The original α3 KO mice, however, showed delayed growth and typically did not survive longer than 7 days after birth. This model would not have allowed the investigation of input elimination, which requires at least 3 weeks to occur in the SCG. Our group needed a longer-living knock-out to test the stability of the α3 KO synapses. The mice had an inbred C57BL/6/J genetic background. To take advantage of a potential hybrid vigor the group out-crossed the original strain with CD+ mice to yield F1 heterozygotes. The F2 homozygous knockouts from F1 breeders show rapid growth and survive for several months in good health. The group found no synaptic transmission in the SCGs when they tested compound action potentials and EPSPs, so the genetic cross did not transfer any sympathetic synaptic activity either early (P7) or late (P28) in development (Krishnaswamy and Cooper, 2009).

To investigate whether synapse number and morphology were maintained in the new out-crossed knockout, the investigators followed the lead from the immunostaining and electron

microscopy in the neonatal (P7) knockout synapses (Rassadi, et al., 2005). They stained for typical cholinergic synaptic markers, PSD95, PSD93, GKAP/SAPAP and Shank/ProSAP, in knockout and wild-type SCGs (Conroy, et al., 2003). Staining showed no significant difference in the number of synapses in the knockout mice and wild-type littermates up to P28. The staining also showed a parallel postnatal increase in the number of synapses in α3 knockout and wild-type. Older α3 KO mice still lack CHT up until P28. (Krishnaswamy and Cooper, 2009).

Electron microscopy shows persistent silent synapses in 4- and 5-week-old α3 KO mice. There is no significant difference between α3 KO and wild-type littermates in terms of the number of vesicles, the post-synaptic density, thickening of the membrane along the synaptic cleft or alignment of pre- and post-synaptic compartments. There is no difference in the number of synapses found in the α3 KO and wild-type. Interestingly, there are subtle differences in the size of the synapses (α3 KO animals have synapses that are 40% larger, and a subset of synapses that cluster in groups of 2 or 3 and are 3.5X larger) but their morphological characteristics are no different than wild-type. These follow-up studies show synapse numbers and morphology are not significantly different over the first 28 days of life, demonstrating postsynaptic activity is not necessary for synapse maintenance (Krishnaswamy and Cooper, 2009).

These persistent synapses, however, do not indicate the number of inputs to the silent SCG neurons. Furthermore, even though the knockout has silent persistent synapses, it is still not compatible with counting presynaptic inputs. In intracellular recordings, distinct evoked EPSP steps represent the number of inputs (Purves, 1975). In a silent model EPSPs cannot be evoked at all. Some method of counting the inputs was needed to observe input elimination.

The lab has developed an adenovirus that can restore activity to the silent SCGs. The adenovirus drives expression of the α3 sub-unit in SCG neurons off of the synapsin promoter

(Synα3). Considering that morphologically normal - but silent - synapses persist in α3 KO mice, our group assumed that a virally expressed α3 sub-unit would co-assemble with β sub-units to form functional nAChRs and quickly return activity to the already-formed synaptic machinery. The prediction was accurate: when α3 KO animals at P1 or P28 were infected with the virus, they regain synaptic transmission. Compound action potentials and EPSPs in the virus-rescued α3 KO were similar to wild-type controls and the restoration of postsynaptic activity occurs rapidly, within 24 hours after injection (Krishnaswamy and Cooper, 2009).

Furthermore, rescued activity restores some aspects of normal maturation of the circuit, as adenovirus-rescued KOs express CHT a week after infection. To test whether the receptor is responsible for CHT expression, and not activity itself, they expressed a point-mutated, defunct copy of the nAChR. This did not restore CHT expression, therefore the rescue of CHT is dependent on an activity-mediated signal to the presynaptic cell (Krishnaswamy and Cooper, 2009).

This virus-rescued α3 KO model is crucial to my investigation. By rescuing postsynaptic activity to the α3 knockout with the Synα3 adenovirus, I would be able to evoke EPSPs and count inputs to the silent SCG. Once activity is restored, I could count the number of inputs by EPSP steps recorded intracellularly (Purves, 1975). It is important, though, that the virus-rescued activity itself does not induce input elimination. If the rescued activity did cause elimination I would not record an accurate number of inputs on 'silent' SCG cells. With this in mind, if the rescue is brief enough to not cause any input elimination, the Synα3-rescued α3 knockouts will represent the number of synapses on postsynaptically silent neurons. This measurement will help to describe the role of postsynaptic activity in input elimination in the SCG.

1.5 Development and maturation of sympathetic neurons

To investigate postnatal input elimination in the sympathetic nervous system, it is necessary to understand how the system develops before birth and in early life. The development of the sympathetic nervous system and the SCG has been outlined in great detail. Like the CNS, the sympathetic nervous system must generate the appropriate number of neurons proportionate to their targets, guide axons to their signaling partners, grow dendrites and form synapses. Then, the juvenile sympathetic connections mature: the presynaptic neuron eliminates some of its axonal inputs to leave the appropriate number of inputs. Meanwhile, the remaining inputs develop more synapses with the postsynaptic cell (Smolen and Raismen, 1980; Glebova and Ginty, 2005).

1.5.1 Post-ganglionic cell development

In embryo, the sympathetic nervous system originates from the thoracolumbar region of the neural crest. These precursor cells migrate together at embryonic day 12 and shape distinct prevertebral and paravertebral ganglia as they proliferate and differentiate (Smolen, 1988). These cells receive innervation from the developing spinal cord as early as E12, and have recordable synaptic transmission from pre-ganglionic neurons in multiple spinal segments by E14. After innervation starts, synapse numbers on SCG neurons increase until birth (Rubin, 1985b). Post-synaptic sympathetic neurons begin to extend axons as soon as they are differentiated, at E12. Sympathetic axons follow arterial vasculature and begin to reach their targets by E15 (Rubin, 1985c).

About one-third of differentiated SCG neurons die in development - their survival is dependent on the retrograde signal of neurotrophic factors from their targets (Wright, et al.,

1983; Levi-Montalcini, 1987). Specifically, target tissues synthesize nerve growth factor (NGF), which binds to the TrkA receptor on post-ganglionic axon terminals (Oppenheim, 1991). The neuron internalizes the NGF-TrkA complex in a signaling endosome and transports it back to the cell body, where it inhibits pro-apoptotic signals (Riccio, et al., 1997; Riccio, et al., 1999). The NGF survival signal is crucial (NGF knockouts have no observable sympathetic ganglia by postnatal day 14) (Crowley, 1994; Glebova and Ginty, 2005).

Beyond sympathetic neuron survival, NGF mediates post-ganglionic innervation of sympathetic effector organs. Target tissues that express relatively high levels of NGF are more densely innervated than tissues that expressed low NGF levels. This correlation was demonstrated in multiple sympathetic-innervated organs in several mammalian models. The level of NGF expressed by a sympathetic target organ may limit the number of synapses to the target (Shelton and Reichardt, 1984). If the degree of target innervation is mediated by the retrograde signal of a neurotrophic factor, perhaps a similar mechanism governs the innervation of the SCG itself.

1.5.2 Pre-ganglionic axon extension and synapse formation

By E13, initial contacts are made by rostral pre-ganglion neurons (from the T1 segment), but are followed soon after by more caudal segments (T4) (Rubin, 1985a). At E13, Rubin recorded compound action potentials in the internal carotid nerve, the efferent nerve of the SCG, with a suction electrode after stimulating the afferent cervical sympathetic trunk. This indicates synapses are assembled quickly after initial contact. At E14, stimulating different spinal segments (C8 to T4) evokes compound action potentials in the internal carotid nerve. Each segment elicits compound action potentials of different amplitudes, with T1 eliciting the greatest

response. If the amplitude of the response represents the degree of innervation, the embryonic SCG innervation is of the same proportion as the adult and may resemble the mature topographical map (Rubin, 1985b; Nja and Purves, 1977).

1.5.3 SCG dendrite outgrowth

Regulation of dendrite growth in SCG neurons is necessary to maintain the appropriate amount of pre-ganglionic input to provide sufficient activation of the post-ganglionic target. In the context of input elimination, dendritic elaboration is important as it predicts the final number of inputs an autonomic neuron receives. Specifically, ganglion cells with large dendritic arbors receive more synaptic inputs (Hume and Purves, 1981). This topic will be discussed further in section 1.5.4, p. 22. (Hume and Purves, 1981; Purves and Hume, 1981; Glebova and Ginty, 2005). Research has uncovered several details underlying the regulation of dendritic complexity. Voyvodic stained SCG axon terminals innervating the submandibular gland. When he ligated the submandibular duct, which caused the gland to atrophy, he saw an decrease in the size of SCG cells' dendritic arbors. Conversely, when he partially denervated the gland, increasing the relative size of the target for the remaining inputs, the size and complexity of the innervating SCG cell's dendrites increased. Voyvodic proposed that these changes were mediated by decreased and increased retrograde signaling of a neurotrophin relative to controls (Voyvodic, 1989).

In support of this finding, when the postsynaptic cell is completely disconnected from its target by axotomy, its dendritic tree loses 60-70% of its length and complexity (Yawo, 1987). When researchers systemically inject NGF into neonatal mice, they see an increase in the length and complexity of dendrites in the neurons of the SCG (Snider, 1988). Similarly, adult mice systemically injected with NGF show an increase in dendritic length and complexity where mice

injected with NGF-antiserum show a decrease in dendritic length (Ruit, et al., 1990). In normal conditions, target-derived NGF signaling does not initiate dendrite outgrowth, however, as neurons develop dendrites before they reach their targets. Instead NGF causes existing dendrites to grow and branch (Ruit et al., 1990; Rubin, 1985c).

Another potential player in dendrite elaboration is activity. Research by Vaillant et al. demonstrated the synergistic effect of neuron depolarization and NGF signaling on neuron survival in culture (Vaillant, et al., 1999). The group extended their study to investigate the role of NGF on dendrite elaboration. The researchers cultured neonatal SCG neurons first in a medium with NGF, and then switched to a medium with elevated potassium (K^+) to depolarize neurons and mimic activity. The cultured neurons did not grow dendrites in media with NGF alone. When researchers added K^+ to the standard media, the neurons grew robust dendritic arbors. In media with both NGF and K^+, dendritic arbors were larger and more complex than in high-K^+ medium. The group showed these two factors mediated dendrite growth through microtubule-associated protein 2 (MAP2), which stabilizes microtubules. NGF and depolarization increased levels of MAP2 as well as phosphorylation of MAP2. To determine whether phosphorylation of MAP2 was initially caused by depolarization, they investigated the role of calcium/calmodulin-dependent kinases II (CaMK II), which is activated in depolarization by calcium influx into the neuron. They also investigated MAP2 phosphorylation through NGF-TrkA signaling, which the group thought was enhanced by the mitogen-activated protein kinase ERK (Vaillant, et al., 2002). ERK itself is activated by depolarization through calcium-activated Ras signaling (Vaillant et al., 1999) By inhibiting these CaMKII and ERK, the group prevented microtubule stabilization and dendrite outgrowth in NGF, K^+ media. NGF-TrkA signaling promotes the growth of dendrites, but its signal depends on activity in the SCG cell (Vaillant et

al., 2002). This study elegantly demonstrates the synergistic role of NGF and activity in dendrite elaboration.

In contrast to these studies, Voyvodic observed the development of dendrites in SCG neurons after they were denervated at P1. Interestingly, Voyvodic sees no difference in the growth of dendrites in the denervated SCG neurons in the first postnatal month, which would receive no postnatal activity. After the first month, dendritic growth is slightly slower in the denervated SCG, implying that the role of activity in dendrite elaboration is minor (Voyvodic et al., 1987). Because Vaillant and colleagues investigated dendrite outgrowth in culture, it is difficult to say if their results reflect *in vivo* dendritic regulation. If dendritic growth is not dependent on postnatal activity, is it possible that only the initiation of dendritic growth depends on activity? Or is it possible that the role of activity and NGF on dendritic elaboration are not synergistic, but redundant?

