# Diversification of Endosomes in the Nervous System: NEEP21 and TrkA Trafficking

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**Abstract**

Neurons project all over the body to maintain homeostasis and respond to external cues. This results in an intricate network of neurons stretching throughout the body with neurons as long as one meter in humans. Given the polarized state of neurons and the long distance over which proteins must be distributed, neurons have adapted special mechanisms to deal with these challenges. One way neurons can deal with the heightened burden of protein trafficking across long distances is through utilizing endosomal transport. To further interrogate the role of endosomes in the neuronal system, I focused on two proteins: neuron enriched endosomal protein of 21 kDa (NEEP21), and the receptor tyrosine kinase TrkA. NEEP21 is expressed almost exclusively in neurons, and has been implicated in trafficking of multiple receptors in cultured primary neurons. However, its expression pattern in the brain over development was unknown. Here, I found that NEEP21 is developmentally and spatially regulated in the brain, maintaining high expression in adulthood only in Purkinje neurons, hippocampal pyramidal neurons, and Layer V cortical neurons. Interestingly, I also found that it was expressed highly in the superior cervical ganglia (SCG), and co-localized with TrkA in culture. TrkA is a receptor tyrosine kinase critical for the formation and maintenance of the sympathetic nervous system. However, loss of NEEP21 in the knockout mouse did not have any effect on the trafficking or signaling of TrkA. To more closely probe the endosomal proteins responsible for the transport and signaling of TrkA, I performed a detailed analysis of the endosomal proteins associated with the TrkA signaling endosome in the axon and in dendrites, as well as describe the endosomal proteins involved with the novel TrkA trafficking event, retrograde transcytosis. I found that in all aspects of TrkA signaling, TrkA was spread among multiple different endosomal compartments and underwent dynamic movements in the dendrites. In my work here, I describe two detailed studies aimed at more thoroughly understanding how the neuronal endosomal system is adapted to the large size and complex morphology by investigating

the possible roles of NEEP21 and identifying somatodendritic retrograde transcytosis as a mechanism for NGF-TrkA signaling endosome diversification in the peripheral nervous system.

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**Table of Contents**

# Chapter I: The Endosomal System in Neurons: NEEP21 and TrkA

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## Introduction to The Endosomal System

Endosomal sorting and transport are integral processes underlying receptor targeting to the correct surface of the cell, signaling, and termination of signaling. Once internalized, cargos entering newly formed endosomes sort back to the membrane locally, to their final membrane destination (i.e. axon/dendrite), back to the Golgi, or traffic towards degradation (**Figure 1**). This first occurs in a "sorting endosome", where the cargo makes a choice to sort into a compartment that determines its final destination (Jovic et al., 2010). This sorting choice is mediated by association of the receptor with a variety of different membrane-associated regulatory proteins which direct the cargo down its correct path (Grosshans et al., 2006). Some of the best-understood endosomal trafficking regulators are the Rab family of small GTPases, and they are widely considered to be the master regulators of membrane trafficking. Activated Rabs play a critical role in vesicle fusion, transport, and maturation via recruitment of specific effectors, and localize to specific subsets of endomembrane compartments. They are thus frequently used as markers to identify different subsets of endosomes (Grosshans et al., 2006) (**Figure 1**). Rabs specifically associate with an intracellular membrane compartment, and over 50 different Rab proteins are found in mammalian cells to regulate a vast array of trafficking pathways (Bucci et al., 2014). They are thus commonly used as markers to identify different subsets of endosomes (Grosshans et al., 2006). Endosomal Rabs are recruited to the cytoplasmic face of different endosomal subtypes and are involved in endosomal fusion, transport, and maturation (Bucci et al., 2014). Commonly used endosomal markers are Rab5 and EEA1 to mark the early endosome, Rab11 to mark the recycling endosome, and Rab7 to mark the late endosome.

Rab proteins are small GTPases that cycle between active (GTP-bound) and inactive (GDP-bound) states. They are regulated by their cognate guanine-nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine dissociation inhibitors (GDIs) (Barr, 2013; Grosshans et al., 2006). Each GEF activates its partner Rab by replacing GDP with GTP, and each GAP deactivates it by activating the intrinsically low GTPase activity of the Rab, leading to hydrolysis of GTP. GDIs can insert or remove GDP-

bound Rab from different membranes, potentially until the Rab encounters its activating GEF (Barr, 2013). In addition to these regulatory molecules, there are many additional proteins which help Rabs carry out specific functions in their GTP state, the so-called "Rab effectors" (Grosshans et al., 2006). Through regulating the GTP state of their specific Rab protein(s), GAPs and GEFs are critical for determining the location, function, and interacting partners of Rab proteins and the cargo in their vesicle.

Different Rabs associate and dissociate from endosomes in a dynamic sequential fashion which is critical for cargo sorting and endosomal maturation. For instance, the early/sorting endosome is marked by Rab5. Rab5-positive early endosomes frequently contain either APPL1 or EEA1 (early endosome antigen 1) (Wandinger-ness and Zerial, 2015). The early endosome results from the homotypic fusion of multiple Rab5 positive endosomes. In order for the fusion of Rab5 vesicles to occur, Rab5 needs various effectors including Early Endosomal Antigen 1 (EEA1) and syntaxin13 (McBride et al., 1999). EEA1 is used in addition to Rab5 to mark the early endocytic compartment, and the two proteins frequently co-localize (Grosshans et al., 2006). In the early endosome, the first steps of cargo sorting can occur through association of the cargo with distinct subdomains. Additionally, the early endosome has a slightly acidic pH at 6.8-5.9 (Huotari and Helenius, 2011). Oftentimes receptors and their ligands can detach in this slightly acidic environment.

The early endosomal network is thought to be a large compartment with many different tubular subdomains enriched in different proteins (**Figure 1**). Cargo is sorted into one of the subdomains, buds off, and is subsequently trafficked in a distinct type of endosome (D'Souza et al., 2014). How cargo chooses which subdomain to enter is not currently well understood. Typically, cargo can either recycle out of the early endosome through a tubular domain, or remain in the vacuolar portion of the early endosome. Intraluminal vesicles (ILVs) start to be generated in the early endosome initiating its maturation to a predegradative Rab7-positive endosome that will ultimately become a late endosome (Jovic et al., 2010).

Recycling cargos either use a fast recycling route via Rab4 or a slower recycling route through Rab11-positive recycling endosomes. Canonical recycling cargo, such as the Transferrin Receptor (TfR), recycle back to the surface of the cell after endocytosis in as fast as 10 minutes (Hémar et al., 1997). TfR does this through being quickly sorted from the early endosome into Rab4 carriers, or by entering perinuclearly located Rab11 positive recycling endosomes (Sönnichsen et al., 2000). Rab11 plays a major role in the recycling of many receptors to the plasma membrane. This ensures correct delivery of cargo to different parts of the cell and is integral to many developmental processes, such as the development of cell polarity by polarized recycling of integrins or other adhesion receptors to the front of the cell. In neurons, recycling endosomes are critical for many processes including long-range targeting of proteins and synaptic plasticity. Budding and tubulation of carriers from sorting endosomes is thus a well-established mechanism by which recycling cargos are saved from entering degradative compartments and are returned to the plasma membrane instead.

Cargos that do not enter recycling carriers in the sorting endosome will be sorted toward a late endosomal fate. The late endosome is commonly defined by the presence of Rab7. In addition to Rab7, late endosomes/lysosomes also contain other characteristic proteins such as LAMPs (lysosome-associated membrane proteins) and lysosomal hydrolases (such as cathepsins), which are responsible for degradation (Klumperman and Raposo, 2014; Scott et al., 2014). *How does an endosome transition from an early to late stage?* It has been shown that endosomes can undergo a Rab5 to Rab7 conversion event (Bucci et al., 2014; Deinhardt et al., 2006; Rink et al., 2005). The Rab5 to Rab7 conversion event is thought to occur rapidly, with an average of 30 min in A431 cells (Rink et al., 2005). Rab5 can recruit Rab7 onto the membrane, and it is thought that Rab7 inhibits Rab5 GTP exchange to force Rab5 off the membrane and enrich it in Rab7 (Huotari and Helenius, 2011). While the early endosome structurally resembles a network of tubules with various vacuolar domains, late endosomes and lysosomes have a very different morphology (**Figure 1**). Late endosomes are morphologically characterized by having intralumenal vesicles (ILVs) surrounded by a limiting membrane, forming what is called a multivesicular body (MVB) (Klumperman

and Raposo, 2014). ILVs form through portions of the limiting membrane budding into the lumenal space, a process requiring the ESCRT complex proteins (Hanson and Cashikar, 2012; Henne et al., 2013). Late endosomes are also characterized by having a lower pH than early endosomes (6.0-4.9). Late endosomes ultimately fuse with lysosomes, which are filled with electron-dense material and membranes. Cargos delivered to the lysosome are degraded by acidic hydrolases active at the low acidic pH. MVBs are, therefore, typically thought of as intermediate endolysosomal compartments on their progression to the lysosome (Klumperman and Raposo, 2014; Woodman and Futter, 2008).

Intriguingly, while the canonical role of lysosomes is degradation, there is clear evidence that in many cell types, lysosomes can themselves be secretory. Here, lysosomes (MVBs) are able to fuse with the plasma membrane and secrete their ILVs as exosomes. Rab3 and Rab27a have been shown to play a critical role in endolysosomal secretion, including of exosomes (Blott and Griffiths, 2002; Chiang et al., 2011; Lettau et al., 2015). While this has mainly been studied in hematopoetic cell types and cancer, it is likely that this can occur in other cells, including neurons (Arantes, 2006).

**Neuron-Enriched Endosomal Proteins (NEEP21)**

Given the polarized state of neurons and the long distances that endosomes must travel, neurons have adapted special mechanisms to deal with these challenges. One way neurons can deal with the heightened burden of protein trafficking across long distances is through expression of a set of neuronal specific endocytic proteins (Yap and Winckler, 2012). One such protein is Neuron Enriched Endosomal Protein of 21kd (NEEP21, Nsg1). NEEP21 is a small endosomal protein with a single transmembrane domain (Steiner et al., 2002). It is highly enriched in neurons, which makes it a likely candidate to carry out neuron-specific protein trafficking events.

NEEP21 is a member of a family of small single-pass transmembrane proteins including Calcyon (Caly) and P19. The NEEP21/Caly/P19 family is present in all vertebrates, with only NEEP21 and P19 being expressed in bony fish (Muthusamy et al., 2009). Each protein in this family is expressed highly in the brain with low expression levels in peripheral tissues. This family of proteins is present on endosomal membranes, although each member may play its own specific role (Muthusamy et al., 2009). For example, Caly is expressed in axons and the somatodendritic region, whereas NEEP21 is restricted to the somatodendritic region (Xiao et al., 2006). The protein level of P19 does not change in the NEEP21 KO mouse, suggesting that there is no compensation for loss of NEEP21 by upregulating P19 (CCY and LM, unpublished), but whether or not P19 and NEEP21 play distinct or overlapping roles in neurons is not well established.

NEEP21 was identified as being a binding partner for the SNARE Syntaxin 13 (Steiner et al., 2002). Syntaxin13 plays a major role in the fusion of early endosomal vesicles (Brandhorst et al., 2006). In cultured hippocampal neurons, NEEP21 is specific to the somatodendritic compartment and is rarely seen in axons. However, it plays a major role in the anterograde trafficking of L1/NgCAM. L1/NgCAM is a cell adhesion molecule that is specifically localized to the axon. L1/NgCAM is initially sorted to the somatodendritic compartment where it is re-internalized and targeted to the axon from endosomes (Wisco et al., 2003), a pathway referred to as transcytosis. When NEEP21 is knocked down, L1/NgCAM is incorrectly targeted to the soma and dendrites. In addition to causing mis-localization of L1/NgCAM, NEEP21 downregulation also caused missorting of L1/NgCAM endosomes to the lysosome (Yap et al., 2008). Correct transcytosis of L1/NgCAM to the axon is thus dependent on NEEP21.

The importance of NEEP21 in the somatodendritic region is not restricted to transcytosis, but includes regulation of synaptic receptors as well. AMPA receptors (AMPAR) are crucial for long term potentiation (LTP) and long term depression (LTD). AMPARs are composed of different subunits including GluR1-4. AMPAR subunits differ in their cytoplasmic tails, which allows each subunit to be differentially trafficked.

For example, GluR2 binds to the glutamate receptor interacting protein-1 (GRIP1). GRIP1 facilitates the surface expression and recycling of GluR2 (Mohrlüder et al., 2009). NEEP21 was shown to bind GRIP1 and form a complex with GRIP1 and GluR2 (Steiner et al., 2005). NEEP21 downregulation caused suppression of GluR2 recycling to the membrane, showing an importance for NEEP21 in recycling of synaptic proteins (Alberi et al., 2005; Steiner et al., 2005). As a consequence of aberrant GluR2 trafficking, LTP in hippocampal slices is also impaired after NEEP21 knockdown (Alberi et al., 2005).

In addition to L1/NgCAM transcytosis and GluR2 recycling, NEEP21 is also involved in the recycling of the neurotensin receptors (Debaigt et al., 2004). The neurotensin receptors are G-protein coupled receptors that bind the neuropeptide neurotensin (NT). Neurotensin receptor 2 (NTSR2) recycles back to the membrane after binding NT, but neurotensin receptor 1 (NTSR1) is sent directly to the lysosome without recycling (Mazella and Vincent, 2006). NEEP21 downregulation was shown to attenuate NSRT2 recycling, and overexpression of NEEP21 causes aberrant recycling of NTSR1.

While this evidence points towards a role for NEEP21 as a cargo regulator in a specific endosome, potentially analogous to the Rab proteins, our lab recently showed that NEEP21 does not localize to a specific endosomal compartment but rather rapidly traverses early and late endosomes. NEEP21 has a short half-life of only 90 minutes, and moves rapidly through the endosomal pathway towards degradation (Yap et al., 2017). NEEP21 is thus not a resident protein of endosomes, but behaves as a rapidly degrading cargo. How a protein that itself moves so rapidly through the endosomal system to be degraded can affect other proteins is still an open question.

NEEP21 thus appears to regulate the axonal targeting of L1/NgCAM through transcytosis, and to prevent receptors from fusing with the lysosome and instead recycling them back to the membrane. These broad functions are critical for many other receptors as well, including the neurotrophin receptor, TrkA. TrkA is a receptor tyrosine kinase that is critical for patterning of the sympathetic nervous system. TrkA is also

targeted to the axon through transcytosis (Ascano et al., 2009). This pathway might therefore also require specific protein machinery to ensure that a newly endocytosed naïve TrkA receptor is not targeted to the lysosome but sent to the axon.

**The role of TrkA in patterning the sympathetic nervous system**

The sympathetic nervous system is composed of a homogenous group of neurons responsible for the "fight-or-flight" response. These neurons project all over the body to maintain homeostasis and respond to external cues. The cell bodies of sympathetic neurons sit together in ganglia just outside the spinal cord. During development, these neurons extend a neurite out into the periphery until they reach their final target, such as the eye or the heart. Once the final target is reached, the neurites stop extending and begin to branch, leading to full innervation of the target tissue. This results in an intricate network of neurons stretching throughout the body with neurons as long as one meter in humans. The development and maintenance of this system is tightly controlled by many factors. One major player is the neurotrophin family of secreted factors.

Neurotrophins are a family of secreted growth factors that are necessary for the correct patterning of the nervous system (McAllister, 2001), and especially important for the survival of peripheral neurons (Huang and Reichardt, 2001). There are 4 mammalian neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). NGF was the first of this family to be identified, and its function is the most well described. NGF is secreted from a variety of sources including a peripheral neuron's target organ, which ensures survival of its innervating nerves (Patapoutian and Reichardt, 2001). In this way, the target tissue determines its own innervation.

The concept of a target tissue directing its own nerve innervation was first proposed in 1924. S. R. Detwiler used ambylostoma to show that a transplanted limb bud can develop into a functional limb and acquires

peripheral nerve innervation (Detwiler, 1924). This suggested that something was emanating from the transplanted limb to provide a cue for peripheral nerve innervation. This finding was later followed up by Viktor Hamburger who showed that the extirpation of a chick embryo's limb bud resulted in the underdevelopment of that side of the body's spinal nerves (Hamburger, 1934). The existence of a secreted factor which is responsible for this innervation was probable, however it was also possible that either the existing nerves from the transplant or the severed nerves in the amputated limb could cause this hyper- and hypoplasia respectively. It was not until Elmer Bueker and Rita Levi-Montalcini showed in 1948 (Bueker, 1948) and 1951 (Levi-Montalcini and Hamburger, 1951), respectively, that a secreted factor was responsible for nerve innervation. This factor was subsequently purified by Stanley Cohen in 1960 and is named nerve growth factor (NGF) (Cohen, 1960).

The receptor for NGF was identified in a screen for tropomyosin-fused oncogenes and was named Tropomyosin-Related Kinase (Trk). The Trk family of receptor tyrosine kinases (RTKs) include TrkA, TrkB, and TrkC which bind with high affinity to NGF, BDNF/NT-4, and NT-3 respectively. The most widely studied neurotrophin-Trk receptor interaction is NGF and TrkA. The Trk receptors are historically thought of as receptors that provide survival signaling for the cell, while p75 is thought to induce death signaling. However, it is becoming increasingly clear that there are alternative functions of each receptor, including p75's ability to induce cell survival and axon branching, and TrkA's ability to induce cell death (Cheng et al., 2018; Fei et al., 2014; Harel et al., 2010).

Neurotrophins are produced by many different sources including sensory and sympathetic target tissue, fibroblasts, Schwann cells, mast cells, blood vessels, and even peripheral neurons themselves (Patapoutian and Reichardt, 2001). In the case of sympathetic innervation, NGF is secreted in an amount proportional to the final target innervation by peripheral nerves (Yuen et al., 1996). This accounts for the naturally occurring cell death seen in the developing peripheral nervous system: neurons compete for NGF, and only those neurons that receive adequate amounts of NGF and induce TrkA signaling will survive. NGF

production and secretion by the target tissue therefore sets up a model of competition where approximately 50% of sympathetic neurons survive (Deppmann et al., 2008).

NGF is secreted at the final target, but survival signaling occurs in the soma. This means that the NGF signal must travel a great distance retrogradely along peripheral axons, which can be up to one meter long. Evidence is now overwhelming that NGF-dependent signaling is propagated from the distal axon to the soma through physical translocation of the NGF-TrkA complex in an endosome. Upon encountering NGF in the distal axon, TrkA binds to NGF and the complex is internalized into endosomes. This endosome is then transported retrogradely by dynein to the cell body (Heerssen et al., 2004), where it elicits survival signaling (Riccio et al., 1999, 1997). These "signaling endosomes" (SEs) contain activated TrkA and are associated with adaptors and effector proteins that mediate downstream signaling cascades (Delcroix et al., 2003; Grimes et al., 1996; Harrington et al., 2011; Watson et al., 2001).

## Importance of NGF/TrkA Signaling

NGF/TrkA signaling is responsible for the survival of sympathetic neurons in multiple ways. When NGF binds to TrkA, TrkA dimerizes, autophosphorylates, and is internalized into an endosome with NGF still attached. Once internalized, the signaling endosome is trafficked back to the soma. NGF/TrkA is able to promote neuronal survival through transcription-dependent and -independent mechanisms. Many of the transcription-dependent mechanisms are a result of the phosphorylation of cAMP response element-binding protein (CREB). CREB is a transcription factor which becomes phosphorylated and is subsequently able to regulate the expression of a variety of genes. CREB phosphorylation is necessary for NGF-induced survival of sympathetic neurons (Kaplan and Miller, 2000). In addition to CREB, MEFD2 is another transcription factor that is key for NGF/TrkA survival signaling (Pazyra-Murphy et al., 2009). MEF2D mediates the transcription of the anti-apoptotic protein bcl-w, which is a key event in sympathetic neuron survival (Pazyra-Murphy et al., 2009).

Survival signaling involves intracellular and extracellular signaling. Upon NGF stimulation, sympathetic neurons transport new TrkA to the axon (Ascano et al., 2009), as well as increase the transcription of TrkA in a feed-forward mechanism to enhance the availability of TrkA (Deppmann et al., 2008). In this way, TrkA up-regulates itself in response to target-derived NGF; it thus amplifies its own signal. Additionally, TrkA/NGF signaling results in the synthesis and release of BDNF and NT-4 by sympathetic neurons. The only neurotrophin receptors that are expressed by sympathetic neurons are TrkA and p75. Because TrkA doesn't bind either BDNF or NT-4, these neurotrophins will bind to the low affinity receptor, p75, to induce apoptosis in neighboring neurons. In this way, TrkA and p75 signaling antagonize each other. Depending on whether a cell receives more signaling from p75 or TrkA, apoptosis or survival, respectively, will result (Deppmann et al., 2008).

Once a sympathetic neuron's axon reaches its final target, it must first survive and second innervate the target. NGF/TrkA signaling is responsible for axonal branching both *in vitro* and *in vivo*. NGF/TrkA signaling regulates both axonal extension along blood vessels and branching upon reaching the final target tissue. NGF synthesis and release in development of the sympathetic system is confined to the tissues that require sympathetic innervation. Indeed, NGF expression by tissues that do not normally secrete it leads to an inappropriate innervation of the tissue (Edwards et al., 1989). Not only does NGF/TrkA signaling need to be present in the correct locations for target innervation, but its levels are tightly controlled. If the NGF/TrkA signal is degraded too quickly, fewer sympathetic neurons survive (as determined in the Superior Cervical Ganglion, SCG) and innervation is dampened (Suo et al., 2013).

NGF/TrkA signaling is also needed for the neurons of the SCG to make connections with their preganglionic inputs (Lehigh et al., 2017; Sharma et al., 2010). While the axons of SCG neurons are extending and branching in the periphery, the cell bodies and dendrites of SCG neurons are sitting within the ganglia and are being innervated by preganglionic neurons sitting in the spinal cord. The axons of the

preganglionic neurons make synaptic connections with the dendrites of SCG neurons within the sympathetic ganglia. In order for these connections to properly form, zones of post-synaptic density (PSD) must be present on the dendrites of the SCG neurons receiving input. In this case, the PSD is present before the pre-synaptic axon makes contact. As mentioned above, NGF/TrkA signaling endosomes travel retrogradely from the distal axon back to the soma to signal transcriptional changes and survival, but they additionally were found to enter dendrites. Within the dendrite, SEs are actively signaling and are located close to PSD clusters (Lehigh et al., 2017). In the dendrite, TrkA is responsible for organizing pre-existing components of PSDs into clusters within the dendrites to be available for axonal input (Lehigh et al., 2017; Sharma et al., 2010). This is true *in vivo* as well, and inhibition of TrkA signaling in adulthood within the SCG causes loss of PSD clusters and ptosis, or drooping of the eyelid (Lehigh et al., 2017). Thus, NGF/TrkA signaling is responsible for the formation of the whole functional circuit from the spinal cord all the way to the peripheral target organ.

## Endosomal Sorting of TrkA

TrkA trafficking can be subdivided into several distinct events (**Figure 2**). First, TrkA can undergo constitutive endocytosis and recycling in the soma in the absence of ligand. Upon NGF signaling, these endosomes are transported anterogradely to the axon (Ascano et al., 2009). Second, in the axon, TrkA encounters NGF and endocytoses into a retrogradely-transported signaling endosome (Howe et al., 2001; Ye et al., 2003). Lastly, the signaling endosome can be exocytosed on the soma membrane and subsequently re-internalized (Barford et al., 2018; Suo et al., 2013).

TrkA has been shown to be present in a number of endosomal compartments. This is perhaps not surprising considering it undergoes 3 distinct internalization and trafficking events. Given the novelty of retrograde transcytosis (Suo et al., 2013), its specific Rab partners had not been well characterized until just recently (Barford et al., 2018). On the other hand, anterograde transcytosis and activated TrkA/NGF travelling

retrogradely to the soma have both been investigated. Given the different populations of TrkA endosomes, it has been a difficult task to parse apart which endosomes are associated with which trafficking event, especially through endosomal isolation and proteomics.

Upon endocytosis, cargo is initially sorted in an early endosome, which is Rab5-positive. Interestingly, phosphorylated TrkA actively decreases Rab5 activity through activation of RabGAP5. The hypothesis is that TrkA escapes the early endosome network in an effort to continue its signaling and trafficking back to the soma (Bucci et al., 2014). Indeed, a constitutively active Rab5, or the expression of its GEF Rabex-5, inhibits neurite outgrowth in PC12 cells (Liu et al., 2007), an NGF-dependent process. This means that while TrkA/NGF associates with Rab5 endosomes, it does not stay there for long. This is consistent with work done with TrkB, showing that while TrkB is first internalized into a Rab5-positive early endosome, it is sorted into a Rab7-late endosome for retrograde transport (Deinhardt et al., 2006).

In addition to early endosomes, TrkA has been shown to co-localize with recycling endosomes both anterogradely moving towards the distal tip of the axon (Ascano et al., 2009), retrogradely back to the soma (Barford et al., 2018), and in dendrites (Barford et al., 2018). TrkA is transported anterogradely to the axon in a Rab11 positive recycling endosomes. In 2009, Ascano et. al. showed that this transport vesicle originates in the soma in response to neurotrophin signaling (Ascano et al., 2009). TrkA undergoes constitutive ligand-independent endocytosis and recycling in the somatodendritic region, but not in the axon, and this allows many TrkA molecules to be readily available in the soma for transport to the axon. Constitutively recycling TrkA provides neurons with a pool of TrkA that can be rapidly mobilized to enter the axon in response to neurotrophin. This happens through a feed-forward mechanism whereby NGF/TrkA signaling recruits more TrkA to the axon (Ascano et al., 2009), a pathway referred to as anterograde transcytosis. Recently, our lab has shown that Rab11 is also responsible for the retrograde transport of a significant pool of signaling endosomes in axons. This work will be discussed in Chapter 3.

In addition to early endosomes and recycling endosomes, TrkA has also been shown to be present in late endosomes, which are marked by the presence of Rab7 (Zhang et al., 2013). In peripheral neurons, a Rab5 to Rab7 switch appears to underlie this conversion (Deinhardt et al., 2006). Although, while Rab7 compartments are usually trafficked directly to the lysosome, late endosomes containing NGF/TrkA manage to escape acidification and continue signaling. In DRG neurons, Rab7 mutants responsible for Charcot-Marie Tooth Disease Type 2B (CMT2B) are deficient in TrkA trafficking. Retrogradely travelling TrkA co-localizes with Rab7, and Rab7 CMT2B mutants display a delay in TrkA trafficking (Zhang et al., 2013). Interestingly though, retrograde TrkA endosomes do not sort immediately to degradation, because they are able to maintain signaling status in the soma (Reviewed in Barford et al., 2017a). This may in part be the effect of other proteins acting on the TrkA endosome to help it avoid lysosomal fusion. One such protein, Coronin1a, was shown to associate with the TrkA signaling endosome upon arrival in the cell body and prevent its lysosomal fusion (Suo et al., 2013).

The signaling and trafficking events that happen upon TrkA endocytosis are important for multiple reasons. TrkA-NGF binding induces 3 major signaling cascades: PI3K, PLCgamma, and Ras/MAPK (**Figure 3**). Most obviously, the signal from the TrkA/NGF signaling endosome is responsible for keeping a cell alive during a time of neuronal pruning (Huang and Reichardt, 2001). It protects cells against pro-death factors being secreted from neighboring cells as well as providing its own survival signaling (Deppmann et al., 2008). Additionally, the signaling that happens during endocytosis may direct the subsequent trafficking of the receptor. This is most evident in the case of differential signaling between NT-3 and NGF on TrkA. NT-3 is secreted by intermediate targets (most commonly blood vessels), and NGF is secreted by the final target. NGF mediates cell survival and neuronal branching at the final target, whereas NT-3 cannot (Harrington et al., 2011). Interestingly, NGF induces Rac1 activity when bound to TrkA, but NT-3 does not. Rac1 is a small GTPase involved in cytoskeletal rearrangements. This selectivity between NGF and NT-3 is thought to occur due to the fact that the NGF-TrkA association is more stable in the slightly acidic environment of the early endosome, whereas NT-3 becomes dissociated from TrkA under the same

condition (Harrington et al., 2011). In this way, it is thought that TrkA controls its own trafficking depending on the strength of the attachment to its ligand and potentially its activation state.

In summary, TrkA undergoes 3 distinct endocytic events and participates in multiple trafficking events. In addition to association with Rab4, Rab5, Rab7, Rab11, and Rab22, TrkA is also associated with many other trafficking molecules not mentioned above. A major open question is whether and how endosome formation, trafficking, maturation and identity affect the specific signaling outcomes for TrkA, and whether there are functionally distinct sets of signaling endosomes that differ in terms of location, signaling, and associated effectors. In addition, it is largely still unknown what regulators allow TrkA to undergo multiple internalization events with differential outcomes. Since axon extension, survival, and synapse formation are all NGF-dependent in sympathetic neurons, a major challenge in the field is to determine whether particular trafficking or signaling events underlie a particular developmental outcome.

**Effectors and regulators of Rab proteins – why the Rab "flavor" matters**

How is the identity of the signaling endosome functionally relevant? There are many different Rabs, and each one has its own set of effector proteins that can associate with it at a given time (Wandinger-ness and Zerial, 2015). In their active GTP-bound state, they recruit a variety of effectors to the endosome (Bucci et al., 2014; Hutagalung and Novick, 2011), which then can affect the behavior of this endosome. Essentially, each Rab protein can give the same signaling endosome a different set of effector proteins, thus providing a direct way of diversifying the pool of signaling endosomes.

Rab effectors fall into several classes: effectors that recruit proteins that promote tethering and fusion with other compartments, recruit motor proteins that can move the compartment to a different location in the cell, recruit proteins that regulate cargo inclusion and coat protein association for vesicle formation, and recruit proteins that regulate the actin cytoskeleton. Therefore, the Rab that is present on a specific

endosome determines whether or not to include a particular cargo into the carrier, to bud off a carrier, to fuse with another compartment, to recycle or degrade, and to move to another place in the cell. For example: (1) Both Rab5 and Rab7 can link to the retrograde dynein motors via one set of adaptors, or link to kinesin motors via a different set of adaptors (Zhen and Stenmark, 2015); (2) Rab11 can link to kinesin, but also to the actin motor myosin Vb (Schafer et al., 2014) ; (3) Rab5 recruits tethering factors, such as EEA1 (Callaghan et al., 1999; Simonsen et al., 1998); (4) Rab7 recruits HOPS complex and ESCRT to promote maturation of intralumenal vesicles (Lin et al., 2015; Wang and Hong, 2006). TrkA has been shown to traffic in Rab5-, Rab11- or Rab7-endosomes, and TrkA-containing endosomes are thus likely to have different association with SNARE fusion machinery, kinesin or dynein motors, or association with actin filaments (Barford et al., 2018; Delcroix et al., 2003; Suo et al., 2013; Zhang et al., 2013) depending on the associated Rab. An endosome's association with SNARE fusion machinery, motors, and the cytoskeleton can affect its trafficking fate (Wojnacki and Galli, 2016). Signaling downstream of TrkA can thus be regulated in terms of duration and location by means of either avoiding (or not) fusion with lysosomes, moving retrogradely in axons, moving into dendrites, or fusing with other compartments or the plasma membrane. Whereas the association and significance of regulating association with motor proteins via Rabs in terms of regulating neurotrophin signaling is well established, how regulating fusion with other compartments (such as lysosomes or the plasma membrane) requires Rab-mediated regulation of SNARE function is not well established. What is clear, though, is that the regulation of trafficking of TrkA through different endosomal compartments affects its signaling output.

Conversely, TrkA signaling can also affect its trafficking. One example of this is that Rabs are activated and inactivated by proteins which themselves are subject to regulation. When, where and for how long a particular Rab protein is active thus regulates trafficking downstream of signaling. Rabs are activated by GEF proteins (GDP exchange factor) that exchange GDP for GTP on the Rab. They are inactivated by GAP proteins (GTPases activating proteins) that accelerate the intrinsic GTPase activity of Rabs (Barr and Lambright, 2010). There are a large number of both GEFs and GAPs, and while their regulation is currently

poorly understood, they clearly constitute a rich field of exploration for the future. For example, several Rabs are regulated by phosphorylation: Rab5b is a substrate for the kinase LRRK2 (which is implicated in Parkinson's disease) (Yun et al., 2015) and Rab11 is regulated by the kinase LMTK1 in a cdk5-dependent manner (Takano et al., 2012). Kinase signaling cascades are thus upstream of activating Rab proteins.

As described above, several Rab proteins have been implicated in Trk receptor trafficking (Ascaño et al., 2009; Delcroix et al., 2003; Harrington et al., 2011; Suo et al., 2013; Zhang et al., 2013). Even though the precise association both spatially and temporally with the correct Rab protein is critical for Trk receptor function, and disruption of this association causes disruption of NGF-dependent processes, the exact time and place of how various Rabs regulate NGF-TrkA trafficking and signaling are still incompletely understood.