It is interesting that SCG cells start growing dendrites before post-ganglionic axons reach their targets (Rubin, 1985c). If activity initiates dendrite growth, we may assume that initial contacts made by pre-ganglionic inputs onto SCG neurons at E14 have a low level of synaptic transmission (Vaillant et al., 1999; Rubin, 1985a). In a step-by-step explanation, at E13, when pre-ganglionic axons have only begun to reach the SCG, there are no SCG dendrites. At E14 when synapses have established enough functional connections to elicit compound action potentials, dendrites begin to grow (Rubin, 1985b; Rubin 1985c). Whether a low level of prenatal postsynaptic activity affects input elimination to the SCG is unknown, but could be investigated in the embryos of α3 knockout mice, which would lack this activity.

In the developing postnatal circuit, dendrites are the preferred site of input innervation and larger dendritic arbors allow for more persistent inputs to an adult SCG neuron (Forehand, et

al., 1985). Dendrite outgrowth in the SCG is mediated by upstream and downstream factors. Downstream, target-derived neurotrophic factors and upstream, synaptic transmission from pre-ganglionic cells both influence dendritic growth (Ruit, et al., 1990; Vaillant, et al., 1999; Vaillant, et al., 2002). Dendrites may be a coordinating factor to match the number of ganglionic inputs to the needs of targets. For example, a large target that requires strong sympathetic activity from an SCG cell secretes a high level NGF retrogradely. Presynaptic inputs depolarize this SCG cell, which in concert with the NGF signal, causes dendritic elaboration. This SCG cell, with an expansive dendritic arbor can then support several pre-ganglionic inputs, which causes frequent firing and sufficient sympathetic drive to the target organ. If this model is accurate, then what effect do dendrites have on input elimination?

1.5.4 Input elimination

Input elimination has been studied extensively in the neuromuscular junction, the cerebellum and the visual system. In all of these systems, postsynaptic cells are innervated with more axonal inputs at birth than in adulthood. In the NMJ and the visual system, manipulation or blockade of postnatal activity impairs or alters normal input elimination, highlighting the importance of activity in the developmental motif (Sanes and Lichtman, 1999; Katz and Crowley, 2002). Although no experimental study in the autonomic nervous system has blocked postnatal activity, input elimination has been well-documented in wild-type sympathetic and parasympathetic nervous systems. This is because researchers can easily count the number of synaptic inputs. To do this, researchers dissect ganglia, and stimulate the pre-ganglionic nerve through a suction electrode, gradually increasing the current injected into the nerve. As the injected current reaches the stimulation thresholds of different inputs, discrete EPSP amplitudes

are recorded by intracellular electrodes in post-ganglionic cells. Like in the NMJ and cerebellum, the number of discrete EPSP amplitudes represents the number of inputs to the cell. In the parasympathetic nervous system, a neonatal rat submandibular ganglion cell receives an average of 4.7 axonal inputs, whereas a mature ganglion receives 1.3 (Lichtman, 1977). In the rabbit, ciliary ganglion cells have 4.6 inputs from the optic nerve at birth, which is refined to 2.2 in a mature animal (Johnson and Purves, 1981). In the sympathetic nervous system, the hamster superior cervical ganglion cells receive an average of 11-12 axons at birth and 6-7 in adulthood (Lichtman and Purves, 1979). Although the final number of inputs innervating autonomic ganglion cells is variable between species (Purves et al., 1988) we can see that there is a clear motif of input elimination. The exact input numbers in the input elimination in the mouse SCG have not been studied to my knowledge.

The sympathetic nervous system provides quantitative electrophysiological evidence that input elimination can result in a circuit with multiple innervation at maturity. The NMJ and cerebellum show single innervation after elimination by motor axons and climbing fibers, respectively (Sanes and Lichtman, 1999; Hashimoto and Kano, 2005). Quantitative evidence of input elimination in the adult sympathetic circuit expanded the role of developmental elimination that was proposed in earlier studies. Because input elimination occurs in sympathetic circuits, where mature neurons are multiply-innervated, these experiments show that input elimination is not restricted to systems that have only one mature, persistent input. Instead, it is likely a general characteristic of nervous development (Lichtman and Purves, 1979). Purves and Hume investigated the nature of the multiply-innervated adult autonomic neuron. As alluded to earlier, adult ganglion cells with large dendritic arbors can maintain multiple innervation. The group saw that ganglion cells with small or no dendritic arbors have only one input (Purves and Hume,

1981). Furthermore, multiple pre-ganglionic inputs make their synapses in distinct regions of the arbors of multiply-innervated ganglion cells. The authors assert that a larger distance between inputs diminishes the role of competition between them and allows multi-innervation (Forehand and Purves, 1984). Though the complexity of dendrites seems to be an important regulator of input elimination in the SCG, whether or not this is a universal factor in input elimination will be considered in the discussion section of my thesis.

1.5.5 Dendrite growth in input elimination

Purves and his colleagues have suggested that dendrites are preferred synaptic sites in the SCG in neurons that have complex dendritic arbors. Only SCG cells with relatively few dendrites have a significant number of synapses on their cell bodies. Electron microscopy shows that only 8.5% of synapses are formed on the soma of mouse SCG neurons. Dendritic complexity governs the number of synapses an SCG neuron can support: neurons with more elaborate dendritic arbors have more synapses than those with small arbors (Forehand, 1985).

The correlation between the number of inputs and dendritic complexity that is observed in mature cells does not exist in the neonatal SCG cell. SCG cells are multiply-innervated before they elaborate their dendrites (Purves and Lichtman, 1980; Rubin, 1985c). As dendrites develop, the proportion of synapses found on the cell body decreases. Through development, dendritic area increases dramatically, whereas cell body surface area only increases modestly (Smolen and Beaston-Wimmer, 1984). 90% of adult synapses are created within the first postnatal month, though very few of the new postnatal synapses are made on the cell body (Smolen and Raisman, 1980; Rubin, 1985c). A simple idea may be that larger postsynaptic cells, with large dendrites, simply provide more space for synapses from the pre-ganglionic axons. This idea conflicts with

the fact that inputs are being eliminated through early life. If dendrites are growing, why would the number of inputs be decreasing? There must be another factor involved.

Between synaptic formation at E14 and birth, innervation on SCG neurons is maintained on the cell bodies. Through postnatal development, presynaptic inputs form new synapses and postsynaptic dendritic elaboration occur at the same time. Axons preferentially form new synapses on new dendrites, but axons can only innervate dendrites growing close to their initial synapses. (Purves et al., 1985). At the same time, inputs are being eliminated, theoretically through competition. In the theory of competition, competitive factors do not affect synapses from the same axon, only synapses from other pre-ganglionic inputs. Because the different inputs from different pre-ganglionic cells are likely very similar qualitatively, Purves and his colleagues predict that synapses are grouped together by their synchronous activity and competition would begin at birth when inputs became active. In the immature animal, many converging axosomatic synapses could exist. At birth, asynchronous activity among the groups of synapses initiates competition and competitive destabilization of inputs. Some persistent inputs are able to form new synapses on newly formed dendrites, while their cell body synapses are destabilized and eliminated (Forehand, 1985; Forehand, 1987). In summary, the group asserts that input elimination begins at birth, when competition begins between asynchronous inputs. The postsynaptic cell is the mediating player between inputs in this model.

1.5.6 The role of activity in input elimination in the sympathetic nervous system

In an embryonic mouse, there is not strong activity in the sympathetic nervous system, as homeostatic control is maintained by the mother, activity begins at birth (Purves and Lichtman, 1980). Research on input elimination in the sympathetic nervous system suggests that it occurs

when the neonatal sympathetic nervous system becomes active (Forehand, 1985). To my knowledge, there are no studies that have manipulated either pre- or postsynaptic activity in the sympathetic nervous system to support this claim. The novel α3 knockout could be used to investigation this theory.

1.5.7 Factors involved in synaptic maturation, maintenance and competitive elimination

To better understand how the postsynaptic cell might cause destabilization or elimination, we may look to how synapses are normally maintained. A large body of evidence comes from the NMJ. At birth, the NMJ is innervated by up to eleven axons, but many of the inputs 'die-off' within the first postnatal days (Tapia, et al., 2012). By P6, only half of the muscle fibers are multiply innervated (Balice-Gordon and Lichtman, 1993). Most studies investigate muscle fibers over a period when the muscle fiber receives inputs from two or three neurons. The multiple inputs share an intermingled contact with the muscle on the plaque-like postsynaptic apparatus (Wyatt and Balice-Gordon, 2003). Over the first two postnatal weeks, in fibers with two inputs, one evokes progressively stronger end-plate potentials (EPPs) as its postsynaptic apparatus on the muscle expands, while the other is weakened and eliminated (Colman and Lichtman, 1997; Gan and Lichtman, 1998). At the synapse of the to-be-eliminated synapse, nAChRs are lost first, leaving the axon terminal hovering over the muscle fiber. The axon terminal then disappears from the former synapse (Balice-Gordon, 1993a).

Experiments in the NMJ show that when activity is inhibited pre- or postsynaptically, input elimination does not occur and multiple innervation persists (Thompson, et al., 1979; Favero, et al., 2009). Balice-Gordon and Lichtman blocked a fraction of postsynaptic receptors on single immature NMJs with multiple-innervation, causing the retraction of the input above the

blockade. When they blocked all of the postsynaptic receptors, the corresponding input was not eliminated (Balice-Gordon and Lichtman, 1994). Buffelli et al. were able to differentially change the activity of motor axon inputs to muscle fibers. By postnatally administering a 'low dose' of tamoxifen to mice with a conditional knockout ChAT, researchers were able to removed the ChAT gene through CRE-recombinase in a 20% minority of motor axons, knocking out ChAT activity while expressing yellow fluorescence protein at the same time. Without ChAT activity, axonal inputs to the NMJ were silenced. When two silent inputs innervated the same muscle fiber, multiple-innervation persisted. When a silent and active input both innervated the same fiber, the active fiber occupied more territory and had a thicker axonal caliber. Because inputs were silenced after several postnatal days, some fibers were already singly-innervated, either by active or silent inputs. The authors explain that activity would only mediate development competition, but not maintenance of the 'winning' input. When a high dose of tamoxifen silences many inputs, multiple innervation persists in the innervated muscle fibers (Buffelli, et al. 2003). These studies of the NMJ have directly explored the role of activity in competition. When some neural activity is blocked, silent inputs are eliminated. When all activity is blocked, all inputs persist. This is the basis for the model of activity-driven competition: an inactive axon will retract only when an active axon is present.

Other studies suggest that when competing axonal inputs to the NMJ are stimulated synchronously, input elimination does not occurs. When the same stimulation frequency is asynchronous in the inputs, only one input persists. The 'winner' is distinct from the 'loser' based on its level of activity. When activity in the two inputs is identical, no losing input can be eliminated (Busetto, et al., 2000; Wyatt and Balice-Gordon, 2003).

In a recent study of the NMJ, McCann and colleagues showed the elimination of motor neuron input to the NMJ within 12 hours of inhibiting protein synthesis in the muscle. This process does not directly relate to competition in input elimination, as inhibiting protein synthesis in muscle fibers causes the elimination of all innervating inputs. The group proposes that a constitutively expressed factor maintains inputs at the NMJ and a similar factor may be involved in input elimination (McCann et al., 2007). Whether this is true or not, target-derived NGF increases the number of synapses the target organ receives, so perhaps an analogous SCG neuron-derived signal mediates the number of inputs it receives.

The NMJ is a classic model of input elimination, and the dynamics of activity-mediated competition have been studied in great detail. However, the NMJ is highly-specialized and quite distinct from neuron-to-neuron synapses. Further studies of neuron-to-neuron connection may help determine which components of input elimination in the NMJ are universal to circuit development and which are specific to neuron-to-muscle circuits.

1.5.7.1 Possible factors involved in maintenance or elimination

BDNF is released by SCG neurons to bind to its receptor, TrkB, on pre-ganglionic cells. The retrograde BDNF/TrkB signal causes hypertrophy of the pre-ganglionic cells and increases the number of synapses formed with their post-ganglionic partners. When researchers increase BDNF levels in transgenic mice, they observe larger pre-ganglionic cells that innervate post-ganglionic neurons to a greater degree compared to normal mice. BDNF, however, does not increase the number of axons that form connections with SCG neurons. In BDNF knock-out, both size and synaptic innervation density are decreased (Causing, et al., 1997). NT-4 also exerts a proliferative role on pre-ganglionic cells: adult NT-4 knockout mice have about 25% fewer pre-

ganglionic cells, axons and synapses on the SCG, but neonatal mice are unaffected (Roosen, et al., 2001). BDNF and NT-4 have both been suggested as retrograde supportive factors that might be expressed by a postsynaptic cell to stabilize inputs at the NMJ (Sanes and Lichtman, 1999). BDNF also matures visual circuitry through mediation of developmental input elimination (Hensch et al., 1998) although these roles for BDNF may be quite different between these two models. Though the identity of a retrograde signal is uncertain, research on the NMJ and SCG suggests that it depends crucially on postsynaptic activity.

1.6 Summary of rationale and research questions

The α3 knockout model presents an opportunity to study input elimination in a post-synaptically silent neuron-to-neuron circuit. In these knockout mice, morphological synapses exist after a month of life. Further, our lab has observed the same number of synapses in knockout and wild-type animals through the first month of life (Krishnaswamy and Cooper, 2009). Unfortunately, we cannot determine the number of axonal inputs using electrophysiology without postsynaptic activity. We need to introduce activity for a short enough period to record the number of inputs. At the same time, we cannot cause input elimination. To test the number of inputs in the knockout, the animal would live without postsynaptic activity until past the normal time course for input elimination in the mouse SCG, about 21 days. After P21, I can restore postsynaptic activity to the SCG with the Synα3 virus (Krishnaswamy and Cooper, 2009). Within two days of infection, I can then count the number of inputs intracellular recordings in the Synα3-infected α3 knockouts.

The α3 knockout presents a novel way to investigate circuit development in the absence of postsynaptic activity. Other models where postsynaptic receptors have been knocked out or

activity has been blocked were not appropriate to study input elimination. In agrin knockout mice, for instance, all AChRs disappear from the NMJ. The agrin knockout, however, is lethal as it eliminates all nAChRs and prevents respiration (Gingras et al. 2002). In non-genetic manipulations, postsynaptic activity has been blocked in several ways in the NMJ, but generalizations from this highly-specific synapse may not apply to neuronal synapses (Sanes and Lichtman, 1999). Outside of AChRs, other postsynaptic receptors have also been inactivated by researchers. For instance, Reiter and colleagues temporarily inactivated the visual cortex by applying muscimol to inhibit action potentials in cortical neurons in month-old kittens. In this study, the researchers suggest certain limitations. The block may occur after input elimination is initiated, action potentials were not completely blocked in the visual cortex and that presynaptic activity may be affected by cortico-subcortical feedback (Reiter, et al, 1986; Reiter and Stryker, 1988). In contrast, the α3 knockout presents a distinct model: a non-lethal, mono-synaptic, neuron-to-neuron circuit that lacks postsynaptic activity from synapse formation in embryogenesis.

The α3 knockout eliminates all excitatory fast synaptic transmission in the sympathetic nervous system, but is not lethal (Rassadi, et al., 2005). It has been possible to eliminate other post-synaptic receptors, for modulating inhibitory and non-essential excitatory (NMDARs) neurotransmitters (Holmes et al, 2004; Mihalek et al, 2001; Li et al, 1994). Systemically eliminating all excitatory glutamate or acetylcholine transmission would likely result in non-viable model. It might be possible to conditionally knockout excitatory synaptic transmission to non-crucial brain areas, but this type of model would require a long list of genetic manipulations to eliminate all of the subtypes of excitatory receptors in the brain circuit.

Finally, the sympathetic nervous system is ideal because there is already a significant amount of evidence from dendritic innervation studies and dendritic outgrowth studies implicating the role of activity for input elimination in the SCG. There is already evidence about blocking activity in the NMJ and some qualitative evidence from the visual system. The α3 knockout, as outlined above, provides a postsynaptically silent and viable neuron-to-neuron model.

1.6.1 The role of postsynaptic activity in input elimination in the sympathetic nervous system

In my master's project I addressed the following questions:

1. How many axons innervate a wild-type mouse SCG in adulthood?
2. How many axons innervate a wild-type mouse at birth, and, if the number is different from the adult, is there a transition? Does the strength of the inputs change during development?
3. Will the absence of activity in α3 KO mice affect the normal axonal refinement in the SCG?

1.6.2 Experimental Approach

It was important first to quantify what axonal input elimination looks like in the mouse SCG. I used three age categories to quantify the progression of input elimination, P1-3, P7-9 and P21+. I recorded intracellularly from SCG neurons while I gradually increased current injected through the pre-ganglionic nerve with a suction electrode. Increasing the current through the pre-ganglionic nerve showed an addition of discrete 'steps' in the recorded EPSP. This is to say, as stimulus strength increased, the neuron was depolarized in distinct, cumulative EPSPs until the maximal response was reached. The discrete step-increases in the cell recording reflect the number of inputs to the SCG neuron. After isolating and counting the number of inputs, I

analyzed the amplitude of each discrete EPSP step to assess the disparity between signal strength, to verify if or how inputs change in strength through development.

To measure the number of axonal inputs in postsynaptically silent SCGs, I rescued synaptic activity in α3 knockout mice by infecting them with the Synα3 adenovirus. I grew several batches of Synα3 adenovirus in cultured HEK 293 cells, in which the virus can replicate. I titered the adenovirus stocks using the cytopathic method, again in HEK 293 cells, to control the amount of virus injected into the knockout animals. After intravenously injecting the knockouts with the adenovirus, it was important to record from neurons with little delay to ensure that rescued activity did not cause input elimination. The virus shows expression after 24 hours, so I recorded between 24 and 48 hours after injection, the timing will be further discussed in the discussion section on page 53. If activity was rescued, it was immediately evident by measuring the compound action potential from the entire SCG. Input numbers were recorded using the same intracellular protocol as the wild-type animals. With restored activity, I was able to quantify the number of inputs connecting to a silent SCG as well as their strength and relative contribution to the maximal evoked EPSP.

2. METHODS

2.1 Mice

An outcrossed colony of a3 knockout mice was generated by breeding heterozygous a3 +/- males from an inbred C57BL/6 strain (from Dr A. Beaudet) with females from a CD1 strain (Charles River, St. Constant, Québec)(Krishnaswamy and Cooper, 2009). The resulting outcrossed heterozygotes bred to yield wild-type (a3 +/+), heterozygotes (a3 +/-) with wild-type phenotype, and knockout (a3 -/-) populations. All genotyping of mice was done by PCR (Xu et al., 1999). Wild-type mice were sacrificed in three age groups, 'P1-3', 'P7-9' and 'P21 and older'. Homozygous a3 knockout mice were IV injected with virus at P21 or later and sacrificed one or two days after injection. All experiments used the superior cervical ganglia of these mice.

2.2 Electrophysiology

Mouse SCGs were rapidly dissected in oxygenated Ringer's solution. SCGs were removed from both sides of subject animals within 20 minutes of sacrifice. To do this, mice under the age of P7 were stunned and then decapitated, older mice were anesthetized in a cage filled with carbon dioxide and then decapitated. The head, ventral side up, was placed and secured with needles in a dissection dish, perfused with oxygenated Ringer's solution. Using fine-tipped scissors and forceps, the skin, muscle layer and trachea were removed. Then I located the carotid artery and the SCG, which is found near the carotid bifurcation. The SCGs, with both the pre- and post-ganglionic nerves intact, were dissected from surrounding tissue in the neck and placed in a small Sylgard-coated recording dish containing 1mL of oxygenated Ringer's solution perfusion. The connected tissue of the carotid artery, on which the ganglion sits, was

used to pin the preparation down with minutien pins, avoiding damage to the ganglion. Tissue around the nerves was carefully removed to ensure a good seal for suction electrodes in the recording bath. The recording dish was mounted on the stage of a dissecting microscope (Nikon, Tokyo, Japan) and perfused with Ringer's solution maintained at 35-37°C at 3mL/min.

Extracellular Recordings

The cervical sympathetic trunk was suctioned with a glass electrode connected to a stimulator (365R Isolator, World Precision Instruments, Sarasota, FL). The post-ganglionic nerve was suctioned with a glass electrode connected to a differential amplifier (DP-301, Warner Instrument Corp, Hamden, CT). To measure compound action potentials in SCGs, the pre-ganglionic nerve was stimulated and the output from the recording electrode amplified (by 1000x) and then digitized by an input/output NAT NI-PCI-6259 data acquisition card (National Instruments, Austin, TX) and filtered between 10Hz and 3kHz, recorded, displayed and analyzed off-line on a Pentium II-based computer using Igor (Wavemetrics, Lake Oswego, OR) (Krishnaswamy and Cooper, 2009).

Intracellular Recordings

To count the number of inputs to SCG cells, the preganglionic nerve of the dissected SCG preparation was held in a suction electrode. The electrode was connected to the stimulator (World Precision Instruments) and the pre-ganglionic nerve was stimulated with depolarizing current at 0.8-1 Hz through an input/output data acquisition card (NI-PCI-6259, National Instruments) and Igor software (Wavemetrics). To record intracellularly, I used sharp glass microelectrodes (G150-F, Warner Instruments), filled with 1M potassium acetate, with

resistances between 70-100 MΩ that were pulled by a DMZ puller (Zeitz Instruments, Munich, Germany). A high inertial precision microdrive (Inchworm 8200, EXFO, Vanier, QC) held by a micromanipulator (Narshige, Tokyo, Japan) held the sharp electrode over the SCG preparation. To record from individual neurons, the microdrive drove the electrode down into the SCG to pierce and stably hold the electrode in individual neurons. Membrane potentials from individual neurons were recorded by electrodes connected by a silver-chlorided wire to the head stage of an amplifier (Axoclamp 2A, Axon Instrument, Union City, CT) set to current clamp. Membrane potential was digitized with a data acquisition card (National Instruments) and sampled at 10 kHz recorded and analyzed offline on a Pentium II-based computer with Igor (Wavemetrics) (Krishnaswamy and Cooper, 2009).

SCG neurons were found by passing a small hyperpolarizing current train through the recording electrode. When the electrode reached a neuron, the resistance through the electrode increased. Once the electrode impaled the neuron, I stopped the current train. Then, the neuron was directly depolarized through the intracellular electrode to fire an action potential to verify the integrity of the neuron. This depolarization also served as a control in knockout SCG neurons. A direct action potential shows that a neuron is being recorded from, even if there is no evoked postsynaptic response. Neurons with potentials greater than -60mV were injected with a hyperpolarizing current via the amplifier to prevent them from firing APs during stimulation. (APs obscure the measurement of discrete steps in EPSPs.)

When the electrode pierced a neuron and the recording was stable, represented by a stable recorded membrane potential, I began to inject current. Gradually, I increased the stimulus strength to the pre-ganglionic nerve through the suction electrode. I recorded evoked EPSPs with the intracellular electrode. Discrete steps in the evoked EPSP were counted by carefully

increasing current in the pre-ganglionic nerve to the first EPSP threshold, where the injected current evokes an EPSP in about 50% of recordings. This EPSP represents the response from the first recruited axonal input. After this point, 100 recordings of the oscillating EPSP/no-response membrane potential were taken. After the first EPSP was recorded, the stimulus was increased and a second 100 recordings were taken at the second threshold, where current evokes the first EPSP in 50% of the recordings and a second, larger EPSP amplitude in the other 50%. This represented the first and second inputs, respectively. This protocol was continued until maximal EPSP response was recorded, after which increases in stimulus do not elicit changes in EPSP amplitude.