There are over 50 Rabs in the mammalian genomes and little is known about most of them in any cell type. The best studied endosomal Rabs (Rab4, 5, 7, 8, and 11) have mostly been investigated in fibroblasts. In neurons, somatic endosomes resemble the endosomes in fibroblasts in many respects. Even though endosomes in axons and in distal dendrites are presumed to be similar to the somatic ones, there are some clear differences between axonal and somatodendritic endosomes. Little is known about the functional consequences of these differences. One of the known differences is with respect to early endosomes. In the soma and dendrites, Rab5 and EEA1 are present. In the axon, on the other hand, EEA1 is not present, but Rab5 is still associated with early endosomes (Wilson et al., 2000). Some neuronal-specific proteins, such as the small transmembrane protein NEEP21, are also excluded from axonal endosomes and only found in somatodendritic endosomes (Lasiecka et al., 2014; Yap et al., 2008).

Endocytosed cargo first enters the early endosome before being sorted to its ultimate fate. This remains true for TrkA as well. NGF-bound TrkA might use multiple endocytic entry routes, including clathrin-mediated endocytosis (Howe et al., 2001) as well as an unusual route mediated by the EHD family member Pincher (also known as EHD4) (Valdez et al., 2005). Rab5 and EEA1 are common markers of the early endosome, and TrkA has been shown to associate with Rab5 (Valdez et al., 2005). In the case of Pincher-mediated endocytosis in the axon, TrkA/NGF was shown to be present in multivesicular bodies (MVBs) together with Rab5 (Valdez et al., 2005). Interestingly, others have also shown TrkA to travel in the axon in MVBs (Bhattacharyya et al., 2002; Ye et al., 2018), however MVBs are not typically marked by early endosomal proteins such as Rab5. Some unresolved questions thus remain with regard to the identity of the retrograde carrier that transports activated NGF-TrkA to the soma, and these will be explained and explored in Chapter 3.

**Conclusion**

It appears then that a multitude of TrkA trafficking events contribute to NGF-TrkA function. A major open question is whether and how endosome formation, trafficking, maturation and identity affect the specific signaling outcomes for TrkA, and whether there are functionally distinct sets of signaling endosomes that differ in terms of location, signaling, and associated effectors. The endosomal system is complex, and even more so in neurons. Neurons are exceptionally dependent on endosomal trafficking, and many neurological diseases have their bases in dysfunction of the endosomal system. Examining “neuronal endosomology” through neuron-specific components, such as NEEP21, and receptors, such as TrKA, are critical to understanding the basic biology behind neurons’ longevity and polarity.

**Figures**

**Figure 1.** An overview of the endosomal system.

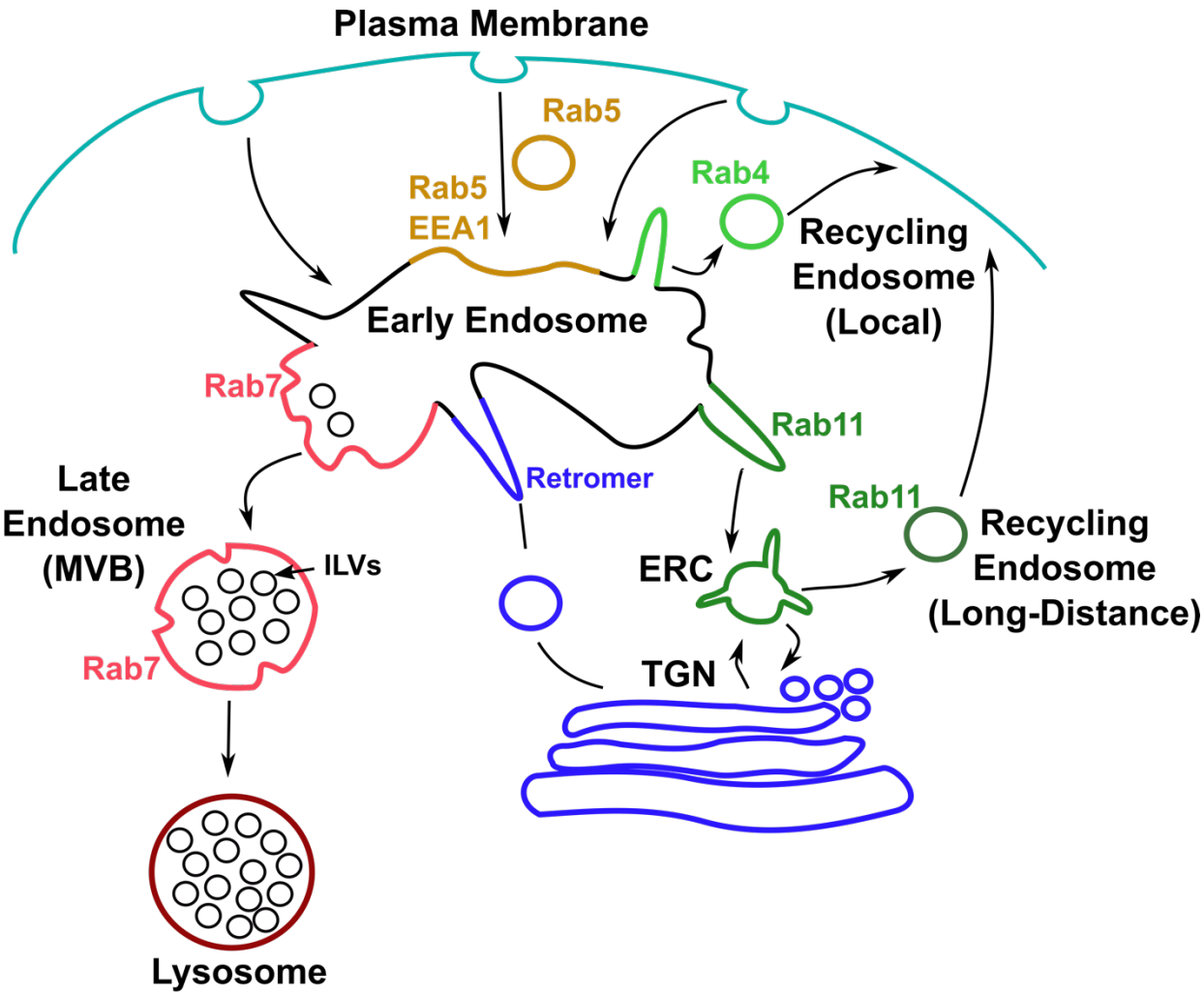


**Figure 2.** TrkA undergoes complex trafficking events. (1) After synthesis, TrkA can be deposited on the somatodendritic membrane where it undergoes constitutive endocytosis before anterograde transcytosis to the axon. (2) Upon NGF binding, TrkA and NGF are internalized into a signaling endosome and transported retrogradely back to the soma. (3) The TrkA signaling endosome can be exocytosed onto the somatodendritic surface and subsequently re-endocytosed.

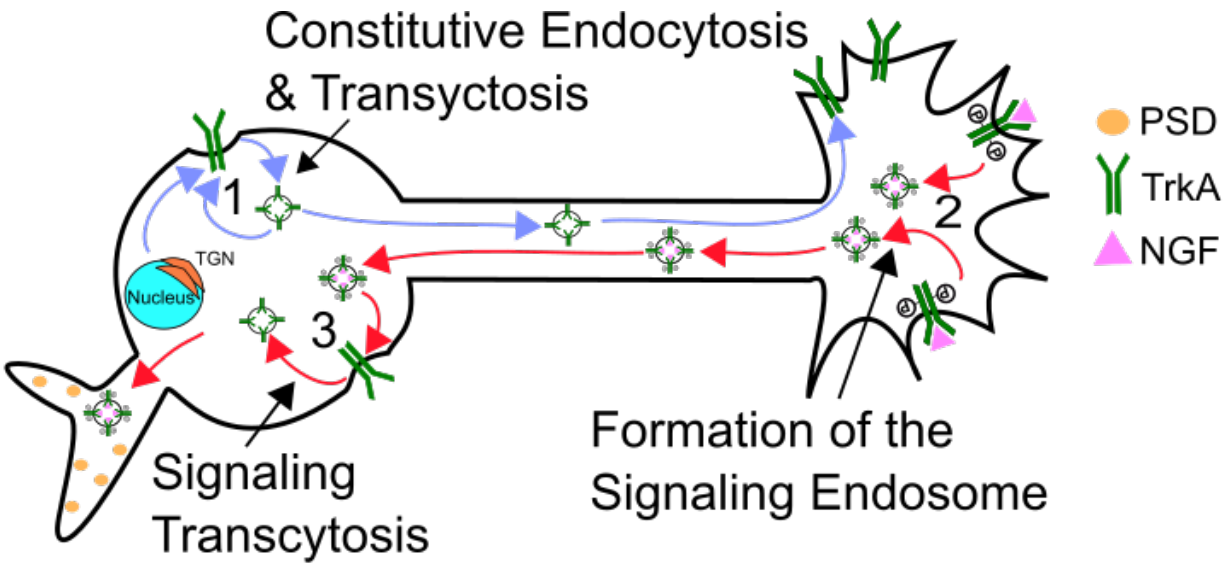
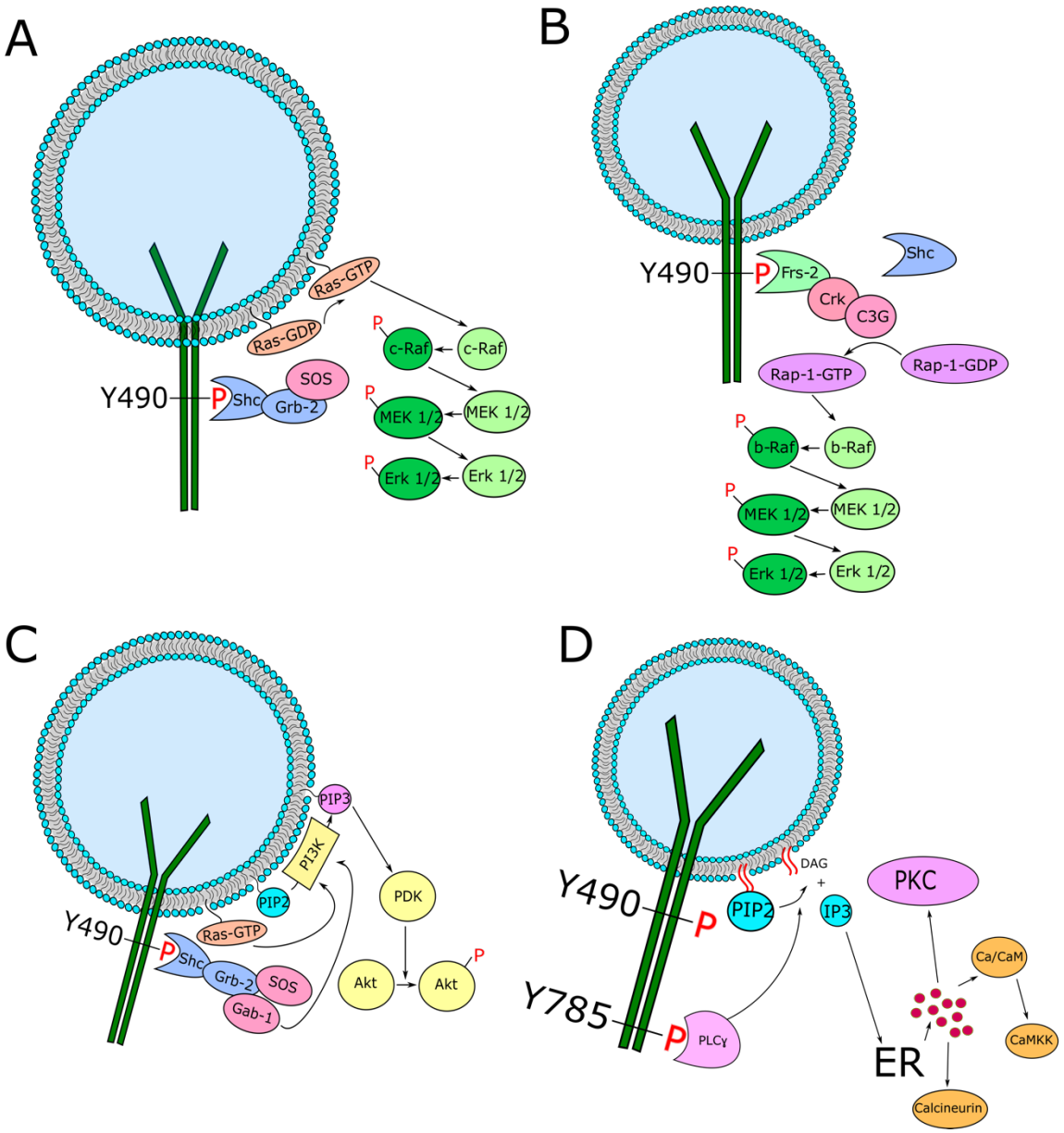


**Figure 3.** TrkA signaling cascades. (A) Transient Ras/Erk signaling. (B) Prolonged Ras/Erk signaling. (C) PI3K signaling. (D) PLC-gamma signaling.



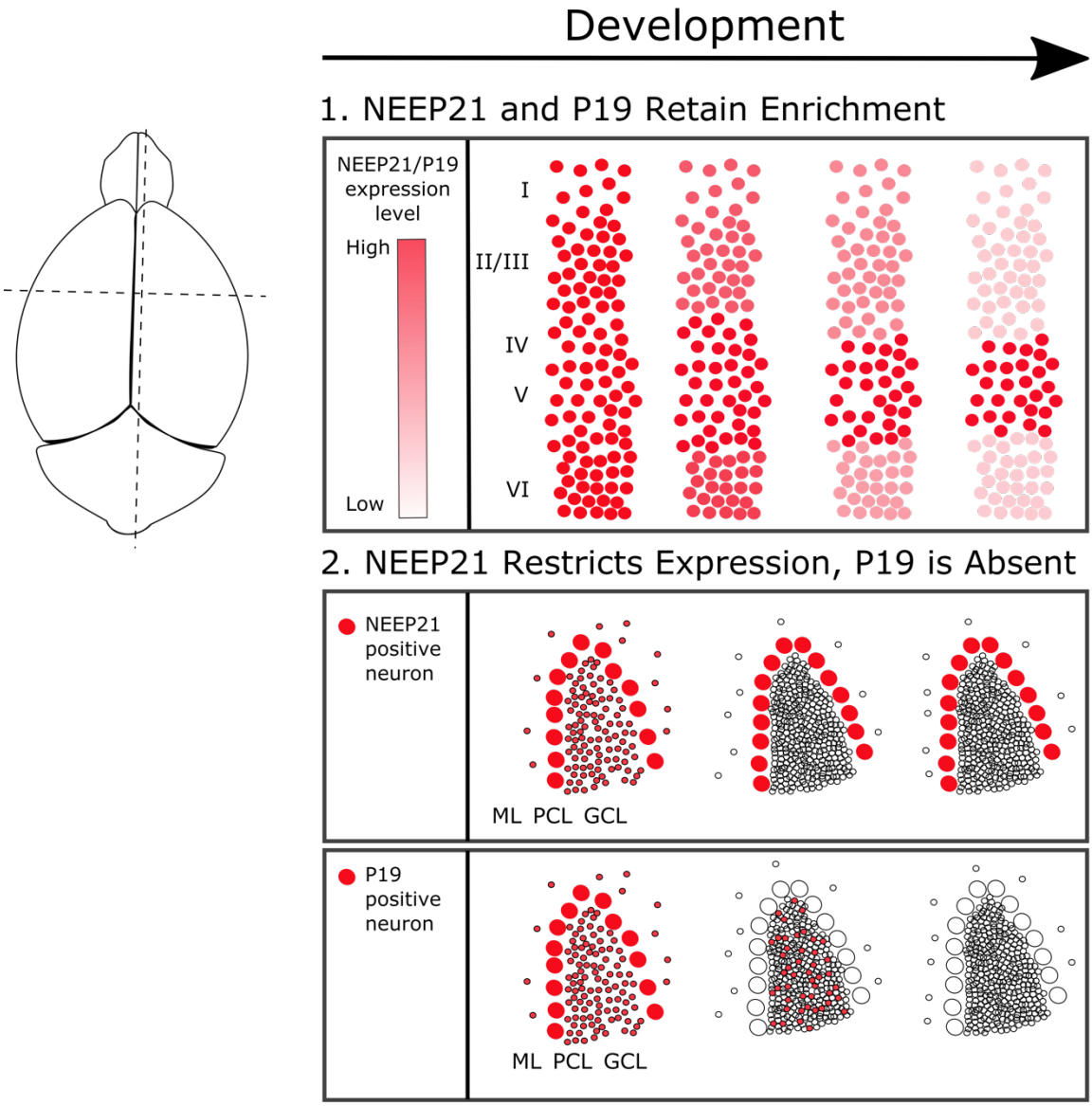
# Chapter II: The related neuronal endosomal protein NEEP21 (Nsg1) and P19 (Nsg2) have divergent expression profiles *in vivo*

This chapter is published as "The related neuronal endosomal proteins NEEP21 (Nsg1) and P19 (Nsg2) have divergent expression profiles in vivo". Kelly Barford, Chan Choo Yap, Noelle D. Dwyer, Bettina Winckler. *Journal of Comparative Neurology.* 2017;525(8):1861-1878. All experiments except Figure 1B were carried out by me.

**Abstract**

Endosomal maturation and transport constitutes a complex trafficking system present in all cell types. Neurons have adapted their endosomal system to meet their unique and complex needs. These adaptations include repurposing existing proteins to diversify endocytosis and trafficking, as well as preferential expression of certain regulators more highly in neurons than other cell types. These neuronal regulators include the family of Neuron-Specific Gene family members (Nsg), NEEP21 (Nsg1) and P19 (Nsg2). NEEP21/Nsg1 plays a role in the trafficking of multiple receptors, including the cell adhesion molecule L1/NgCAM, the neurotransmitter receptor GluA2, and β-APP. Recently, we showed that NEEP2/Nsg1 and P19/Nsg2 are not expressed in all neuronal cell types *in vitro*. However, it is not known where and when NEEP21/Nsg1 and P19/Nsg2 are expressed *in vivo,* and whether both proteins are always co-expressed. Here, we show that NEEP21/Nsg1 and P19/Nsg2 are present in both overlapping and distinct cell populations in the hippocampus, neocortex, and cerebellum during development. NEEP21/Nsg1 and P19/Nsg2 levels are highest during embryonic development, and expression persists in the juvenile mouse brain. In particular, a subset of layer V cortical neurons retains relatively high expression of both NEEP21/Nsg1 and P19/Nsg2 at postnatal day 16 as well as in the CA1-3 regions of the hippocampus. In the cerebellum, NEEP21/Nsg1 expression becomes largely restricted to Purkinje neurons in adulthood whereas P19/Nsg2 expression strikingly disappears from the cerebellum with age. This divergent and restricted expression likely reflects differential needs for this class of trafficking regulators in different neurons during different stages of maturation.

**Graphical Abstract**



## Introduction

Neurons are among the most morphologically complex cells in the body. This complexity manifests on two fronts. First, neurons are extremely large in size and extend axons and dendrites over long distances. A neuron's soma is roughly the size of an epithelial cell, and neuronal axons can extend up to 1 m in length in humans. Second, neurons have a highly polarized morphology with distinct functional domains, axons and dendrites, which are molecularly distinct. Many proteins are found in one domain but not the other (Barnes and Polleux, 2009). This complexity requires both that proteins be transported over long distances during development and throughout life, and also that proteins need to be accurately sorted to the correct location in this very large cell (Winckler, 2016). These special requirements for protein transport have resulted in many neuronal adaptations in terms of cytoskeleton and membrane transport in order to meet a neuron's specific needs (Yap and Winckler, 2012). Lastly, neurons are post-mitotic and among the longest lived cells in the body. This long lifetime means that any problems due to mis-trafficking or dysregulation of recycling and degradation have particularly devastating effects.

Many proteins are highly enriched or even specifically expressed in neurons. These include proteins that fundamentally underlie neuronal synaptic function, such as neurotransmitter receptors, but also cytoskeletal proteins and proteins regulating membrane transport. One such protein is Neuron Enriched Endosomal Protein of 21kDa (NEEP21/Nsg1), a small single-pass transmembrane protein that is highly enriched in neurons (Ohnishi et al., 2010; Sabéran-Djoneidi et al., 1998). Interestingly, NEEP21 is restricted to the somatodendritic domain (Steiner et al., 2002; Yap et al., 2008).

NEEP21 has been shown to play a critical role in the trafficking and polarization of a variety of proteins including the axonal cell adhesion molecule L1/NgCAM, β-APP, GluA2, and neurotensin receptors

(Debaigt et al., 2004; Norstrom et al., 2010; Steiner et al., 2002, 2005; Yap et al., 2008). When NEEP21 is knocked-down in cultured neurons, L1/NgCAM becomes mislocalized to the somatodendritic region and to LAMP2-positive endosomes (Yap et al., 2008). Missorting of cargo into LAMP2-positive endosomes in the absence of NEEP21 is also observed for neurotensin receptor and for GluA2. This dependence of correct protein trafficking on neuronal proteins specific to certain domains of the neuron highlights the complexity of the endosomal sorting machinery in neurons.

NEEP21 belongs to a family of endosomal proteins including Calcyon (Caly) and P19 (Nsg2) (Muthusamy et al., 2009). NEEP21 and P19 show approximately 50% amino acid sequence homology to each other, and 30% to Calcyon. NEEP21 and P19 were both identified as being highly enriched in the brain and developmentally regulated (Sabéran-Djoneidi et al., 1998). NEEP21 has been detected in rat brains at high levels up to P14, at which point the protein levels decline greatly (Steiner et al., 2002).

We recently showed that, surprisingly, NEEP21 and P19 were not expressed in all neurons cultured from embryonic rat hippocampus. Virtually all CTIP2- and Satb2-positive neurons in hippocampal cultures expressed both NEEP21 and P19 robustly. In contrast, both NEEP21 and P19 were expressed at very low levels in Prox1-positive cells, which are likely derived from the dentate gyrus (Digilio et al., 2015). The differential expression found in cultured neurons thus prompted us to ask 1) if NEEP21 and P19 expression levels were similarly low in the dentate gyrus in vivo, 2) at what age NEEP21 and P19 expression disappeared, and 3) if NEEP21 and P19 expression profiles were always identical in vivo. In this study, we examined three brain regions: neocortex, hippocampus, and cerebellum for NEEP21 and P19 expression at several time points during development (E17), at early postnatal ages (P0, P8) and in young adults (P16). We find that NEEP21 and P19 are largely co-expressed with striking exceptions (intermediate progenitors and Purkinje neurons) where neurons selectively express only one of the two distinct endosomal proteins

during development. Secondly, we find that expression of both proteins is broad earlier in development and restricts to small subpopulations where expression is maintained into adolescence and even adulthood.

## Materials and Methods

### Animals

All animal use was approved by the University of Virginia IACUC. E17 mouse brains were harvested from a timed pregnant female C57BL/6NCrl mice from Charles River. E18 primary rat neurons were harvested from a timed pregnant female Sprague Dawley rat from Charles River (RRID: RGD_737891). All other mice were derived from WT C57BL6 mice after IVF for knockout NEEP21 mice from KOMP (RRID: SCR_007318). The NEEP21 allele is on chromosome 5 from nucleotides 38137193-38159467. The gene was targeted by KOMP and is cleaved from the 5' UTR to the last exon of the coding region, exon 6, and replaced with LacZ and Neo genes. Mice aged P8 and above underwent cardiac perfusion before removal of the brain. For cardiac perfusions, mice were anesthetized with a 0.1 cc/25 gm dose of anesthetic (7 cc NaCl + 2 cc Ketamine 100 mg/mL + 1 cc Dexdomitor). Phosphate-buffered saline (PBS) was automatically pumped through the heart by use of a variable flow perfusion pump (Fisher Scientific, Cat #: 13-876-2) until there was no more blood in circulation. After this, 50-100 mL 4% paraformaldehyde (PFA) was pumped through to fix the brain. The brain was removed and stored in PFA at 4°C for at least 24 hours.

### Cryosectioning

All brains were removed from PFA and placed in 30% sucrose at 4°C for at least 24 hours before sectioning. Brains were removed from sucrose, patted dry with a kimwipe, and placed in Tissue Freezing Media (OCT Embedding Compound, VWR Catalog Number: 25608-930). Brains were frozen in isopentane surrounded by dry ice and cut immediately at -20°C into 20 μm thick sections. Sections were placed on gelatin coated slides (1% gelatin, 0.05% chromium potassium sulfate) and kept at -80°C.

**Immunohistochemistry**

Sections were warmed to room temperature and washed with 1 X PBS 3 times for 5 minutes each. Antigen retrieval was performed for all antibodies by transferring slides to sodium citrate buffer (10 mM sodium citrate, pH 6.0) and microwaving until boiling. Sections were then cooled to room temperature, the sodium citrate buffer was replaced, and the sections were microwaved until boiling a second time. Sections were then rinsed 3 times with 1 X PBS and incubated with blocking solution (0.2% Triton X-100, 3% normal donkey serum) for 1 hour at room temperature. Sections were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The next day, sections were washed with 1 X PBS three times and incubated with secondary antibodies for 1 hour at room temperature protected from light (secondary antibodies in antibody Table 1). Sections were then incubated with DAPI (4',6-Diamindino-2-Phenylindole, Dihydrochloride, ThermoFisher Scientific, Cat 3: D1306) for 10 minutes at room temperature. Slides were washed 3 times with 1 X PBS and mounted with ProLong Diamond antifade mounting media (ThermoFisher Scientific, Cat: P36970). Antibodies used can be found in Table 1. Imaging was performed on a Zeiss AxioZoom Observer.Z1 with Apotome 3.1 structured illumination. All images were taken with a 40x objective lens.

**Primary neuronal culture and transfection**

Primary rat neurons were dissected and cultured from E18 rat brains as our lab has previously described (Wisco et al., 2003). Briefly, hippocampi from E18 rat brains were dissected and dissociated, plated on coverslips, and cultured for 8-12 days. Transfections were performed as previously described by our lab (Yap et al., 2008). The P19-sh plasmid and the random control plasmid were purchased from SABiosciences, and the constructs are expressed from the U1 promoter cassette of the hMGFP vector under the control of the CMV promoter. Briefly, 8-12 DIV neurons were transfected using Lipofectamine 2000 and incubated for 72 hours before fixation.

**Characterization of antibodies**

CTIP2 is a transcription factor that is specific to developing corticospinal motor neurons in the mouse embryonic neocortex (Arlotta et al., 2005), using an antibody to the same clone that was used to make the BioLegend antibody used in this study (RRID: AB_10896795) (Senawong et al., 2003). The antibody recognizes 150 amino acids located at the N-terminus of the protein (Senawong et al., 2003). The antibody was originally described as being specific in western blots showing CTIP2 co-immunoprecipitating with a binding partner, but no band in the control immunoprecipiptation (Senawong et al., 2003). This commercial antibody is now quality tested by BioLegend through western blotting and immunofluorescence. The antibody shows characteristic staining of layer IV and layer V neurons in the neocortex as had been described in the literature (Molyneaux et al., 2007).

SatB2 is a transcription factor which marks postmitotic neurons in the upper layers of the neocortex (Britanova et al., 2008). The commercially available antibody that we use shows this stereotypical staining. The antibody used is commercially available through Abcam (RRID: AB_882455) and recognizes the C-terminal segment of SatB2. The antibody was shown to be specific on a western blot where it recognizes a single band for SatB2 which is not recognized upon shRNA knockdown (Asanoma et al., 2012).

NeuN, also known as Fox-3, is a transcription factor found broadly in CNS and PNS neurons. This particular antibody shows a broad distribution of neurons (Lacoste et al., 2014), and antibodies used against same clone commercially available from Millipore are also characteristically absent from Purkinje neurons (Kim et al., 2009) (RRID: AB_11205592). This antibody was shown to be specific to neurons in IHC where it does not co-localize with blood vessel markers (Lacoste et al., 2014). The antibody is a version of the A60 clone which has been shown to be specific to neurons in IHC through lack of staining in the corpus callosum and lack of co-localization with glial subtypes (Mullen et al., 1992).

Calbindin is a calcium-binding protein present in a variety of CNS neurons. It is highly expressed in Purkinje cells, as shown through staining with the antibody available through Sigma Aldrich (Shakkottai et al., 2011) (RRID: AB_2314065). This antibody was verified in IHC by lack of staining after preadsorption with the peptide (Celio, 1990).

Sox2 is a transcription factor which marks progenitor cells in the mouse neocortex. The antibody is produced by Santa Cruz against the C-terminus end of Sox2 and recognizes a single band in mouse stem cell lysates (SantaCruz, RRID: AB_2286684). This antibody was shown to be specific by western blotting where it recognizes a single band (Chew et al., 2006). This antibody has been shown to specifically mark

progenitor cells in the brain (Mathews et al., 2010), and our staining is consistent with previous literature showing Sox2 to be expressed in the VZ (Hutton and Pevny, 2011).

The Tbr2 chicken antibody was purchased through EMD Millipore (RRID: AB_10615604). This antibody was validated by IHC whereby it shows identical staining to a $Tbr2^{Cre}$ knock-in mouse (Vasistha et al., 2015). Tbr2 is a transcription factor that has been shown to be expressed in intermediate progenitors but not apical progenitors/stem cells (Sessa et al., 2008). In our sections, we see Tbr2 staining in the SVZ but not the VZ, consistent with this literature.

MAP2 is a microtubule bundling protein found at high levels in the somatodendritic region. MAP2 chicken antibody was purchased from EnCor Biotechnology (RRID: AB_2138173). This antibody shows a single band on western blots from rat and mouse brain lysates (EnCor). We have previously validated this antibody by showing co-localization with neuronal marker NeuN, and no c-localization with the glial marker GFAP in cultured neurons (Digilio et al., 2015). Here, we again find that the antibody specifically recognizes neuronal soma and dendrites (Figure 1B).

Parvalbumin, a calcium binding protein, is used to identify subclasses of cortical interneurons (Rudy et al., 2011; Rymar and Sadikot, 2007). The antibody used was raised in mouse against purified carp muscle Parvalbumin (Sigma, RRID: AB_477329). The antibody recognized a single band on western blots and does not react with other EF-hand family members (Sigma).

Antibody characterizations against the small endosomal proteins P19 and NEEP21 can be found in the results section. The P19 antibody was purchased from Abcam and is targeted against the C-terminus of the

protein. It has been tested by Abcam on human neuronal cell line and rat brain lysates. Additionally, an immunocytochemistry image from a mouse neuron has been provided on the Abcam website and shows correct endosomal and trans-Golgi network staining. The NEEP21 antibody was raised in rabbit and is not commercially available (RRID: AB_2572209).

## Results

### NEEP21 and P19 antibodies are specific

Recently, antibody specificity has become a concern in publications (Baker, 2015; Prassas and Diamandis, 2014), and we thus first validated the antibodies against NEEP21 and P19. We raised an anti-NEEP21 antibody in rabbits to the same peptide used previously in studies by Steiner et al. in 2002. Since we wished to characterize the staining pattern in the mouse brain, we validated the anti-NEEP21 antibody on cryosections in two ways: by blocking with the immunizing peptide and by staining sections from the NEEP21/Nsg1$^{-/-}$ mouse as controls. NEEP21 shows high staining in cerebellar Purkinje cells in P0 and adult brains, but this staining is absent in NEEP21 knockout sections as well as when the antibody was pre-incubated with the peptide, demonstrating antibody specificity (Figure 1A). To test the expression pattern of P19, we bought a commercially available rabbit monoclonal antibody to P19 from Abcam. Since no P19 knockout mouse exists, we were not able to validate the antibody on knockout sections. We were also not able to use peptide block to validate the anti-P19 antibody, since the amino acid sequence used for immunization was not disclosed by the company. Thus, we were unable to purchase or synthesize the peptide that this antibody was raised against. Therefore, to test antibody specificity we transfected dissociated E18 rat hippocampal neurons with short hairpin plasmid against P19 (shP19-GFP) and observed a distinct loss of staining in transfected cells versus non-transfected cells (Figure 1B). A sh-plasmid

containing a random hairpin was used as a control and did not diminish P19 staining. Overall, our NEEP21 and P19 antibodies are specific to their respective targets.

**NEEP21 and P19 are similarly expressed in the hippocampus throughout development, but not uniformly in all regions.**

Given that NEEP21 and P19 are in the same family, we hypothesized that they would be similarly expressed. Indeed, NEEP21 and P19 are both expressed in the hippocampus throughout development (Figures 2 and 3). We did not observe any differences along the rostral-caudal axis in the neocortex (not shown) and are showing equivalent sections for all ages and markers. NEEP21 and P19 are present in CTIP2-positive, SatB2-positive, and CTIP2/SatB2-double positive hippocampal neurons in vitro (Digilio et al., 2015). Similarly, NEEP21 and P19 are both expressed highly in double-positive cells in vivo as well as in single positive CTIP2- or Satb2-neurons at E17 (Figure 4A). Interestingly, neurons in the CA1 region have higher expression of NEEP21 (Figure 4B) and P19 (Figure 4C) than neurons in the dentate gyrus (DG) at P0. This accords with our ex vivo data showing lower expression of NEEP21 and P19 in Prox1-positive cells, which are enriched in the DG. Unfortunately, anti-Prox1 antibodies did not produce convincing staining on cryosections.