Measurements in neonatal animals were difficult because of the small size of SCG neurons. To record from these cells, we used very fine intracellular electrodes with resistances of 100-130 MΩ. Current thresholds in P1-3 animals were close together, and sometime recordings oscillated between three or four distinct EPSP amplitudes.

2.3 Adenovirus

The Syna3 adenovirus is a replication-deficient virus containing full-length neuronal nAChR a3 sub-unit cDNA ligated to the synapsin 1 promoter. Stocks of the replication-deficient adenovirus were generated from the existing Syna3 adenovirus (Krishnaswamy and Cooper, 2009). Existing virus was amplified in colonies of HEK 293 cells, which contain the necessary components for the virus to replicate (He et al, 1998). Purified viral stocks were titered in duplicate with cytopathic method (Nyberg-Hoffman, et al. 1997) to yield titers between 0.78-1.912 $x10^{10}$ pfu/mL. Purified virus was injected intravenously to the tail vein of adult mice (P21 and older) at 0.001-50 μL in dilutions of 150 μL of L15 or PBS for a final adenoviral

concentrations between 10^4 pfu and 10^{10} pfu. Adenoviral concentrations were adjusted in between attempted viral infections to try to restore wild-type-level postsynaptic activity in the knockout SCGs. Dexamethasone, an immunosuppressant, was added to some injections at 2 μg/kg with no effect on viral expression in the animal (Xu, et al., 2000). Knockout animals injected with adenovirus were sacrificed 24-48hr after injection.

2.4 Analysis of disparity between inputs

Disparity index, modified from Hashimoto and Kano, 2003, was calculated to measure the relative difference between input strength of multiple inputs (Hashimoto and Kano, 2003). This was calculated by subtracting the amplitude of an input from the next most depolarized input. The difference was then divided by amplitude of the maximum evoked response to yield a percentage of contribution. These percentages were then averaged together to give the disparity ratio.

If one input is dominant, the difference in amplitude between it and the preceding input will be great. When the difference is divided by the maximum response amplitude, it will be close to 1. If an input contributes relatively little, there will be a small difference between it and the preceding input. When divided by the maximum response amplitude, the percentage will be close to 0. Therefore, disparity ratios closer to 1 represent cells with relatively dominant inputs, those close to 0 represent relatively small inputs.

The equation for the calculation is: $D.I. = [(A_2 - A_1) + (A_3 - A_2) + ... (A_N - A_{N-1)})/A_N] / (N-1)$

3. RESULTS

3.1 Normal input elimination in the wild-type mouse SCG

We first needed to characterize normal axonal refinement in the mouse SCG. To measure the number of inputs to a SCG neuron, I gradually increased presynaptic current while recording membrane potential with a sharp intracellular electrode. To count inputs, I isolated discrete summative responses that compose a maximal EPSP. Most neurons in adult wild-type mouse (P21 and older) SCGs were depolarized by several inputs. Recordings showed 1-5 discrete steps. The mean number of inputs innervating a wild-type adult SCG neuron was 3.25 (Figure 3.1, A; n=36).

I used the same procedure on younger animals (P1-3) and observed that neonatal SCGs are innervated by many more inputs. The thresholds of the different inputs were also very close together: even when pre-ganglionic stimulus current was held constant, the recording showed up to 3 discrete depolarizations. Neonatal SCG neurons were innervated by 4-10 presynaptic axons; the mean number of inputs to P1-3 SCG neurons was 7.41 (Figure 3.1, C; n=39).

This experiment was repeated in an intermediate age group (P7-9) to see whether there was sudden shift in innervation pattern right after P3 or right before P21. P7-9 SCG neurons were innervated by 1-9 inputs, with a mean of 5.14 inputs (Figure 3.1, B; n=41). Because the range was so large, it seemed some SCG neurons had likely already completed input elimination and had only a few inputs, while others may not have begun input elimination, and still maintained 7 to 9 immature inputs.

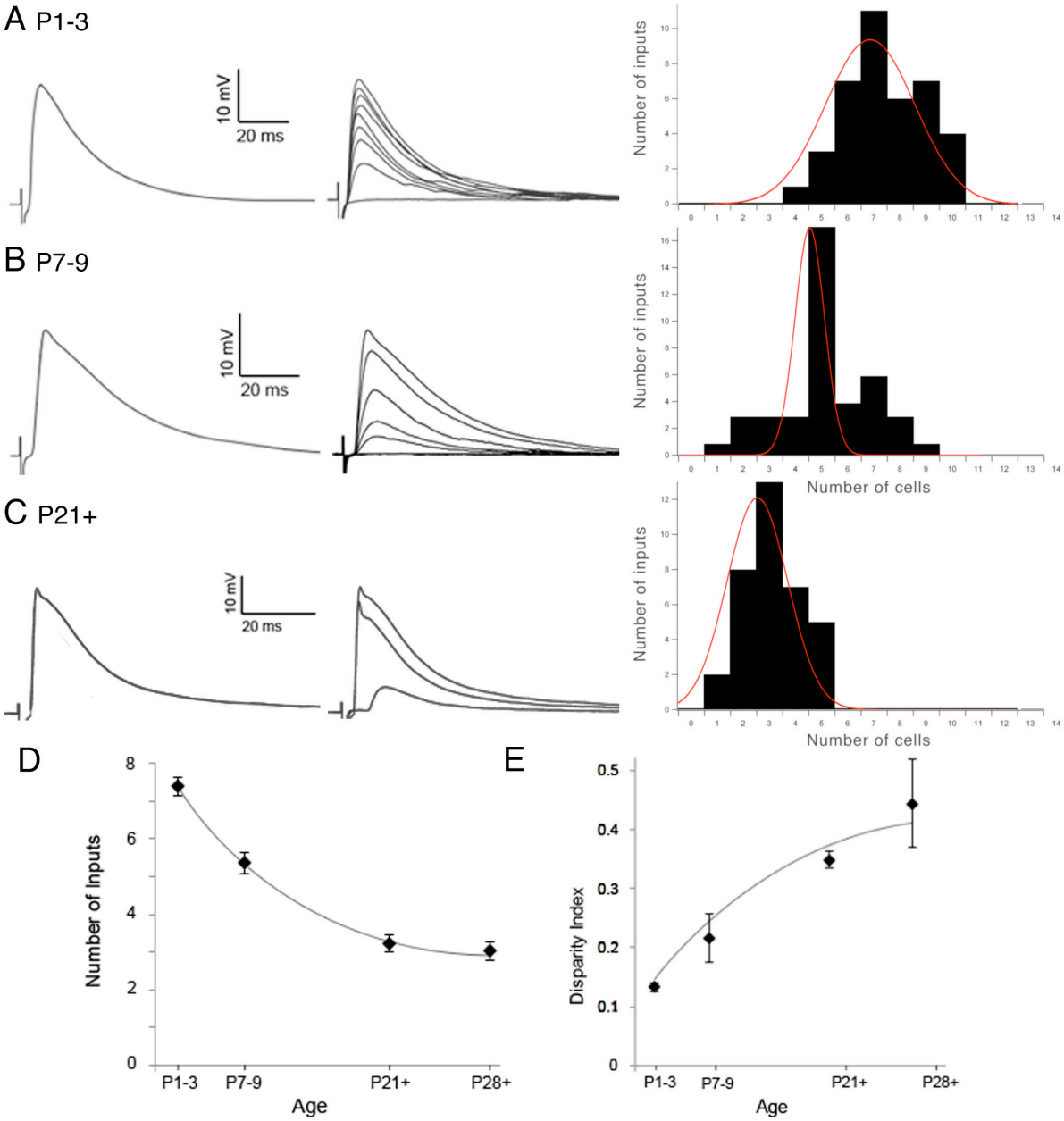
A P1-3
10 mV
20 ms
Number of inputs
B P7-9
10 mV
20 ms
Number of inputs
Number of cells
C P21+
10 mV
20 ms
Number of inputs
Number of cells
D
Number of Inputs
P1-3
P7-9
P21+
P28+
Age
E
Disparity Index
P1-3
P7-9
P21+
P28+
Age

Figure 3.1 *Input elimination in wild-type mouse SCG.* **A, B and C** SCG EPSPs evoked by a current injected through the cervical sympathetic trunk represent the postsynaptic response to synaptic transmission from several inputs. These inputs were counted by gradually increasing stimulation to the excitation threshold of the inputs one-by-one. The different EPSP steps are shown in composite traces in the second column, and histograms of the number of inputs received by SCGs are shown in the third column. (A) SCG neurons in neonatal mice are typically innervated by many inputs of similar strengths (7.41 inputs; n=39). (B) SCG neurons in week-old mice receive fewer inputs than newborns (5.14 inputs; n=41), and some inputs begin to contribute dominantly to the maximal EPSP. (C) By adulthood, SCG neurons are innervated by only a few inputs (3.25 inputs; n=36) and maximal EPSP are made of one or two dominant contributions and one or more weak contributions. **D** To investigate the trend in input elimination through development, I created another data point (P28+; n=16) from data collected from older mice in the P21+ group. Input elimination occurs quickly perinatally and inputs numbers are stable after postnatal day 21. **E** Disparity Index compares the strength of constituent EPSP amplitudes to each other, relative to the maximal EPSP amplitude. This number does not reflect the number of inputs. The DI of the experimental groups increases through P1–3 (0.144), P7–9 (0.217), P21+(0.343) to P28+ (0.445). Over the developmental period of input elimination, SCG cells are primarily driven by one or two strong inputs. Data points for P1, P7–9 and P21+ represent the mean of 36–41 experiments (P28+ is a subset of P21+, with 16 experiments) and error bars represent SEM.

These results showed a distinct difference between the number of inputs that innervate an adult and a neonatal SCG neuron and a gradual elimination of inputs in development. To observe if there was a trend in input elimination, I analyzed an older sub-group of P21+ neurons, which were recorded from wild-type animals between P28 and 2 months. The developmental pattern was hyperbolic, with input numbers dropping quickly soon after birth and stabilizing at around 3 inputs after P21 (Figure 3.1, D). Though the number of inputs to the adult and neonatal SCG neuron varied, there was little overlap in the range of possible input numbers. It was rare to find an adult SCG neuron with more than 4 inputs and conversely, it was rare to find a neonatal SCG neuron with fewer than 4 inputs.

3.2 Dominant inputs emerge through input elimination

To assess whether some inputs were dominant, I analyzed the relative contribution of the inputs in each age group. To do this, I used a disparity index, based on Hashimoto and Kano's analysis in the cerebellar input elimination, to show dominance of developmental inputs in the SCG (Hashimoto and Kano, 2005). I compared the differences between consecutive EPSP amplitudes in respect to the maximum EPSP. In the adult mouse, the disparity index was high: it was common to see one or two inputs contribute large components of the maximum EPSP while two or more other inputs contribute relatively little. In the neonatal animal, the disparity index was low, indicating that there was little difference between the contributions of any of the inputs. These recordings from young animals generally consisted of many small, similarly sized inputs. The disparity index of the intermediate age group falls between the adult and newborn. These

animals showed both small similarly-sized inputs and sometimes showed dominant inputs (Figure 3.1, E).

3.3 Restoring activity to silent synapses

We were able to restore activity to the α3 knockout mice by injecting a replication deficient adenovirus expressing the α3 sub-unit off of the synapsin promoter (Synα3). Expression of the virus took 24 hours in adult knockouts injected with Synα3 virus intravenously to the tail. To test whether activity had been recovered in injected α3 knockout animals, we tested for compound action potentials (CAPs) in the SCG one or two days after infection. Approximately 65% of the SCGs in animals injected with adenovirus showed CAPs. In these animals, however, we saw a low level of restored activity, from 0.1-1.2 mV, but the average CAP amplitude was 0.2 mV, (under 10% of adult CAP in control mice, which is about 2.6 mV) (Figure 3.2). This result may indicate that only a certain number of SCG neurons expressed α3 and assembled functional nAChRs. Because CAPs only measure response from cells that fire action potentials, it may be that many cells assembled functional nAChRs, but fewer had strong enough synaptic transmission to fire an action potential. In rescued knockout SCGs that did show CAPs, we proceeded to measure EPSPs intracellularly.