**NEEP21 and P19 are differentially expressed in the embryonic neocortex**

Given the similar NEEP21 and P19 expression in the hippocampus, we hypothesized that they would be expressed in similar spatial and temporal patterns in the developing neocortex. NEEP21 and P19 are both highly expressed in the cortical plate at E17.5 (Figure 5). However, many different cell types reside within

the cortical plate, so we asked which cells expressed NEEP21 and P19. In particular, we focused on layer V neurons marked by CTIP2, and superficial layers marked by SatB2. Consistent with in vitro data (Digilio et al., 2015), NEEP21 and P19 are both highly expressed in CTIP2-positive, SatB2-positive, and CTIP2/Satb2-double positive cells in the E17 neocortex (Figure 6).

While NEEP21 and P19 both showed high expression in the cortical plate, they showed different expression patterns in the progenitor layers. NEEP21 was highly expressed in the cortical plate and subplate, with low expression in the intermediate, ventricular, and subventricular zone (Figure 5). By contrast, P19 expression is higher in the SVZ (Figure 7A). As the neocortex develops, newborn neurons migrate from the ventricular and subventricular zones to their final destination in the cortical plate with earlier born neurons forming the innermost layer. We sought to determine the identity of P19-positive cells in the progenitor layers by marking the SVZ with Tbr2 and the VZ with Sox2. Interestingly, all Sox2-positive cells in the VZ were lacking P19, however the basal population of Tbr2-positive intermediate progenitors were positive for P19. Examining the basal-most area of the SVZ more closely (Figure 7B), both Sox2 and Tbr2 expressing cells appeared positive for P19. These pools of progenitor cells were all at the basal edge of the SVZ, suggesting that newly born neurons that have yet to turn off Tbr2 and Sox2 express P19. Interestingly, the Tbr2-positive cells appears to be in metaphase while the Sox2-positive cell is in prophase indicating that P19 is expressed prior to the final mitotic division. Therefore, NEEP21 and P19 have similar expression patterns in the cortical plate, but differential expression in the lower zones of the developing neocortex.

**NEEP21 and P19 remain enriched in layer V neurons in the neocortex during development**

Clearly, NEEP21 and P19 are both highly expressed throughout the cortical plate during embryonic development (Figure 5). However, previous immunoblot studies have shown that expression levels of NEEP21 in the rat neocortex dramatically decrease postnatally (Steiner et al., 2002). Therefore, we tested whether the entire neocortex loses expression of NEEP21 and P19 during development or if the expression levels simply became restricted to certain cell types. We examined NEEP21 and P19 immunohistochemistry in the mouse neocortex through development using CTIP2 as a marker for deeper layer neurons and SatB2 as a marker of neurons in the superficial layers. Already by P8, NEEP21 and P19 expression levels were lower than they were in the P0 brain in a majority of neurons (Figures 8-9). Indeed, by P16 NEEP21 and P19 were expressed at low levels in all neurons in the neocortex but maintained high expression in layer V neurons. These neurons expressed CTIP2 or Satb2, and this expression is consistent along the rostral caudal axis of the neocortex. This shows that higher NEEP21 and P19 levels are maintained in a subpopulation of cortical neurons at later ages, as opposed to a general reduction in protein levels.

**NEEP21 and P19 are expressed in some sets of interneurons in the neocortex**

In vitro, we have seen that NEEP21 and P19 are both present in interneuron populations as well as subcortical projection neurons (Digilio et al., 2015). While we were unable to detect convincing staining using anti-GAD65 antibodies on tissue sections, we asked whether or not we could detect NEEP21 and P19 expression in other subsets of interneurons. Specifically, we looked at inhibitory interneurons that are positive for Calbindin or Parvalbumin at P8, where expression is already being restricted. Interestingly, both NEEP21 and P19 show expression in interneurons, but expression levels are not high in all populations of interneurons. Instead, we see a subset of Calbindin and Parvalbum positive neurons that express NEEP21 and P19 at either low or high levels (Figure 10). This shows that there is a range of NEEP21 and P19

expression levels in interneurons in the neocortex, with large interneurons showing higher levels of P19 (Figure 10B) and NEEP21 (not shown) than interneurons with smaller cell bodies at P8.

**NEEP21 becomes restricted to Purkinje cells during cerebellar development**

The cerebellum develops at a later time point than the neocortex, with much of it developing postnatally. Therefore, we stained mid-sagittal sections of cerebellum against NEEP21 in early development (at P0), mid-development (at P8) and fully developed (at P60). Initially, NEEP21 was expressed in both NeuN-positive granule neurons and Calbindin-positive Purkinje cells. However, by the end of cerebellar development at P60, NEEP21 expression had become restricted to only Calbindin-positive Purkinje cells and was essentially undetectable in NeuN-positive neurons (Figure 11).

**P19 expression in the cerebellum decreases during development**

NEEP21 and P19 were both expressed highly in the developing neocortex, therefore we hypothesized that P19 would also be present in the developing cerebellum where NEEP21 was highly expressed. At P0, however, P19 was only expressed at fairly low levels in a subset of neurons, including in some but not all developing Purkinje cells (marked by Calbindin expression) (Figure 12). By P8, P19 expression was very low in Purkinje cells, but present in the external granule layer of neurons migrating past Purkinje cells to form the granule layer (Figure 12). This staining was noticeably different from NEEP21 staining in cerebellum at P8 where the Purkinje cells were the brightest NEEP21-expressing neurons. It should be noted that NEEP21 expression in the cerebellum at all times was much higher than P19 expression. NEEP21 images of the P8 cerebellum were taken with an exposure time of 40ms and the P19 P8 images with an

exposure time of 490ms. This low expression of P19 was maintained throughout adulthood, and P19 was undetectable in either Calbindin-positive Purkinje cells or NeuN-positive neurons in the adult cerebellum, demonstrating a clear and surprising difference in expression between NEEP21 and P19 in the cerebellum (Figures 11-12).

## Discussion

In this work, we analyzed the protein expression levels of the related family members NEEP21/Nsg1 and P19/Nsg2 in developing mouse brain between E17 and P16. Initial papers describing NEEP21 and P19 expression levels described the two proteins as developmentally regulated. However, the spatial aspects of these changes were not explored, and many questions were left unanswered including: Do expression levels decrease across the brain as a whole? Does expression become restricted? Do all neurons lose expression of these two protein? Here, we set out to answer these questions. We showed that NEEP21 and P19 are expressed in distinct spatial and temporal patterns that differ according to brain region by identifying two unexpected aspects of how the NEEP21 and P19 expression profiles change in time and space. First, we show that both proteins are expressed broadly in all brain regions early in development and then become more restricted in their expression at older ages. Contrary to previous reports, expression does not completely disappear but instead is simply reduced in most neurons while a subset of neurons retain expression. In particular, both NEEP21 and P19 remain expressed in layer V of the neocortex and in CA1 and CA3 of the hippocampus. The second conclusion is that there are some cell types where only one of the two proteins is expressed. P19 is expressed in the progenitor cells of the subventricular zone whereas NEEP21 is not expressed in this region. NEEP21, on the other hand, retains high expression in Purkinje neurons in P60 cerebellum whereas P19 is strikingly absent from the adult cerebellum. Our findings are summarized in Table 2.

**NEEP21 and P19 are expressed highly in CA1 and CA3, but not in dentate gyrus throughout development.**

We showed that in the hippocampus, NEEP21 and P19 are widely expressed in the pyramidal cells of the CA1-3 regions with expression remaining high throughout development. This is consistent with the published role of NEEP21 in hippocampal LTP (Alberi et al., 2005; Steiner et al., 2005). The hippocampus is well known as a brain region responsible for learning and memory. These processes are dependent on the induction of long term depression (LTD) and long term potentiation (LTP) (Malenka and Bear, 2004; Sweatt, 2016). In LTP, AMPA receptors are recruited to the synapse and inserted into the membrane (Jurado, 2014; MacDougall and Fine, 2014). At the molecular level, NEEP21 has been shown to facilitate recycling of the AMPAR subunit GluR2 in hippocampal slices, and reduction of NEEP21 levels leads to a decrease in the surface levels of GluR2 containing synapses (Steiner et al., 2005). Interestingly, the two proteins were relatively less abundant in the granule cells of the DG. This observation agrees with expression of NEEP21 and P19 in hippocampal cultures (Digilio et al., 2015), and perhaps suggests a differential need for endosomal transport in CA1 and CA3 neurons versus DG neurons. One explanation for this is the anatomy of each hippocampal region. Pyramidal cells make up the majority of the CA1-3 regions of the hippocampus (Dale et al., 2016) and maintain extensive axons and dendritic arbors, while the DG is composed mainly of small granule neurons. We speculate that these large neurons might require specific neuronal endosomal regulators more than smaller, less expansive granule neurons do.

**NEEP21 and P19 expression is broad during development but persists into adulthood only in a subset of neuronal cell types with large dendritic arbors**

Maintenance of NEEP21 expression in pyramidal neurons is also seen in the neocortex. The neocortex is composed of 6 layers with many different neuronal subtypes. Pyramidal cells are the primary cell type in cortical layers III and V, and these neurons have expanded dendritic arbors (Spruston, 2008). While the majority of neurons in the neocortex express NEEP21 and P19 at embryonic ages, layer V pyramidal neurons maintain expression of NEEP21 and P19 through development (Figures 8-9). This may suggest that NEEP21/P19 are important for excitatory pyramidal cells, especially those in layer V which can have very large dendritic trees, extending in some cases all the way to the pial surface.

However, expression of NEEP21 and P19 is also maintained in GABAergic interneurons positive for Calbindin or Parvalbumin. Interestingly, only a subset of Parvalbumin and Calbindin positive cells retain NEEP21 or P19. There are two types of Parvalbumin-positive interneurons in the neocortex: basket cells and chandelier cells (Rudy et al., 2011). Basket cells can vary in size, with large basket cells containing large axonal and dendritic arbors (Markram et al., 2004). Many large basket cells are dually positive for calbindin and parvalbumin. This characteristic, as well as their large size, leads us to hypothesize that these are the interneurons positive for NEEP21 or P19. However, additional studies will have to be done to further delineate the many interneuron subtypes and how NEEP21 and P19 are expressed in them.

NEEP21 is once again seen to be developmentally restricted in the cerebellum. Embryonically, all cerebellar cells contain NEEP21, but only Purkinje cells maintain NEEP21 into adulthood while granule cells lose expression (Figure 11). Granule neurons are much smaller than pyramidal neurons and Purkinje neurons, pointing perhaps to increased need for NEEP21 in larger neurons. This would be in line with the expression of NEEP21 in layer V cortical neurons and large interneurons.

**NEEP21 and P19 are largely co-expressed, but certain cell types express only one of the two proteins**

Interestingly, NEEP21 and P19 expression patterns were not identical. NEEP21 and P19 are closely related family members, and we hypothesized that they would have the same expression pattern. This was true in culture (Digilio et al., 2015) as well as for most neurons in situ. However, there were a number of striking exceptions. For instance, the expression patterns diverged early in development (E17 neocortex). At this point during cortical development, P19 was highly expressed in the progenitor layers including the SVZ, which is well known for its role in neurogenesis, while NEEP21 was mainly restricted to the cortical plate. Radial glia reside in the VZ and can generate neurons and glia, whereas intermediate progenitor cells (IPCs) reside in the SVZ and produce only neurons (Englund et al., 2005; Miyata et al., 2001; Noctor et al., 2001; Paridaen and Huttner, 2014). IPCs express the transcription factor Tbr2, which is down-regulated once the cell's neurogenic fate is determined (Englund et al., 2005; Ochiai et al., 2009). Interestingly, P19 was expressed in a subset of Tbr2 positive cells in the SVZ (Figure 7B). Even though P19 was first described as being enriched in neurons (Saberan-Djoneidi et al., 1995), our data show expression of P19 in Tbr2-positive IPCs and thus point towards a potential role for P19 just prior to neural specification. NEEP21, on the other hand, is not expressed in the IPCs and may not function until later in neuronal maturation. This divergent expression suggests some non-redundant functions for NEEP21 and P19, a notion we will test in the future.

Whereas in the neocortex, NEEP21 and P19 had different expression patterns during development but eventually restricted similarly to layer V (Figures 8-9), they did not have overlapping expression patterns at any point during development in the cerebellum (Figures 11-12). P19 was expressed at low levels in many different cell types in the cerebellum at P0 whereas NEEP21 was expressed at a much higher levels. By P8, NEEP21 had overwhelmingly restricted expression to Purkinje cells, whereas P19 levels were low

in Purkinje cells and higher in the migrating external granule layer. By P60, P19 was undetectable in the cerebellum, but NEEP21 levels remained high in Purkinje cells. This suggests non-overlapping roles for NEEP21 and P19 in the cerebellum, and specifically in Purkinje cells which maintain a high expression of NEEP21 even after development has completed.

Restriction of protein expression during development was also observed in the third Nsg family member, Calcyon (Caly). Caly was more highly expressed in younger rats as opposed to adult rats, similar to the reduction in NEEP21 and P19 protein levels (Heijtz et al., 2007). This points to a key role in brain development for the Nsg family of proteins, and perhaps a continued requirement for these family members in a subset of large adult neurons. We were not able to carry out more detailed comparisons with Caly since the available antibodies do not recognize mouse Caly.

**Layer V cortical and Purkinje neurons – special needs for endosomal trafficking?**

NEEP21 and P19 are both neuronal-enriched endosomal proteins. From their expression patterns, we can infer that they are important during development of the neocortex, hippocampus and cerebellum. Consistent with this observation, we previously showed that NEEP21 regulates recycling pathways of the cell adhesion molecule L1. Other developmentally important guidance receptors might also be regulated by NEEP21 and this might explain why most neurons express Nsg family proteins developmentally. We have previously observed dendrite and axon growth defects in cultured neurons grown on L1 substrate when NEEP21 is knocked down, consistent with a role in process elaboration during development (Lasiecka et al., 2014).

In addition to high expression universally during development, NEEP21 remains enriched in distinct areas of the brain, including layer V cortical neurons, the CA1-3 region of the hippocampus, and Purkinje cells in the cerebellum. Considering the complex endosomal system that is present specifically in neurons, the restriction of these neuron-specific endosomal proteins suggests a high necessity for endosomal trafficking in these neurons. Indeed, layer V cortical neurons and Purkinje Cells have extraordinarily long axons, as well as complex dendritic trees. Complexity of neuronal processes and a large soma is a feature that is common to all pyramidal cells, as well as large basket cells. Thus, persistent expression of NEEP21 endosomal protein in these neuronal cell types suggests that these neurons might need additional proteins to maintain complex endosomal trafficking over long distances in large dendritic arbors. The function of P19 is very poorly understood and no functional data have been published. The differential expression suggests that P19 and NEEP21 might not perform redundant functions, at least in some cellular contexts, i.e. IPCs for P19 and Purkinje neurons for NEEP21. More work is needed to understand the significance of the observed differential expression. The persistence of expression in the hippocampus, layer V projection neurons, and the very large Purkinje neurons raises many questions about what special needs these neuronal subclasses have that requires continued expression of this interesting, but poorly understood family of proteins.

## Figures

**Figure 1**. NEEP21 and P19 antibodies are specific to their intended targets. (A) WT cerebellar sections from P0 or P60 mouse brains stain positive for NEEP21, however there is no staining in the KO sections or when the primary antibody is incubated with its immunizing peptide for 1 hour before application. Calbindin marks Purkinje neurons, and NeuN marks granule neurons.

(B) The P19 antibody purchased from Abcam is specific. Primary neurons were transfected with either shP19-GFP or shControl GFP (arrowhead) and fixed 72 hours later. P19 staining (red) is greatly diminished in the neuron expressing shP19 (arrow) as opposed to its neighboring untransfected cell (arrowhead). shControl did not diminish P19 staining (arrow).