To quantify input elimination, I counted discrete EPSP thresholds in the adenovirus-rescued α3 knockout SCGs.

In SCGs with CAPs, about 7% of neurons showed evoked EPSPs. In early experiments, cells without EPSPs were not recorded, because rate of recovery was not initially an important factor to my study. Of neurons with recovered activity, about half (3%) had responses that were

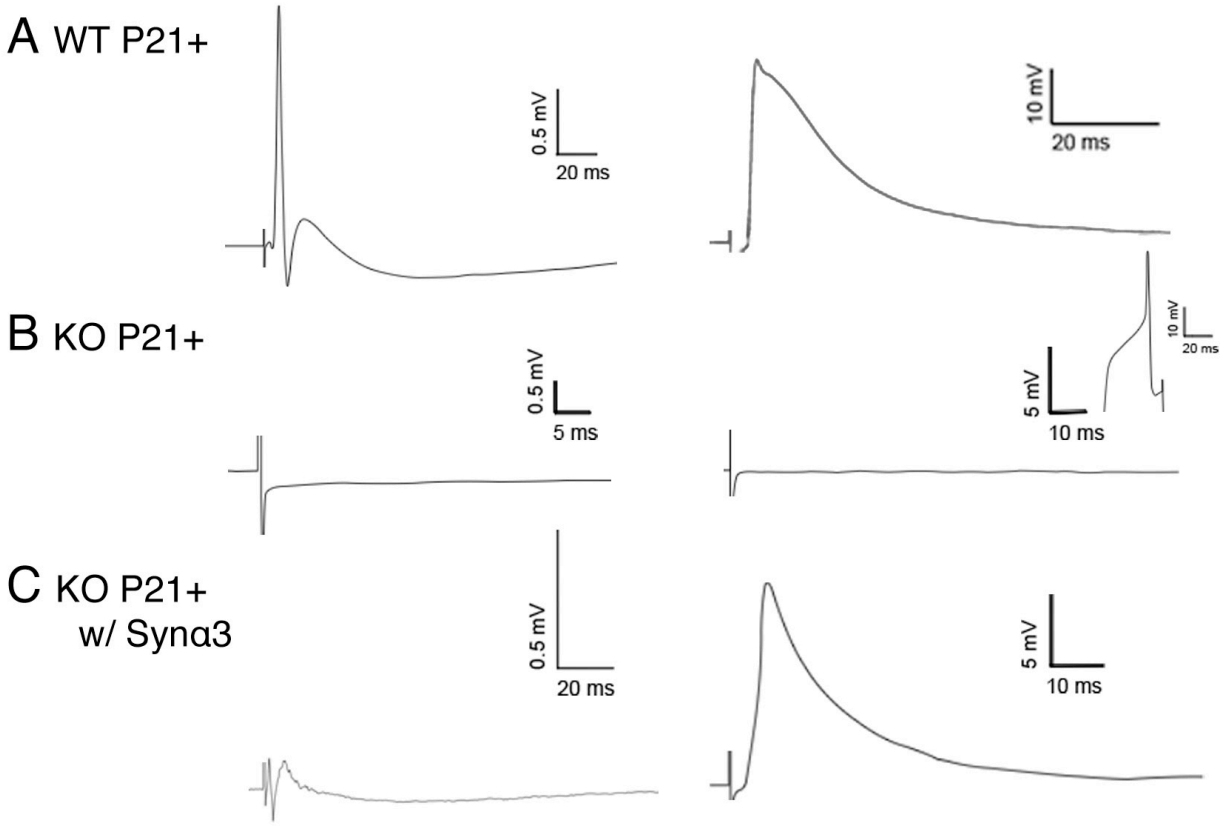

Figure 3.2 *Synα3 adenovirus recovers some postsynaptic activity to SCG neurons.* **A** Robust CAPs (average amplitude 2.6 mV) and EPSPs (average amplitude 27.8 mV) are relatively simple to find in wild-type adult SCGs. **B** Sympathetic ganglion neurons in α3 knockout animals do not assemble functional nAChRs and show no nerve-evoked extracellular or intracellular responses. Although the cells are silent in response to synaptic transmission, they are intact as shown by action potentials induced by direct intracellular depolarization. **C** CAP and EPSP traces showing typical responses of ganglia and SCG neurons that showed rescued activity, respectively. 24–48 hours after injection with Synα3 adenovirus, SCG neurons show recovered postsynaptic activity. Some level of extracellular activity was recorded in 64.7% of injected animals. In rescued SCGs with evoked response, recovered activity varied but was generally low (CAP average = 0.21 ± 0.26 mV; n=68). In SCGs with CAPs, 7.3% of cells show EPSPs (n=329). Of cells with EPSPs, 3.4% showed EPSPs that were too small to count inputs, the rest of the cells had large enough EPSP to isolate contributions from different inputs (n=24).

too small to resolve different inputs. On the other hand, some SCG cells showed large depolarizations up to 25mV and higher. Because of different levels of recovered activity, cells with EPSPs smaller than 6mV were excluded (at 6mV, it is possible to identify at least 5 inputs). In the group of SCG neurons that were not excluded, the average maximum EPSP was 16.1mV (compared to 27.8mV in adult wild-type and 17.4mV in the neonatal wild-type). In this group, 27% of neurons fired action potentials when stimulated from resting potential.

3.4 Input elimination does not occur in SCG neurons lacking postsynaptic activity

Because I was able to rescue activity in some SCGs cells, I was able to count the number of inputs to silent neurons. Because the rescue was not always robust, I excluded rescued SCG neurons whose EPSP signals were too small to distinguish discrete activation thresholds. In the group of cells with maximum EPSPs larger than 6mV, I was able to identify distinct stimulation thresholds and count the inputs (Figure 3.3, A). Still, in some of the cells with EPSPs between 6 and 10mV (about 25% of the cells), input contributions were less distinct, so in these cells the number of inputs may have been underestimated. This describes 5 of 31 cells whose inputs were counted.

In the rest of the recorded cells, I was able to identify multiple excitation thresholds, meaning I could count the number of inputs innervating silent SCG neurons after P21. I was able to isolate 4-12 different inputs to the neurons using the same gradual stimulation protocol as detailed in the wild-type experiment. The average number of inputs innervating an α3 knockout SCG neuron was 6.9 (n=31). In virally-rescued α3 knockout SCG neurons, the innervation

pattern more closely resembled the neonatal wild-type SCG than the age-matched, wild-type SCG (Figure 3.3, B).

Also like neonatal SCGs, excitation thresholds for different inputs were very close together, and a constant injection of current could sometimes evoke several discrete EPSPs.

The disparity index for the inputs to the a3 knockout SCG also resembled the neonatal wild-type (Figure 3.3, C). The disparity index is low, indicating there are no particularly dominant inputs contributing the EPSP recordings.

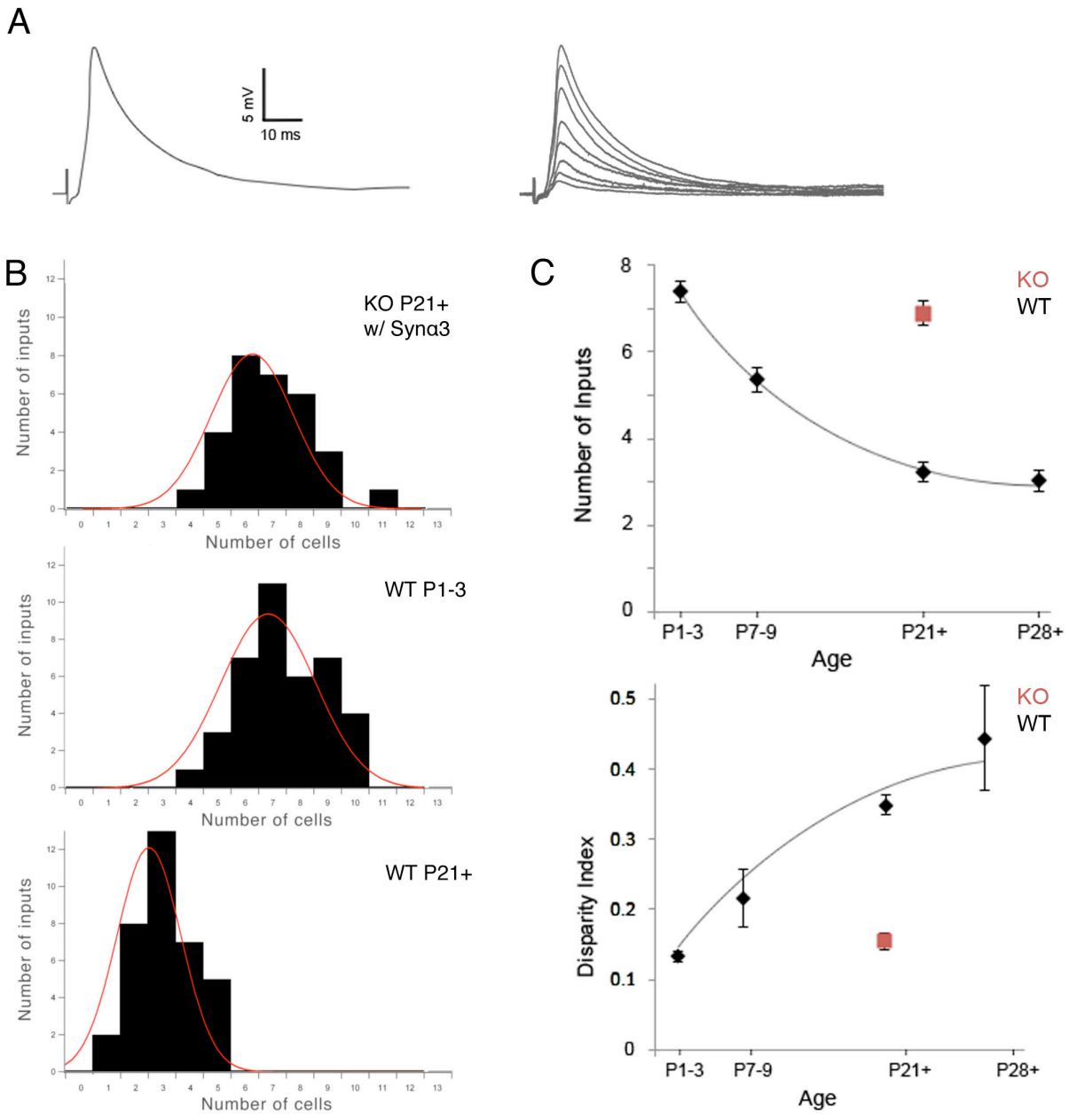
A
5 mV
10 ms
B
KO P21+
w/ Synα3
WT P1-3
WT P21+
Number of inputs
Number of cells
C
KO
WT
Number of Inputs
Disparity Index
P1-3
P7-9
P21+
P28+
Age

Figure 3.3 α*3 knockout SCG cells do not show input elimination through development*. **A** Some rescued knockout cells in the SCG were large enough to clearly show distinct EPSP amplitudes at different stimulus thresholds. The left trace is an example of a maximal EPSP and the left trace is a composite of all of an example neuron's distinct EPSP potentials. **B** The number of inputs to rescued knockout neurons are recorded in the histogram. The average number of inputs is 6.9 (n=31). Comparing the innervation histograms from P1–3 and P21+ show that adult α3 knockout SCG have innervation that resembles the neonatal innervation pattern. **C** In the top graph, the average number of inputs to the adult knockout SCG neuron (red) stray significantly from normal input elimination seen in wild-type littermates (black). In the bottom graph, the disparity index (0.154) shows inputs to the adult knockout SCG neurons have similar strengths and contribute equally to the maximal EPSP, contrasting with the adult wild-type disparity index, which indicates the dominance of one or two inputs. Data points for P1, P7–9 and P21+ represent the mean of 31–41 experiments (P28+ is a subset of P21+, with 16 experiments), and error bars represent SEM.

4. DISCUSSION AND CONCLUSION

4.1 Discussion

Input elimination in the mouse SCG

I show that the mouse SCG is a good model to investigate input elimination. At birth, SCG neurons receive on average 7 axonal inputs, and by maturity at P21 they receive on average 3 inputs. At an intermediate developmental stage, P7-9, SCG neurons are innervated by around 5 inputs though the innervation to P7-9 SCG cells varies from adult to neonatal levels. This suggests some SCG cells eliminate inputs earlier than others. Like autonomic ganglia in other rodent models (Lichtman, 1977; Johnson and Purves, 1981, Lichtman and Purves, 1979), the mouse SCG circuit clearly shows developmental input elimination within the first 21 days of life.

Furthermore, I measured the relative strength of the persistent inputs to the SCG, and show that the relative strength of inputs to SCG neurons diverges. At birth, EPSPs are composed of many small input contributions that are similar in strength. By P21, one or two dominant inputs evoke most of the postsynaptic response, with several smaller inputs adding relatively little to the maximum EPSP amplitude. Again, the intermediate stage of development, P7-9, shows an intermediate level of dominance. This finding reflects the divergence of input strengths through development in the NMJ and the cerebellum (Colman and Lichtman, 1997; Hashimoto and Kano, 2003). One difference between the SCG and the NMJ or cerebellum is that I observed two dominant inputs in a mature SCG neuron, instead of just one dominant input.

Input elimination does not occur in postsynaptically silent SCGs

Despite my difficulties optimizing the rescue of postsynaptic activity in knockout animals to wild-type levels, I show that ganglia in α3 knockout mice do not undergo input elimination. Adult knockout SCG neurons receive approximately 7 inputs. Moreover, in these knockout rescues the disparity index remains low, many small responses with similar amplitudes summate to the maximal EPSP recorded similar to neonatal SCG neurons. Adult SCG innervation in knockouts that lack postsynaptic activity resemble neonatal innervation in wild-type with respect to the number of inputs they receive and the relative dominance of the inputs. It seems the presynaptic cell waits for activity-dependent retrograde signals before inputs are eliminated. Without the feedback of the retrograde signal, there is no elimination.

Adenoviral rescue of activity in the silent a3 knockout mouse

One limitation to the larger design of my study was that I was not able to achieve high levels of adenoviral expression of α3 in knockout rescue trials. In earlier work in our lab, the same stock of Synα3 adenovirus injected into α3 knockout animals led to better recovery of synaptic activity (Krishnaswamy and Cooper, 2009). In our previous work with adenovirus, injections led to strong synaptic transmission, detected by compound action potentials, in over 90% of ganglia, where my injections led to modest synaptic transmission in around 50% of ganglia. Furthermore, previous injections were able to restore strong EPSP amplitudes of around 10mV in over 85% of α3 knockout SCG cells, where my trials restored strong EPSPs in only 7% of knockout SCG neurons. I would require better recovery of postsynaptic activity in the ganglion to investigate other aspects of input elimination in the SCG.

I spent much of my time in the lab trying to optimize adenoviral expression and restoration of postsynaptic activity. In previous studies, a total of 10^7 plaque-forming units (pfu)

of synα3 adenovirus were dissolved in 100 μL injections (Krishnaswamy and Cooper, 2009). In my study, I diluted 1 μL of viral stock titered between 0.78-1.912 x10^{10}pfu/mL in 100-150 μL injections. This should have produced a similar 10^7 pfu per injection, like the previous study. I thought that the low recovery of activity could be caused by several possibilities: the virus did not express α3 after several years in storage, my injections did not deliver the virus properly, the virus was being blocked by the immune system or the amount of virus injected was inadequate. First, I tested the functionality of my copy of the adenovirus by infecting α3 knockout SCG neurons in culture. I infected SCG cultures (1.5 mL) with 1 μL of the adenovirus and incubated them for 24 hours. In a patch-clamp preparation, my colleague Aliona Rudchenko recorded currents in SCG neurons in the infected knockout cultures after puffing ACh onto them. The shape and amplitude of currents in virus-treated knockout SCG neurons resembled ACh-evoked currents in wild-type SCG cultures. No currents were found in uninfected knockout cultures. This experiment showed that the copy of the Synα3 adenovirus did indeed drive expression of the α3 sub-unit in SCG neurons.

To investigate whether I was successfully injecting the virus into the mice, I asked technicians from the Animal Care Centre at the McIntyre Medical Building to inject the virus for me. The technicians' injections yielded no change in the recovery of activity. I also added a small amount of the marker Evan's Blue to some injections. The skin of white mice turns blue when their blood is dyed by Evan's Blue. My injections were successful at delivering the vehicle to the animals' circulation, as I saw mice turn blue. Despite successful injections, activity was still low.

In early trials, some knockout mice died soon after injections: they stopped breathing within 30 minutes. I was more careful to purify the adenovirus from the medium that it was grown in, which likely contained debris of infected HEK 293 cells, and saw a marked decrease in

the mortality of the injected animals, but no improvement in activity expression. Injecting high concentrations of adenovirus to nervous tissue can also lead to inflammation that reduces adenoviral expression (Thomas et al., 2001). In an attempt to prevent shock, reduce inflammation and promote adenoviral α3 expression, I added dexamethasone to the PBS vehicle (Xu et al., 2000). The immunosuppressant, however, did not increase the frequency or amplitude of CAP or EPSP measurements in injected knockouts.

Because the stocks I used had been amplified from the original copy of the adenovirus, and because the titrations I achieved were always within the same range as other viruses that had been produced in our lab, I did not know what was causing this low expression of the α3 sub-unit. Since expression was low, I thought that the concentration of the stored purified adenovirus was actually lower than the titration, and I had damaged the integrity or lost the concentration of the adenovirus in processing, freezing and thawing or purification - some point after I titered the virus. To resolve this issue, I amplified several batches of adenovirus in colonies of HEK293 cells, attempting to increase the concentration of adenovirus (He et al., 1998). I also added more purified adenovirus stock to injections. Increasing concentration and amount of adenovirus injected did not change the typical amount of expression: CAPs in these rescued-knockout ganglia were the same size and the number of cells with EPSPs were low.

In a study investigating the efficiency of the synapsin 1 promoter in adenoviral gene expression in neurons, Kugler and colleagues show that injections containing 10^7 pfu of adenovirus produce the strongest and longest expression patterns (2.5 months) in various, directly-injected areas of the brain in rats. At 10^6 pfu, expression of the reporter gene is less profound, and at 10^8 pfu expression is initially no different than 10^7 pfu, but is weak after a month (Kugler et al., 2003). To explore the ideal concentration of my adenovirus, I injected

knockout animals with 0.1μL and 0.01μL of 10^{10} pfu/mL stock again in 100-150 μL of PBS, to produce injections with 10^6 pfu and 10^5 pfu, respectively, but observed no better rescue of activity in SCGs. Nearing the end of my time in the lab, however, I injected a few knockout animals with 1 nL of 10^{10} pfu/mL stock and saw slight improvements in the amplitude of compound action potentials (which averaged about 0.25mV, which is still low compared to wild-type CAPs, which are about 2.6mV) and the proportion of SCG cells with evoked EPSPs (about 30%). I finished collecting my virus-rescue data with these injected animals. It seems that the concentration of adenovirus injected into the animal is absolutely crucial to gene expression. If I had had more time, I would have continued to explore the lower range of adenoviral concentrations to optimize activity recovery.

I would have liked to complete other control experiments if I had achieved a more robust restoration of postsynaptic activity. Firstly, I would have liked to determine whether the virally-expressed nAChRs, and a newly increased level of activity, would cause changes to the number of inputs connecting to a SCG neuron. On several occasions, I injected wild-type animals with the Synα3 virus and tested their CAPs and EPSPs. Anecdotally, I saw no difference in the amplitude of CAPs or the number of inputs to an SCG neuron. Unfortunately, because I had a low rate of successful infections, I cannot safely comment on whether the virus injection did or did not affect the connection pattern in wild-type animals. Secondly, I would have also liked to control for the short-term time scale of the infection. McCann et al. show that input elimination can occur in as short as 12 hours in the NMJ (McCann, et al., 2007). If I had had a more reliable infection rate, I would have liked to test the number of inputs to virally-recovered α3-KO SCG neurons between the time of injection and infection at 24 hours, when I normally collected data.

Complementary Studies

Considering the amount of research devoted to the relationship between dendrite elaboration and input elimination, I would have liked to investigate the pattern of innervation (whether mature inputs target dendrites or soma) and the extent of dendrite growth in the wild-type and α3 KO models (Hume and Purves, 1981; Purves and Hume, 1981). If I had finished my electrophysiological studies earlier in my degree, I would have liked to investigate the morphology of dendrites and innervation pattern on wild-type and α3 KO SCG neurons. To accomplish this, I would have injected SCG cells with a fluorescent dye, Lucifer Yellow. Further, I would stain ganglia for co-incident markers of presynaptic terminals (VAChT) and postsynaptic terminals (postsynaptic density protein 93, PSD93). I would then visualize whole-mount ganglia with confocal microscopy (Krishnaswamy and Cooper, 2009).

In these imaging experiments, it would be important to image the ganglia of mice of different ages to see how activity can change neuron morphology over time. This time-course could then also be compared with the time course of the synaptic refinement. It is also possible that the disparity in strength between inputs would be observed in synaptic size or input caliber in the imaging data, which could be compared to the disparity found in the electrophysiological data.

Hebb's Rule

My results relate to the classical Hebbian axiom "cells that fire together, wire together." This common adage simplifies plastic changes in neural circuitry to the general rule that synaptic inputs that are strongly active and drive postsynaptic activity are potentiated (Wyatt and Balice-Gordon, 2003). The results from my study challenge the converse assumption, "inputs that do

not drive postsynaptic activity are eliminated". A lack of postsynaptic activity may not destabilize a connection in-and-of-itself. Destabilization may require the presence of another active input for competition, or 'heterosynaptic suppression', (Lo and Poo, 2001) while no elimination occurs without competition.

In the NMJ the observation that input elimination coincided with the postnatal increase in activity in developing rats, led to studies focusing on the role of activity in the process. When researchers block EPPs in the NMJ with tetrodotoxin (TTX) they do not observe the normal shift from poly-neuronal innervation to single innervation (Thompson et al, 1979). Researchers also specifically blocked postsynaptic activity with α-bungarotoxin (α-bgt), which irreversibly binds and blocks nAChRs, and observed no input elimination (Duxson, 1982). In a recent study, a group over-expressed inward potassium rectifier channels in the soleus muscle of mice and thereby blocked nerve-evoked action potentials. Without action potentials, the muscle fibers remained multiply-innervated in adulthood (Favero, 2009). Therefore, blocking pre- or postsynaptic activity in the NMJ prevents the elimination of non-active inputs.

In prototypical studies of the visual system, Hubel and Wiesel first occluded vision to one eye (monocular deprivation, MD) in a new-born kitten and recorded from layer IV of the visual cortex after 3 months. The investigators found that very few cortical cells were driven by the previously-deprived eye. Moreover, more neurons respond to the non-occluded eye. This phenomenon fits with Hebb's rule well: inputs that drove postsynaptic activity were maintained while inputs that did not drive postsynaptic activity, were eliminated (Hubel and Wiesel, 1963). From these results, Hubel and Wiesel expected that if they obstructed vision in both eyes (binocular deprivation, BD) cortical cells would not respond to stimuli in either eye. Instead, they found that a majority of the cortical cells were driven by both eyes (Hubel and Wiesel,

1965). Blocking activity in some axons causes elimination of their inputs, while the remaining active inputs proliferate and innervate new territory. When all activity is blocked, however, all inputs persist.