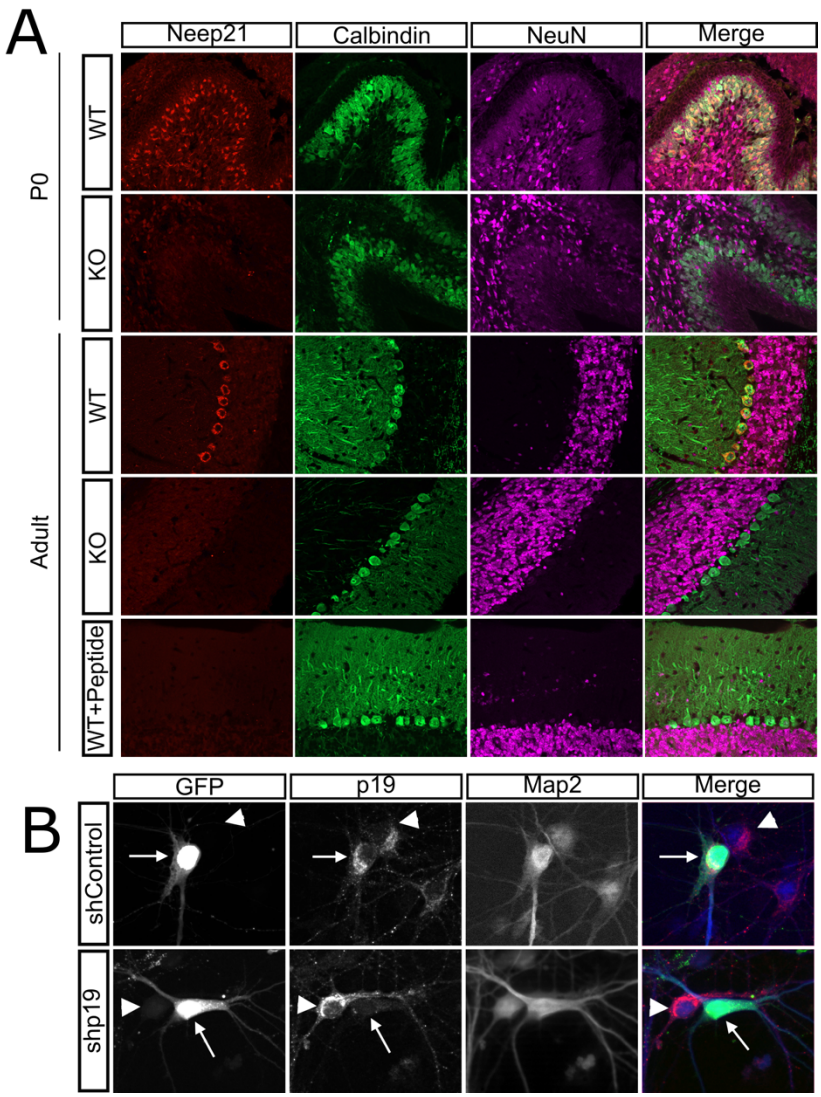


**Figure 2**. NEEP21 is expressed throughout the hippocampus during development. NEEP21 is highly expressed in CA1-CA3 regions of the hippocampus, but less highly expressed in the dentate gyrus (DG). CA1-CA2 and DG are marked by CTIP2, with CA3 showing characteristic lack of CTIP2.

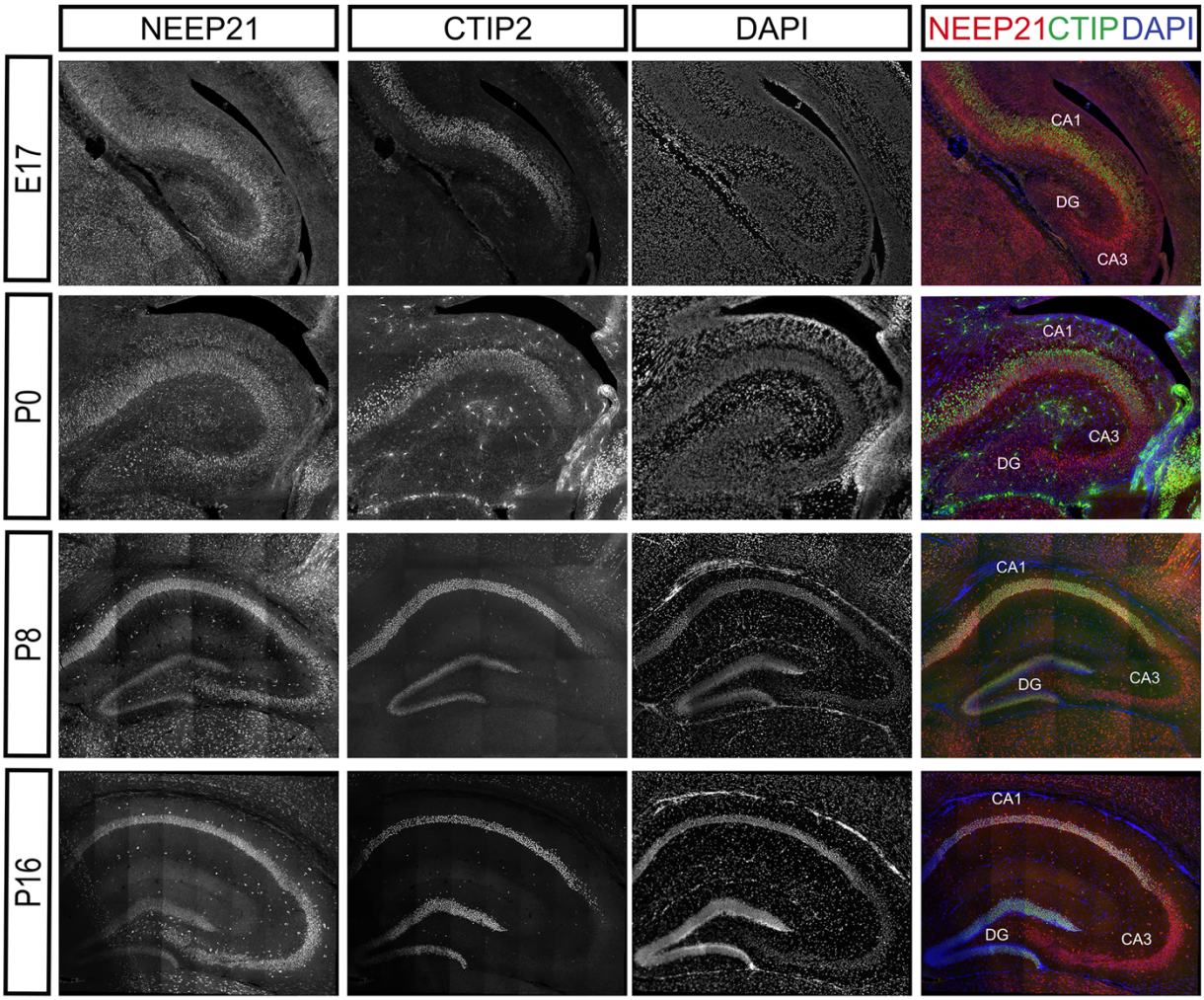


**Figure 3.** P19 is expressed throughout the hippocampus during development. P19 is highly expressed in CA1-CA3 regions of the hippocampus throughout development. Ages and antibodies are indicated in the figure. CA1-CA2, DG marked by CTIP2. CA3 shows characteristic lack of CTIP2.

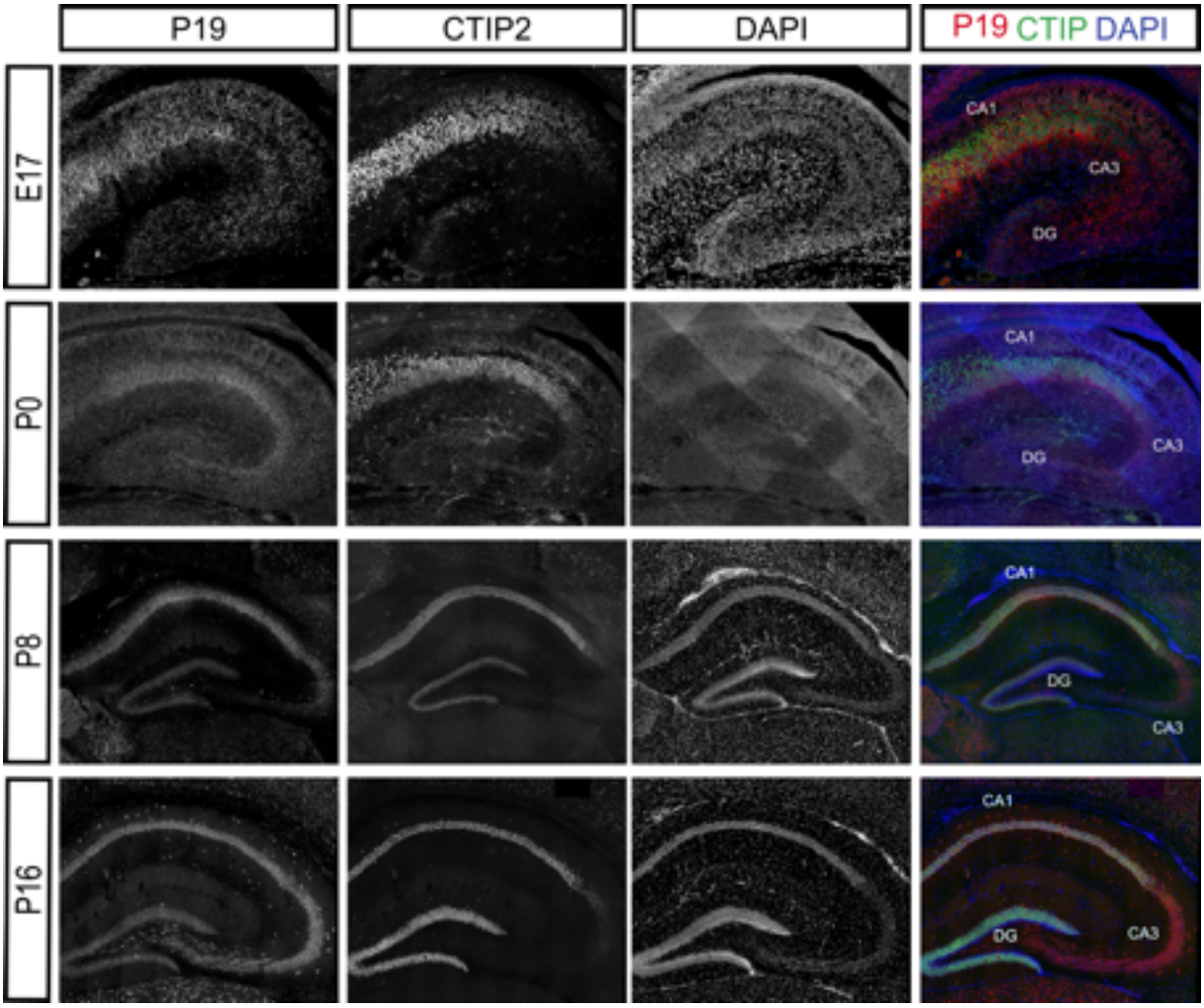


**Figure 4**. NEEP21 and P19 in the developing hippocampus. (A) CTIP2 positive (encircled/boxed), SatB2 positive (arrowhead), and double positive (arrow) neurons in the CA1 region of the hippocampus at E17 express NEEP21 and P19. (B, C) NEEP21 (B) and P19 (C) are both expressed at higher levels in the CA1 region of the hippocampus than in the dentate gyrus (DG) at P0. All imaging and processing was identical between brain regions.

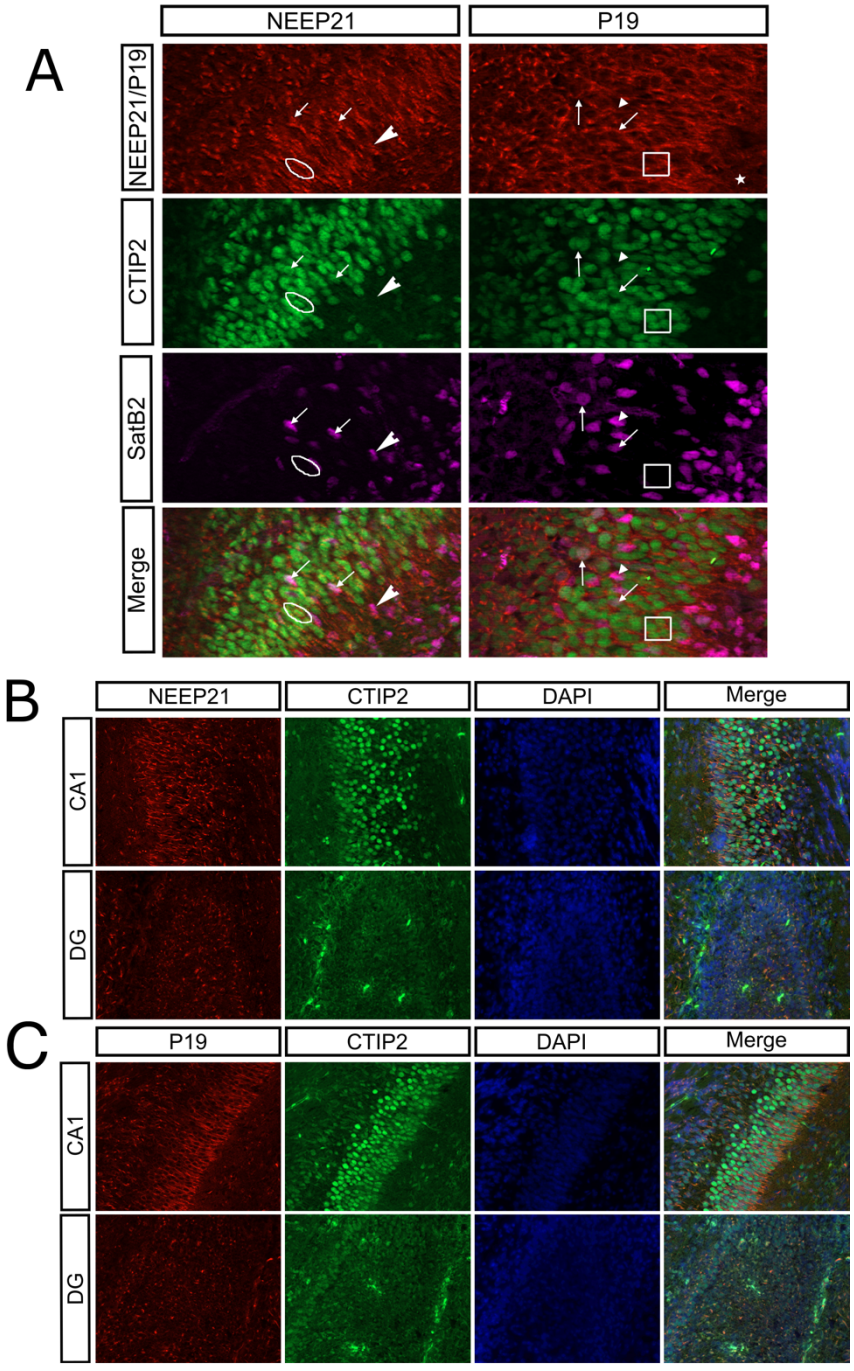


**Figure 5**. Expression patterns of NEEP21 and P19 in the mouse embryonic cortical plate. NEEP21 and P19 are both expressed at high levels in the cortical plate (CP) at E17.5, as marked by SatB2- and CTIP2-positive neurons. CTIP2 marks layer V strongly and layer VI weakly at this age. NEEP21 is also highly expressed in the subplate (SP). P19 is expressed throughout the developing neocortex, and highly in the subventricular zone (SVZ) where NEEP21 is expressed at a much lower level.

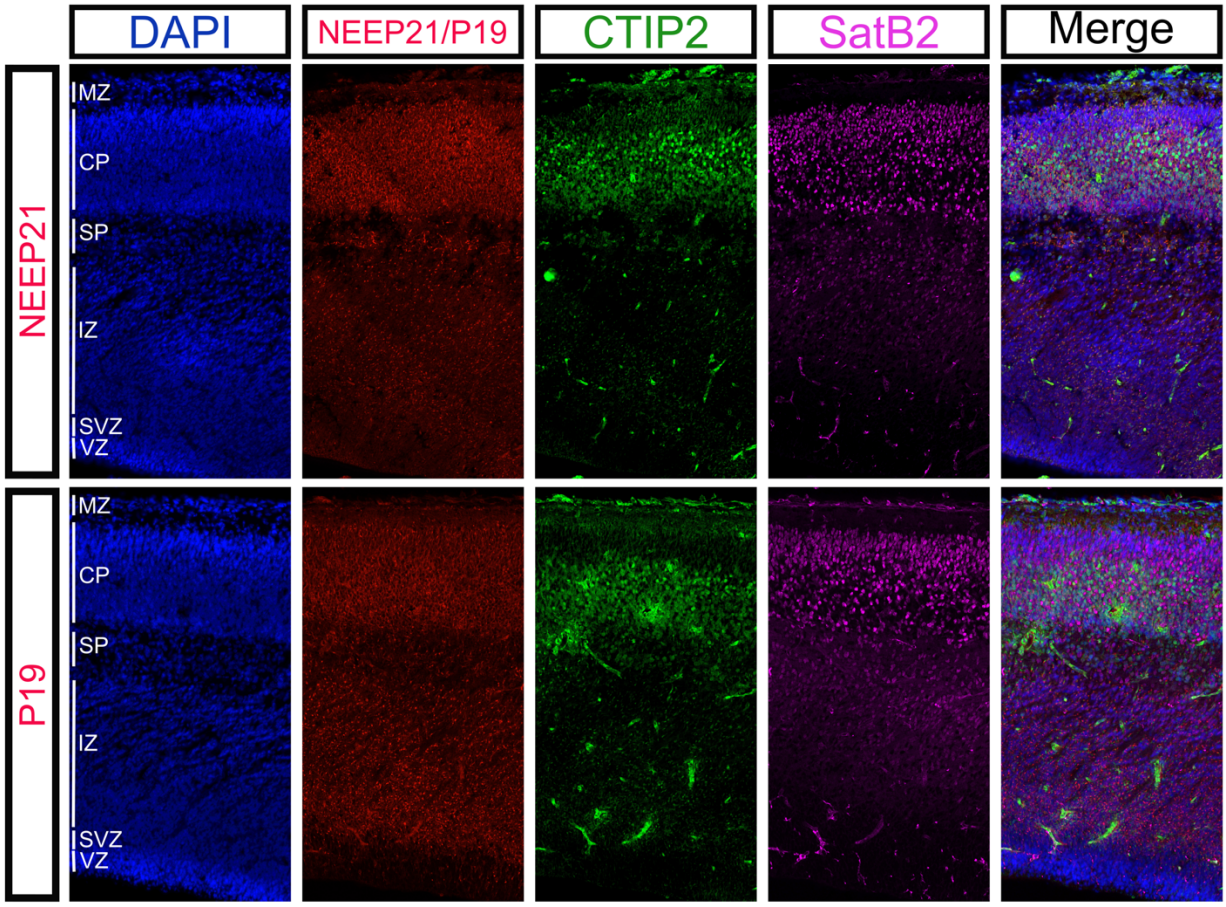


**Figure 6.** NEEP21 and P19 are expressed in multiple cell types in the embryonic neocortex.

NEEP21 and P19 are present in CTIP2 positive (star), SatB2 positive (large arrow), and double positive (small arrow) cells in the E17.5 neocortex.

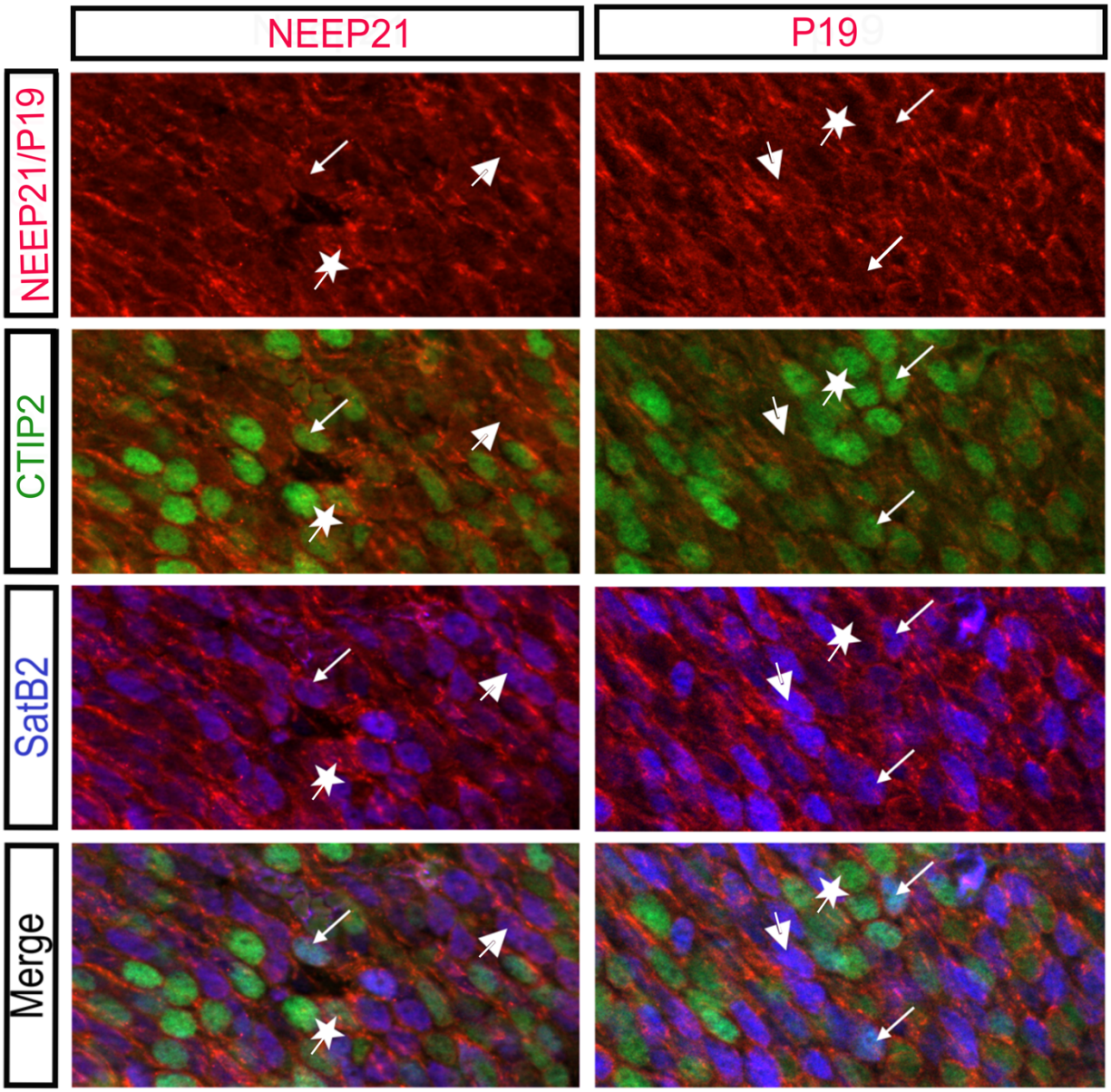


**Figure 7.** P19 is expressed in SVZ progenitors. (A) At E17.5, P19 is absent from the ventricular zone (VZ) progenitors as marked by Sox2, but highly expressed in the basal SVZ, as marked by Tbr2 positive cells. The area in the box is expanded in (B) and shows both Sox2 positive cells (arrowhead) and Tbr2 positive cells (arrow) can be found in the basal aspect of the SVZ. P19 is expressed in both of these cell types.

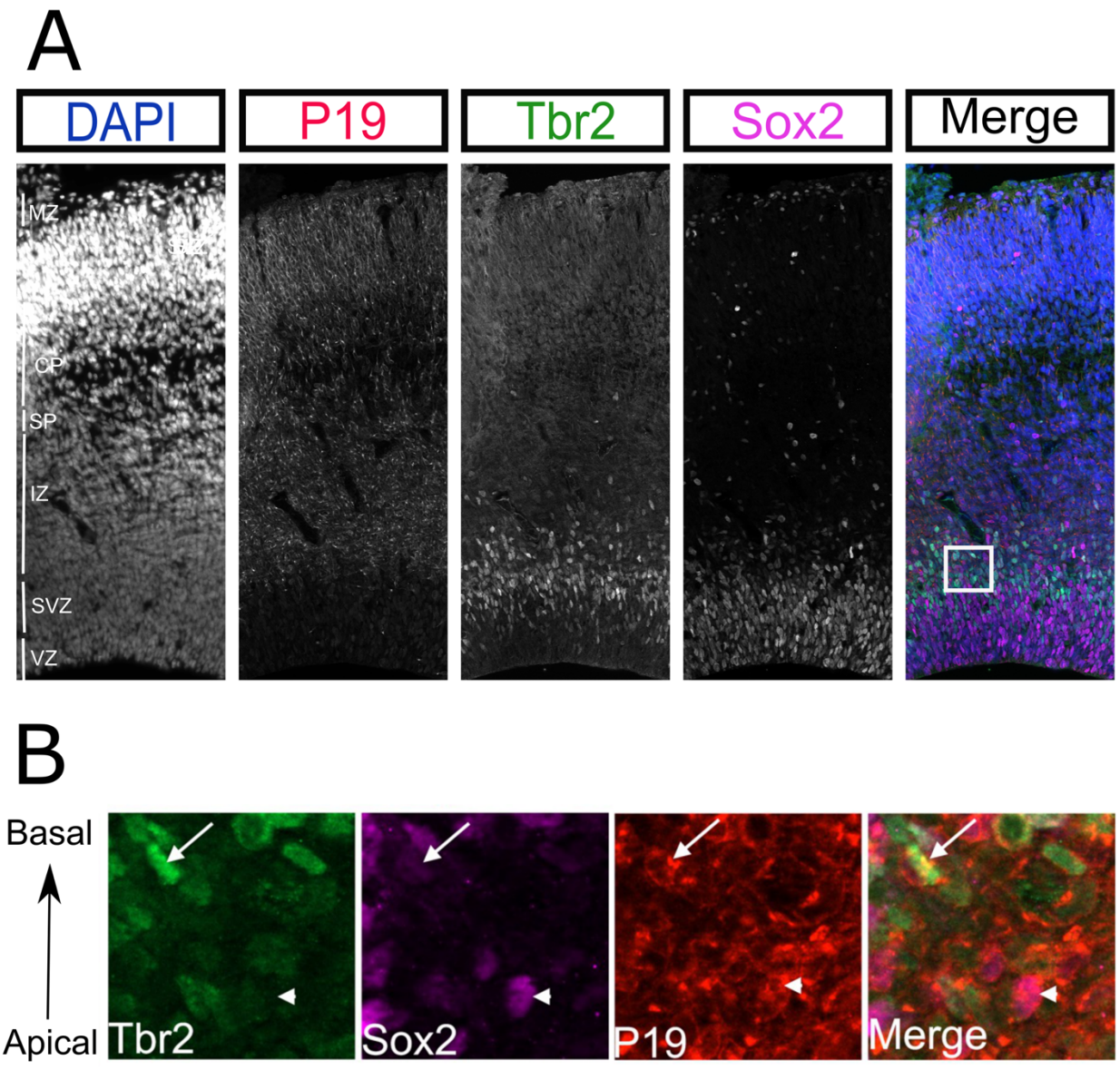


**Figure 8**. NEEP21 expression remains enriched in layer V cortical neurons during development. NEEP21 is highly and broadly expressed throughout the cortical plate at P0, but is downregulated overall at P8 and P14, but remains relatively enriched in layer V.

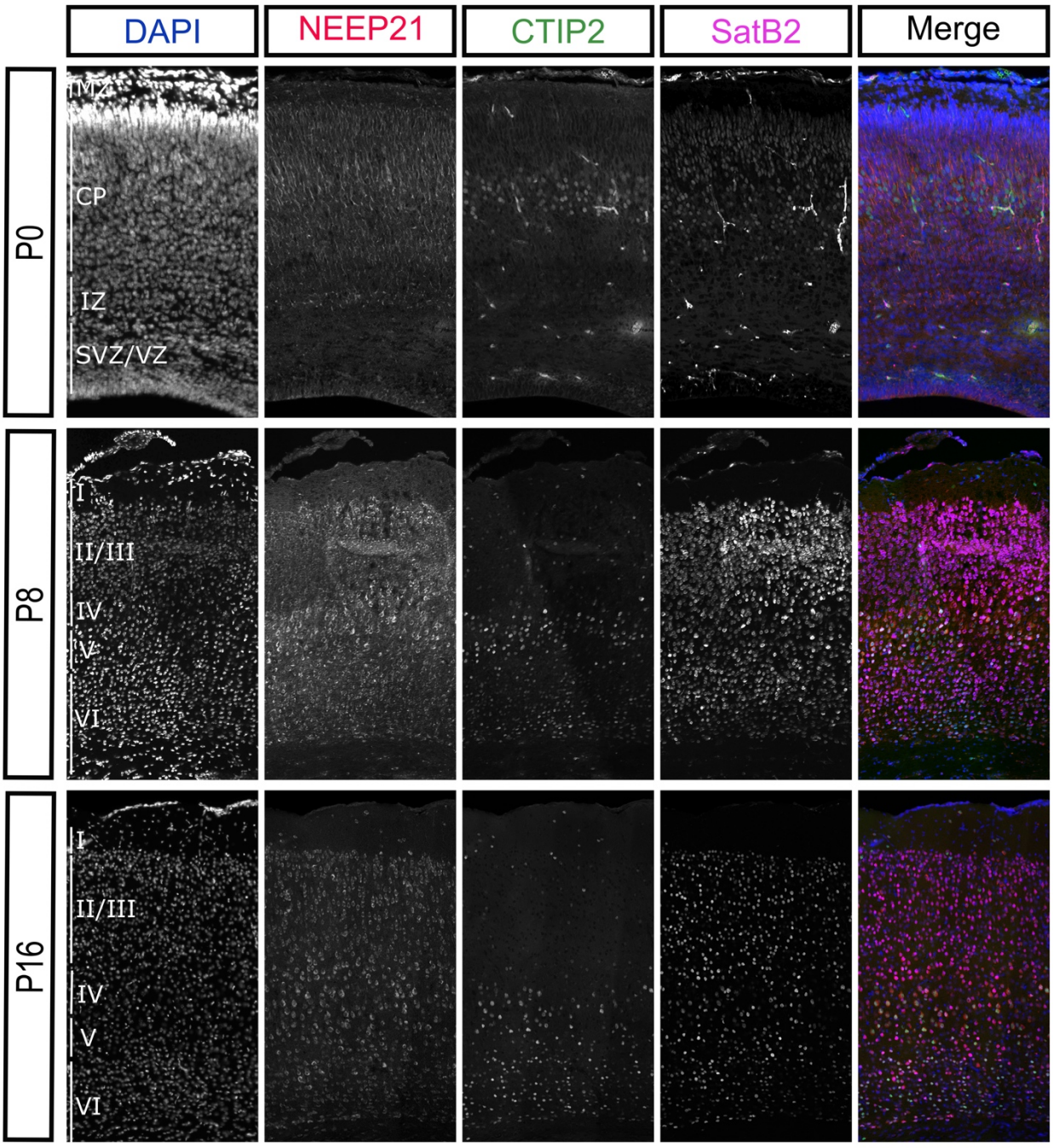


**Figure 9**. P19 expression remains enriched in layer V cortical neurons during development. P19 is also broadly expressed in the P0 neocortex. Similarly to NEEP21, P19 is downregulated overall at P8 and P14, but remains relatively enriched in layer V.

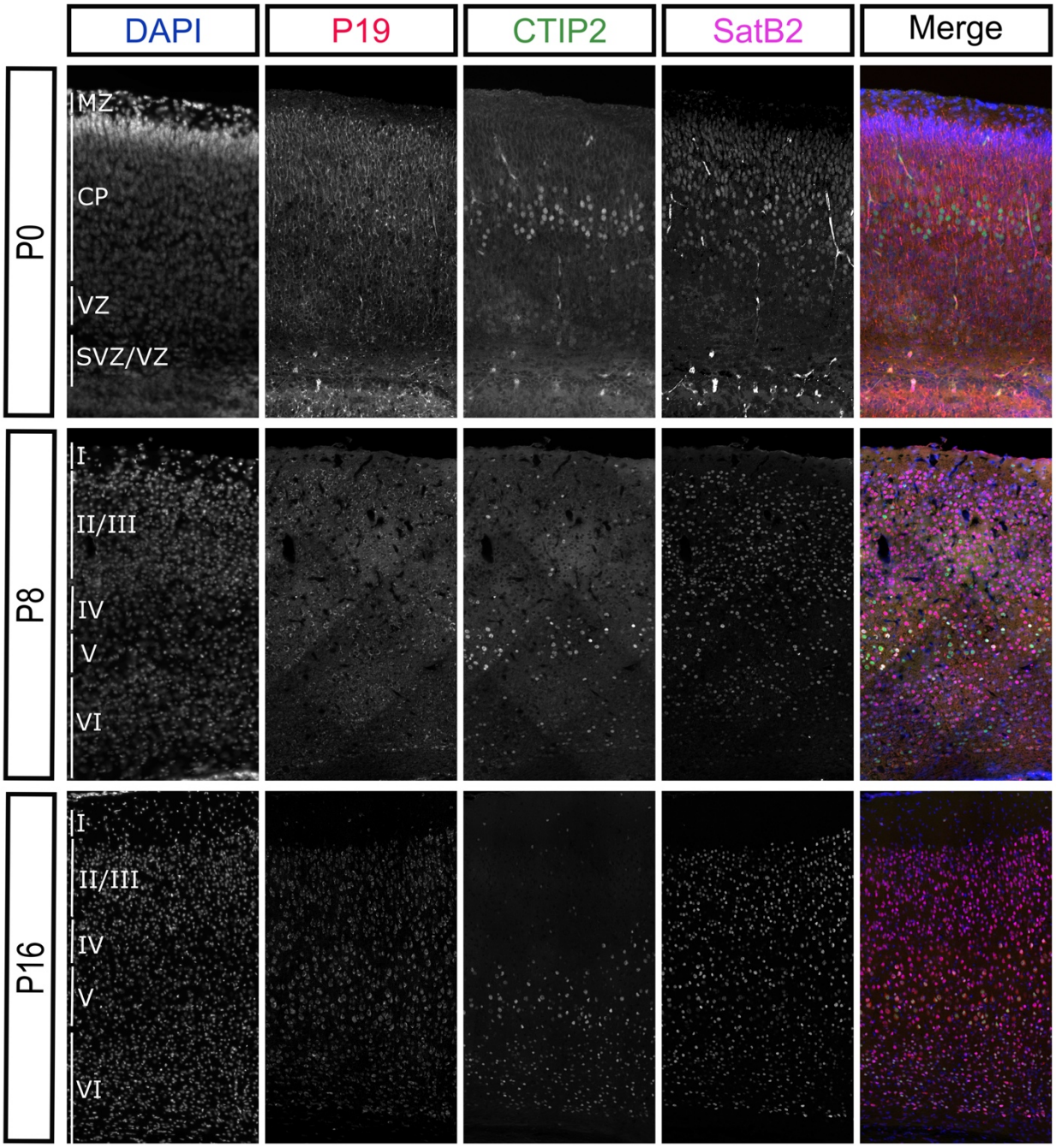


**Figure 10.** NEEP21 and P19 have varied expression levels in interneurons. (A) NEEP21 and P19 are both expressed in Calbindin positive interneurons (arrows) in the P8 neocortex, and their expression levels vary between cells. (B) NEEP21 and P19 are both expressed in Parvalbumin positive interneurons (arrows). Expression levels are higher in larger Parvalbumin cells (larger) than small Parvalbumin cells (small). Arrows: Parvalbumin positive cells, Arrowheads: CTIP2 positive cells.