Results from the NMJ and visual system and my results from the silent SCG challenge one assumption about Hebb's rule - that inputs that do not evoke postsynaptic responses are eliminated. If this were true, all of the silent synapses in blocked visual cortical cells, NMJs and α3 KO SCGs should be removed. Instead, all of these circuits maintain presynapstic inputs. In the context of developmental input elimination at least, we might add a modifier to Hebb's Rule: cells that fire together wire together, *but silent cell populations do not change their wiring*. My study provides quantitative evidence for the persistence of silent inputs in a neuron-to-neuron circuit.

Although the SCG circuit is a neuron-to-neuron connection, the conclusions of my study concerning the role of postsynaptic activity may not apply directly to more complicated CNS circuitry. Some factors that might complicate similar experiments in central circuits. For instance, convergent inputs from different areas of the nervous system connecting to one postsynaptic cell. The fact that several input elimination patterns may occur simultaneously between inputs of different origins or different classes (excitatory, modulatory and inhibitory) might produce a more complex competitive model. Some experiments have examined this paradigm in the visual system with interesting results. Reiter and colleagues first observed that when they completely block cortical activity with TTX in kittens, they prevented shifts in ocular dominance in response to MD. This protocol, however, blocks both presynaptic and postsynaptic activity in the cortex. To isolate the role of postsynaptic activity, the group inhibited the activity of cortical cells with the GABA-R agonist muscimol. In this condition, there is no change in the

cortices of animals with non-occluded vision. Surprisingly, the ocular dominance of the inactivated cortex shifts to favour the deprived eye, and away from the open eye when exposed to MD (Reiter, et al, 1986; Reiter and Stryker, 1988). In kittens with normal vision and muscimol-inhibited postsynaptic activity, inputs to silent postsynaptic cells are not eliminated. When only half of the presynaptic inputs are active on silent postsynaptic cells in monocular deprivation, active inputs are preferentially eliminated. These authors speculate that only active afferents respond to a putative repulsive signal from the postsynaptic cell, leaving unaffected inputs from the deprived eye to innervate the cortex, but this phenomenon remains poorly understood. This complicates Hebb's rule. When the entire postsynaptic cell population is inhibited in the visual cortex there is no input elimination despite incoming activity. However, when half of the presynaptic inputs are active and others are inactive, *active* inputs are preferentially *eliminated*. The postsynaptic cell may detect which inputs should be maintained, but which inputs - if they are especially inefficient at evoking a postsynaptic response - should be eliminated (Reiter and Stryker, 1988). Essentially, the postsynaptic cell may eliminate inputs based on their relative efficacy. Further investigation of this model may help explain the signals exchanged between the pre- and postsynaptic cells that lead to input elimination.

Competition among active and inactive postsynaptic cells

The low recovery of activity to knockout SCG neurons is a limitation to my study. Although there was some activity recorded by both compound action potential and intracellular recordings, they were weak compared to wild-type responses. Only a small population of cells in SCGs with CAPs showed rescued activity. So these SCGs were mixed, having both active and inactive neurons. Other experiments have shown that unequal presynaptic activity skews

innervation to favour active inputs (Crair, 1999). If the traditional version of Hebb's rule applies to the small population of activity-rescued knockout SCG neurons (inputs that cause a post-synaptic action potential persist) there is a possibility that the number of inputs counted from rescued activity is inaccurate. It is possible that cells with activity restored are suddenly preferentially innervated. This is improbable; if this were the case, we would expect more inputs than in neonatal wild-type SCG neurons. This possibility is also unlikely because of the brevity of the activity rescue. In 7 days of normal input rearrangement in the SCG (from P1 to P7), average input number is reduced by 2 (shifting from 7 to 5), showing that changes in input number occur relatively slowly. The time it takes to show innervation shifts in the visual system is also at least a week (Katz and Crowley, 2002). The adenovirus takes 24 hours to rescue activity and I recorded between 24 and 48 hours after injection. The activity rescue is too brief to change input number, so the rescue accurately reflects the activity-null SCG.

Beyond the time course of changes in innervation, it might be possible that the virally-rescued SCG neurons receive preferential innervation from spinal inputs. If there is a preferential innervation of virally-rescued SCG neurons, it is possible that the average number of inputs in the virally-rescued KO SCG cells at P28 resembles the neonatal wild-type average innervation because there is a maximal number of inputs that can be sustained by an SCG cell (around 7-10). This number may be governed by the size and dendritic elaboration of the cell, as suggested in Hume and Purves' work (Hume and Purves, 1981). This possibility could be investigated if a more effective virus was used. If the virus restored activity to most α3-KO SCG cells, no preferential effect should exist among postsynaptic neurons. The number of inputs would more accurately represent KO innervation pattern, without any possible skew caused by preferential innervation to more-active SCG neurons.

It is also possible that the α3 virus may cause a gain-of-function in the rescued KO neurons, where all cells with virally-expressed α3 sub-units see an increase in innervation. This possibility could be investigated by infecting aged matched (P28) wild-type animals with the virus to see whether there is an increase in the average input number.

The low level of viral expression in my experiment is not ideal to investigate whether input elimination occurs normally in the knockout after longer periods of restored activity. It is possible that differential activity in the postsynaptic cells over longer periods could lead to atypical innervation created by competition among neurons in the SCG. This scenario in itself may be useful to investigate the role of competition in input elimination. The shifts of innervation in response to blocking half of the visual system's presynaptic activity are a staple in studies of input elimination in that system (Shatz and Katz, 1996). Blocking half of the activity in a postsynaptic population has not been studied to my knowledge. The α3 knockout is a first step to an interesting opportunity to investigate input elimination in a half-postsynaptically-active SCG. To address this question, our lab has developed a mosaic mouse model where half of SCG neurons lack postsynaptic activity. Krishnaswamy and Cooper designed a transgenic copy of the α3 sub-unit driven by the ubiquitinC promoter and recombined the gene onto the X chromosome in mice. Male transgenic mice were bred with α3 heterozygotes, to yield α3 +/- $X^{\alpha3}Y$ males. The lab generated another α3 knockout colony expressing red fluorescent protein (RFP) on the X chromosome. These males were bred with α3 -/- X^{RFP} females to produce α3 -/- $X^{\alpha3}X^{RFP}$ mosaic mice. Because only one copy of the X chromosome is expressed in each cell through X inactivation, half of the cells in the SCG will express α3 off the X chromosome and the other half will express RFP as a reporter (Krishnaswamy and Cooper, unpublished).

The imaging experiments detailed on page 54 would provide further insight into the innervation in the mosaic model. Imaging dendrite elaboration, input targeting and synaptic numbers would help consolidate the findings of the electrophysiological data.

This model will provide more insight on the nature of postsynaptic activity in input elimination. If presynaptic cells follow Hebbian selection only when there is activity in the postsynaptic population, we might expect to see active cells (those without RFP) maintaining innervation while postsynaptically inactive cells (those expressing RFP) lose their innervation. This would suggest inputs that cannot evoke postsynaptic activity are eliminated in the presence of stronger, postsynaptic potential-evoking inputs. This result would also suggest an activity-dependent retrograde supportive factor released by the postsynaptic cell in normal input elimination. If a presynaptic input does not receive the support factor from a silent postsynaptic cell, while receiving the factor from active neighbouring neurons, it retracts its input to the silent cell. Another possible outcome is that inactive postsynaptic cells maintain neonatal numbers of inputs while inputs to active neurons are eliminated normally. This would suggest that without a postsynaptic signal to destabilize connection, it persists, regardless of the local activity. This would suggest the role of an activity-dependent retrograde punitive factor in normal input elimination. The mosaic model will elaborate the role of the postsynaptic cell in input elimination and help explain why certain inputs persist while others are eliminated. Does the presynaptic cell eliminate inputs to a silent postsynaptic cell and opt for a nearby active postsynaptic cell, or can a silent postsynaptic cell maintain its inputs independent of the activity in neighboring postsynaptic cells?

Competition and dendritic outgrowth in the silent SCG

In my data, the emergence of dominant inputs in the wild-type SCG and the absence of dominant inputs in the α3 knockout is supported by evidence from the NMJ and cerebellum (Colman and Lichtman, 1997; Hashimoto and Kano, 2003). In the NMJ, activity causes the gradual potentiation of a "winner" input. Colman et al. measured EPPs in the NMJ while individually stimulating two inputs innervating one muscle fiber. Through the first two postnatal weeks, the synaptic strengths of the two inputs diverge, as one becomes stronger and the other becomes weaker. To isolate mini-EPPs from each input, the group repetitively stimulated them separately, after which mEPPs are more frequent. The increased strength (the ability to evoke postsynaptic EPPs) of the winner and decreased strength of the loser is reflected in the quantal content. The reduction in the losing input is attributed to a reduced quantal content, both decreased neurotransmitter release and postsynaptic response. After the initial imbalance of input strength, the divergence accelerates until one input is ultimately eliminated. The authors posit that the synaptic efficacy of one input increases its ability to cause the elimination of the other. As the winner becomes stronger, it destabilizes its competitor. This shift increases by feedback as the winner gains more synaptic territory (Colman et al, 1997). These authors offer these results as evidence of competition, though whether inputs compete presynaptically or postsynaptically or otherwise is not clear from their data. In the NMJ there are no dendrites to mediate multiple-innervation, so this model only offers a possible generalization for competition between neighbouring inputs.

In the neonatal cerebellum, at least five climbing fibers with relatively small EPSPs connect to Purkinje cells in young animals, between P4-7. All of the multiple, immature connections evoke EPSPs of similar magnitudes. As the animal develops, one input is 'selected' and the EPSP it evokes grows in magnitude. The other inputs' contributions are reduced and

mostly eliminated by P14. The morphological location of the CF-Purkinje cell synapses migrate through input elimination. Initially, multiple CFs contact Purkinje cells on their perisomatic dendrites. In a mono-innervated Purkinje cell, its partner CF elicits calcium transients across the entire dendritic tree, indicating an extensive network of synapses. If any second CF persists by P14, it elicits only a small calcium transient on a proximal dendrite of the Purkinje cell. (Hashimoto and Kano, 2003; Hashimoto and Kano, 2005). This system of input elimination is also distinct from the SCG, because despite a very complex dendritic tree, only one climbing fiber persists in the adult model. In the SCG neurons with complex dendritic trees can support multiple inputs at maturity. Therefore, dendritic complexity must play a different role in the climbing fiber-Purkinje cell circuit and the SCG circuit.

Because adult SCG neurons maintain multiple inputs, there must be another important factor that prevents one dominant input from eliminating all of the others. Other studies show that spatial separation may dilute competitive signals in refinement (Forehand and Purves, 1984; Gan and Lichtman, 1998). In the SCG, dendrites grow dramatically in the first month of life, the same time that developmental input elimination occurs (Rubin, 1985c). Because postsynaptic activity is involved in the sprouting of dendrites in SCG neurons (Vaillant et al., 1999), it seems postsynaptic activity develops dendrites, allowing multiple innervation, while simultaneously causing other inputs to be eliminated. The interaction between dendrite outgrowth and input elimination could be sequential, where several dominant inputs spur the growth of their postsynaptic partner's dendrites, or conversely that sufficient dendritic territory allows for the persistence of multiple strong inputs. In our knockout mouse, we would presume dendrites would not grow without postsynaptic activity. Measuring dendritic complexity and synaptic connections in the wild-type, the knockout and rescued knockouts may show how these two

factors interact and elaborate the model of spatially-mediated punishment signals. If input elimination is driven by competition, whose factors act through postsynaptic activity, we would expect to see a persistence of somatic innervation in the knockout. If the developmental elimination is paused, we may see dendrite outgrowth and dendritic innervation occur when activity is virally-restored to the knockouts. It is also possible that a plasticity-limiting factor mediated by postsynaptic activity restricts dendritic growth in the knockout.

It may be possible to explore the relationship between dendritic growth and input elimination further in the SCG. Activity causes dendritic outgrowth in SCG neurons *in vitro*, regardless of input synchrony (Vaillant et al., 1999). In other models of input elimination, inputs only seem to be eliminated if there is asynchronous activity. If this is also true in the SCG, what would happen to the SCG circuit with experimentally synchronous activity? Purves and colleagues predict that dendrites are the preferred site for postnatal input innervation in the SCG. They also suggest that distance between inputs, which is provided by large dendrites, mitigates competition (Purves et al., 1985). This experiment would ablate activity from the superior cervical trunk and provide artificial and synchronous activity to the SCG via a transplanted cholinergic nerve, similar to the experiment of Busetto, et al. in muscle (Busetto, et al., 2000). If dendrites grow in response to synchronous activity but inputs are not eliminated from synchronous activity, we might predict SCG neurons with a high number of inputs. If there is no competitive elimination, inputs may also be highly intertwined across the cell body and dendritic arbor of the SCG neurons instead of having distinct dendritic territories (Forehand and Purves, 1984). This may not be the case, perhaps inputs will not innervate newly expanded dendrites if synchronous activity does not drive competition, and inputs will persist on their perinatal territory, near the cell body. This type of experiment may tell us how dendritic length mediates

final input numbers. Does new dendritic growth recruit new synapses or do inputs innervate new dendrites to escape competition?

Is input elimination in the α3 knockout permanently prevented or just paused?

The results of my study show that silent SCG innervation resembles that of neonatal SCG neurons. I have suggested input elimination does not occur in the knockout SCG, but there is another possibility. Because results reflect a snapshot impression of knockout development and not a dynamic impression, it is possible that the high number of inputs reflects a turnover of inputs being eliminated while new inputs are sprouting. In partial denervation studies in the autonomic nervous system, uninnervated postsynaptic cells recruit sprouts from nearby presynaptic terminals. Once regenerated axons contact the postsynaptic cell, the transient sprouts are removed (Lichtman, 1977). This possibility in development seems inefficient and unlikely. Furthermore, there is no evidence for input turnover (sprouting and elimination) in developing NMJs that are postsynaptically inactivated and microscopically observed throughout development (Balice-Gordon and Lichtman, 1994).

The unlikely possibility that presynaptic cells sprout new inputs as existing inputs are being eliminated might be examined by electron microscopy, looking for retracted axonal inputs in the knockout SCG. In input elimination in the NMJ, inputs are eliminated in development by retraction, which is distinct from elimination due to damage or denervated, where the inputs disintegrate by Wallerian degeneration, leaving behind debris (Coleman and Freeman, 2010). Instead, the presynaptic neuron at the NMJ retracts the input by forming a terminal retraction bulb. The retracting inputs become thin and have spherical ‘bulbs’ on their terminals containing vesicles (Riley, 1977). While retracting, axonal inputs also shed membrane-bound debris called

axosomes that are engulfed by local glial cells (Bishop et al., 2004). If the inputs in the knockout SCG are constantly being eliminated, there would be clear evidence in retraction bulbs and axosomes. EM images of the α3 knockout SCG were not screened for retraction bulbs or axosomes, but these formations are quite distinctive and nothing similar was reported (Krishnaswamy and Cooper, 2009).

The Critical Period

Input elimination does not occur in the α3 knockout SCG. If development has been stopped, it would be interesting to determine if the process of input elimination can be restarted or if it necessarily has to occur during a specific period in development - a critical period. It is possible that if postsynaptic activity were returned to the SCG neurons with the Synα3 adenovirus for longer than 2 days, inputs would be eliminated normally.

To investigate a possible critical period in the α3 knockout model, we would infect mice at different ages, for instance P7, P21 and 2 months, with the Synα3 adenovirus and record the number and strength of inputs after the typical 3 week input elimination period. This investigation may not be possible considering the low recovery of postsynaptic activity in my trials. Only around 10% of the rescued knockout SCG neurons showed any EPSP, Of this 10%, even fewer fired action potentials, and early studies in the NMJ show that cells that do not fire action potentials (tonic muscle fibers) do not undergo input elimination (Ridge, 1971; Lichtman et al., 1985). Furthermore, because of the low level of recovery, there would be two populations of postsynaptic cells, active and inactive. This difference in activity status could greatly affect the innervation pattern.

A large amount of research has outlined the critical period in the visual cortex. In the visual system, developmental input elimination is equated to shifts in ocular dominance columns. Cats reared in the dark (presynaptic activity is blocked) can restart input elimination and show ocular dominance shifts when returned to normal light conditions for up to two years (Cynader and Mitchell, 1980). The development of the silent SCG may be paused like in the visual system. Morphologically, all of the important synaptic machinery is assembled, so the circuit may be 'waiting' for postsynaptic activity to provide a feedback signal to the presynaptic cell to start refinement or to identify which inputs to remove. When Krishnaswamy and Cooper restored postsynaptic activity to the α3 knockout, they observed presynaptic CHT expression after 7 days. So, when certain developmental changes in the sympathetic circuit maturation are stalled without postsynaptic activity, they can be restarted (Krishnaswamy and Cooper, 2009). Perhaps this is also true in regards to input elimination.

Restarting input elimination in the SCG may not be possible, however, if it normally occurs during a critical period and activity is recovered after this critical period. In the cat visual system, the effect of monocular deprivation to shift ocular dominance is particularly strong in a critical period between 3 weeks and 2 months of age. Before and after this critical period, there is no significant effect of MD on cortical response specificity. So, if the SCG has an analogous critical period in which developmental plastic changes must occur, restoring activity after 21 days may not restore input elimination.

If postsynaptic activity mediates the critical period, then plastic changes may be possible after the postsynaptic activity is restored. Pre- and postsynaptic activity mediate plastic changes in innervation in layer IV of the visual cortex, but also affect the critical period itself when circuits are susceptible to plastic changes.

The closure of the critical period in the visual system seems partially mediated by postsynaptic activity. Blocking activity in the visual cortex with TTX or rearing animals in the dark reduces mRNA levels of brain-derived neurotrophic factor (BDNF), a factor the matures inhibitory interneurons throughout the cortex. BDNF is expressed by depolarized visual cortical cells to develop surrounding inhibitory interneurons, which control the critical period (Lein et al., 2000; Marty, 1997; Hensch, et al. 1998). In theory, cortical cells release factors to mature inhibitory cells to facilitate spike-timing dependent plasticity between excitatory inputs and the cortical cell. In theory, inhibition ensures cortical cells are not excited when they receive impulses, so their excitatory inputs will be potentiated (Foeller, Feldman 2004; Fagiolini, et al. 2004). There are no inhibitory interneurons in the SCG to coordinate firing of pre- and postsynaptic partners, so if there is a critical period, it is not likely mediated by postsynaptic activity in the same way, if at all.

Other studies in the visual system suggest that presynaptic activity may close the critical period. The homeoprotein Otx2 also matures visual circuitry in the visual cortex. Activity mediates the transport (but not synthesis) of Otx2 down the visual pathway from the retina and LGN. Additionally, Otx2 promotes the growth of peri-neuronal nets in the visual cortex (PNNs). PNNs are made of chondroitin sulphate proteoglycans, which impair morphological changes in the postynaptic cell and close the critical period (Sugiyama, et al. 2008; Pizzorusso, et al. 2002). It is possible that an analogous presynaptic factor is transported to the SCG to similarly impair plasticity and close the developmental critical period. Because α3 knockout mice have normal presynaptic activity, such a factor would prevent rescued activity from restarting input elimination in the α3 knockout SCG.

The retrograde signal

One of the major unresolved questions regarding input elimination is "What is the retrograde signal, passed from the postsynaptic to presynaptic cell, that mediates input elimination?"

Lichtman and his colleagues, who work primarily with the NMJ, suggest there are two possible types of retrograde signal: a supportive factor or a punitive factor. They also suggest several factors may work in concert. The postsynaptic cell could release synapse-supporting factors that preferentially bind to or are taken up by active inputs. Less active inputs would eventually be starved of these factors and retract. Another explanation may be that an activity-mediated signal from the muscle fiber destabilizes the motor axons, and activity protects active inputs from its adverse effects (Sanes and Lichtman, 1999). The nature of the signal (supportive or punitive) is unknown, but several factors could possibly be involved in the signal.

Different groups have studied the effect of neurotrophins on input elimination in the NMJ by injecting BDNF, NT3 and NT4/5 and then imaging and recording from fibers. BDNF causes a transient stabilization in synaptic contacts, but the experimentally persistent synapses were silent (Kwon and Gurney, 1996). BDNF and NT4 also have retrograde effects on pre-ganglionic cell proliferation and post-ganglionic synapse formation in the SCG (Causing et al., 1997; Roosen et al., 2001). Also in the NMJ, a group investigated GDNF in elimination after it was highlighted as a neurotrophic factor for motor neurons. GDNF does not effect elimination of motor neuron inputs to the NMJ, but actually induces sprouting (not stabilization) of inputs to muscle fibers, leaving them hyperinnervated (Henderson, et al, 1994; Nguyen et al, 1998). Other factors have been suggested as molecular mediators, such as ciliary neurotrophic factor, insulin-growth factor

and androgens, but their roles are unclear as all three affect muscle tissue integrity itself (Sanes and Lichtman, 1999).

Other groups show a delay in input elimination when they inhibit proteases. Activity-mediated rises in calcium activate calcium-activated neutral protease (CANP) in muscle, which is proposed as a possible punitive signal. In muscles treated with a calcium chelator, or when CANP is inhibited, muscle fibers maintain polyneuronal innervation during development. In fact, reducing CANP activity increases the complexity of axon terminals (Swanson and Vrbova, 1987). The inhibition of thrombin, another protease, does not prevent but delays input elimination. Prethrombin is found in the muscle and maximally expressed around P7 (Zoubine, 1997). These are interesting leads, but the identity of a definitive synapse-destablizing signal is unknown (Sanes and Lichtman, 1999). Whether or not any of these signals, supportive or deleterious, are conserved between the NMJ and the sympathetic nervous system is also unclear.

Further studies in the SCG indicate how the retrograde signal may be initiated in the postynaptic cell. The postsynaptic signal is likely caused by activity-dependent calcium influx from voltage-gated calcium channels, which then activates a signaling pathway, probably initiated by CaMKII phosphorylation, as we see in dendrite outgrowth and nAChR sub-unit regulation (Vaillant et al., 1999; De Koninck and Cooper, 1995). This calcium signal would lead to the expression or release of a retrograde signal to the presynaptic input to remain or retract from the SCG neuron.

Postsynaptic calcium signaling is supported by evidence of postsynaptic activity destabilizing inputs in the NMJ. External depolarization of myocytes *in vitro* causes depression in the innervating motor axons. Blocking calcium changes with a chelator in the myocyte blocks input depression (Lo et al., 1994). There is also evidence to link the postsynaptic calcium signal

to a retrograde support factor. Calcium influx also causes the expression of certain genes with promoters containing cAMP response elements (CREs). Calcium induces activity in CaMK, which phosphorylates cAMP response element binding protein (CREB) and drives the CRE promoter (Wheeler and Cooper, 2001). In neurons, CRE promoter regions on the *BDNF* gene drive BDNF transcription (Tao et al., 1998) and elevated BDNF levels in the SCG cause increased innervation of the postsynaptic cell by the pre-ganglion neuron (Causing, 1997). Whether the initiating signal in developmental input elimination promotes innervation or removes innervation, it originates from the postsynaptic cell. More work is needed to identify the signal or signals

4.2 Conclusion

There are still many unanswered questions regarding the mechanism of general and sympathetic nervous system-specific input elimination in development. The results from my study show that developmental input elimination in the sympathetic nervous system requires postsynaptic activity. In this experiment, I used a model with no synaptic transmission to SCG neurons. Specifically, I used a knockout mouse with a deletion of the α3 sub-unit of the nicotinic acetyl choline receptor, a mutation which eliminated postsynaptic activity. Inputs to the SCG neurons in the adult α3 knockout do not show the characteristic number and relative dominance that wild-type adults do, but instead resemble immature, neonatal connectivity. From these results, I conclude that postsynaptic activity is necessary for developmental input elimination in the mouse SCG.

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