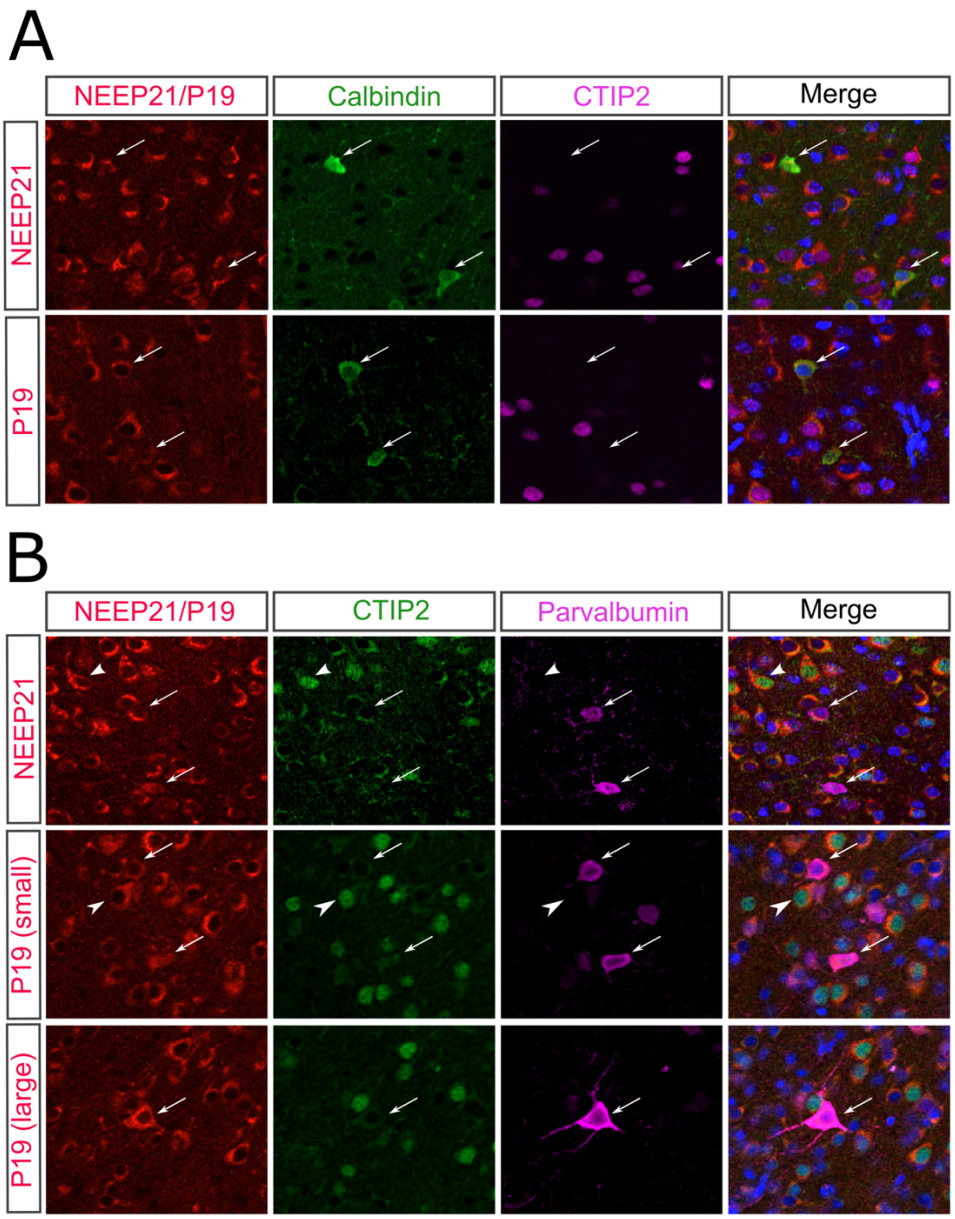


**Figure 11**. Expression pattern of NEEP21 in the cerebellum. NEEP21 is expressed ubiquitously in the developing P0 cerebellum. This includes Calbindin-positive Purkinje neurons as well as NeuN-positive granule neurons. However, it becomes restricted to Purkinje cells by P8. This exclusive expression in Purkinje cells is maintained throughout adulthood as shown in the P60 brain. NEEP21 is not detectable in granule neurons.

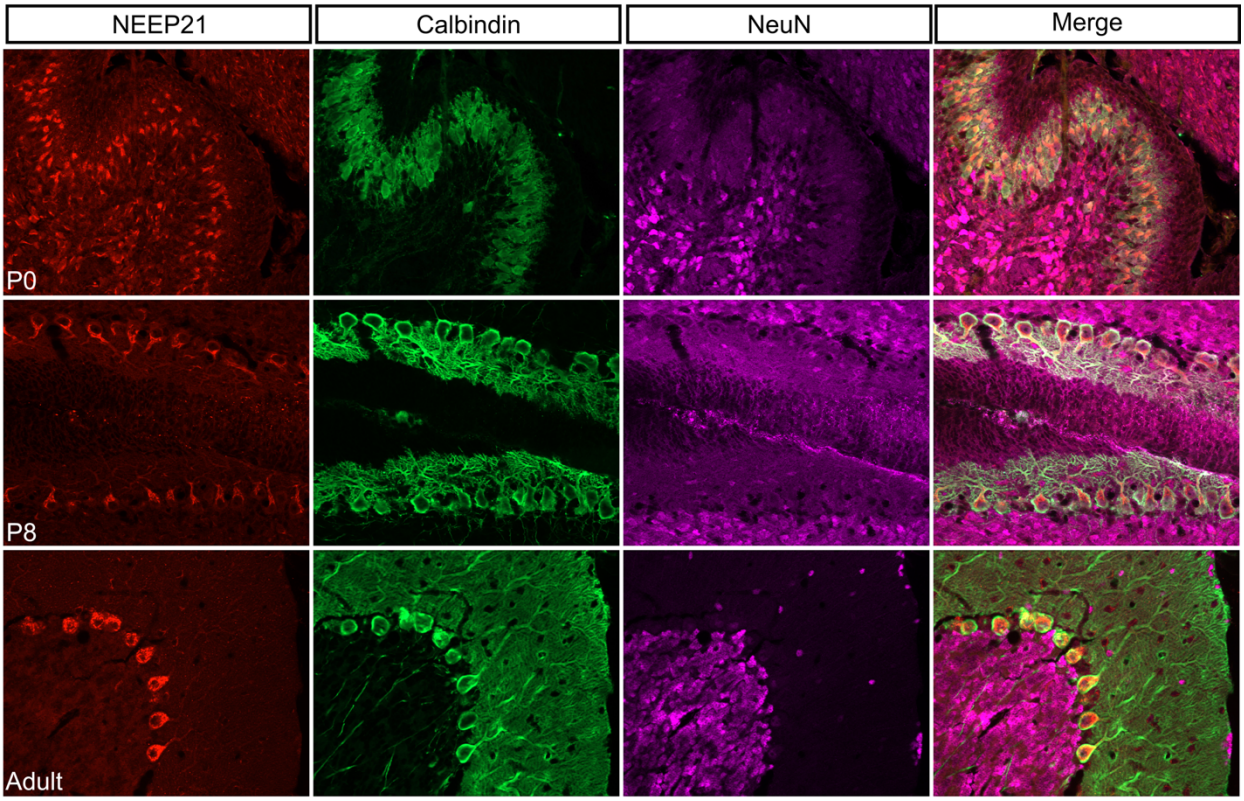


**Figure 12**. Expression pattern of P19 in the cerebellum. P19 is expressed broadly in the P0 cerebellum, but at much lower levels than NEEP21. P19 expression remains low throughout development, and is undetectable by adulthood.

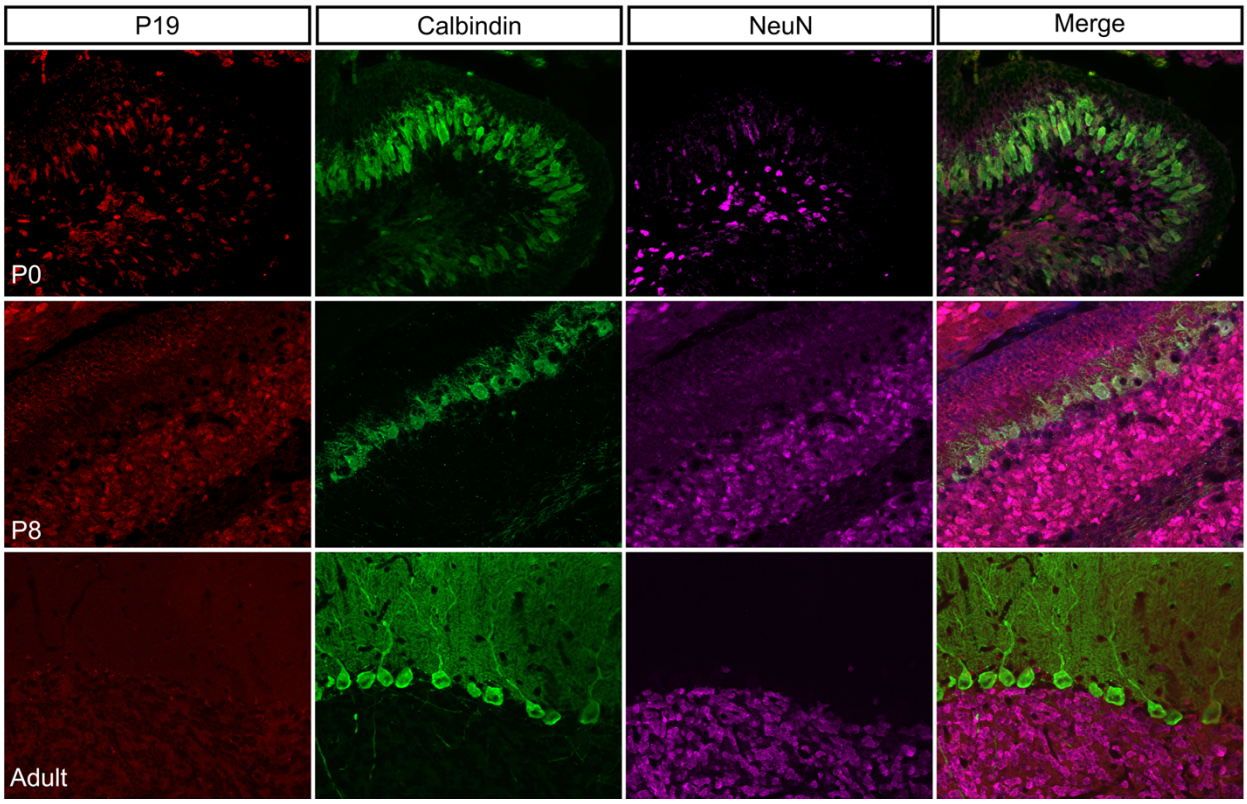


**Table 1**. All primary and secondary antibodies used as well as their dilution and reference information. All antibodies were diluted in blocking buffer described in materials and methods.

| Primary Antibodies | | | | |
|---|---|---|---|---|
| **Target Protein** | **Species** | **Dilution** | **Source, Cat. No** | **RRID** |
| NEEP21 | Rabbit | 1:400 | | |
| P19 | Rabbit | 1:400 | Abcam, ab189513 | AB_2571866 |
| CTIP2 | Rat | 1:400 | Biogend, 650601 | AB_10896795 |
| SatB2 | Mouse | 1:400 | Abcam, ab51502 | AB_882455 |
| Sox2 | Goat | 1:100 | Santa Cruz, sc-17320 | AB_2286684 |
| NeuN | Guinea Pig | 1:1000 | Millipore, ABN90 | AB_11205592 |
| Calbindin | Mouse | 1:800 | Sigma-Aldrich, C9484 | AB_2314065 |
| MAP2 | Chicken | 1:20,000 | EnCor, CPCA-MAP2 | AB_2138173 |
| Parvalbumin | Mouse | 1:2000 | Sigma-Aldrich, P3088 | AB_477329 |
| **Secondary Antibodies** | | | | |
| **Antibody** | **Fluorophore** | **Dilution** | **Source, Cat No.** | **RRID** |
| Donkey anti Mouse | 647 | 1:400 | Life Technologies, A-31571 | AB_2536181 |
| Donkey anti Mouse | 488 | 1:400 | Life Technologies, A-21202 | AB_2535788 |
| Donkey anti Rat | 488 | 1:400 | Jackson Labs, 712-546-153 | AB_2340686 |
| Donkey anti Rabbit | 568 | 1:400 | Life Technologies, A10042 | AB_2534017 |
| Donkey anti Guinea Pig | 647 | 1:400 | Jackson Labs, 706-175-148 | AB_2340462 |
| Donkey anti Goat | 488 | 1:400 | Life Technologies, A-11055 | AB_2534102 |

**Table 2.** Summary of spatial and temporal expression of NEEP21 and P19. More "1" signs indicate higher expression.

| | | E17 | | P0 | | P8 | | P16 | |
|---|---|---|---|---|---|---|---|---|---|
| Brain area | subregion | NEEP21 | P19 | NEEP21 | P19 | NEEP21 | P19 | NEEP21 | P19 |
| Cortex: | Upper layers | +++ | +++ | +++ | +++ | - | - | - | - |
| | Layer V | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ |
| | Interneurons | | | | | ++ | ++ | | |
| | Subventricular zone | - | +++ | | | | | | |
| Hippocampus | CA | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ |
| | Dentate gyrus | + | + | + | + | + | + | + | + |
| Cerebellum | Granule layer | - | - | +++ | + | - | + | - | - |
| | Purkinje neurons | - | - | +++ | + | +++ | - | +++ | - |

# Chapter III: Transcytosis of TrkA leads to diversification of dendritic signaling endosomes

This chapter is published as "Transcytosis of TrkA leads to diversification of dendritic signaling endosomes". Kelly Barford[1], Austin Keeler[2], Lloyd McMahon[1], Kathryn McDaniel[1], Chan Choo Yap[1], Christopher D. Deppmann[2,3,4], Bettina Winckler[1,4]. *Scientific Reports*. 2018;8:4715. All experiments were carried out by me with the exception of the in vivo experiment (Figure 7) which was done by AK. I did the immunocytochemistry of the sections and the microscopy for Figure 7.

**Abstract**

The development of the peripheral nervous system relies on long-distance signaling from target organs back to the soma. In sympathetic neurons, this long-distance signaling is mediated by target derived Nerve Growth Factor (NGF) interacting with its axonal receptor, TrkA. This ligand receptor complex internalizes into what is commonly referred to as the signaling endosome which is transported retrogradely to the soma and also to dendrites to mediate survival signaling and synapse formation, respectively. The molecular identity of signaling endosomes in dendrites has not yet been determined. Here, we perform a detailed analysis of TrkA endosomal compartments and trafficking patterns. We find that signaling endosomes are not uniform but molecularly diversified into Rab7 (late endosome) and Rab11 (recycling endosome) populations in axons and dendrites *in vitro* and in the soma *in vivo*. Surprisingly, TrkA-NGF signaling endosomes in dendrites undergo dynamic trafficking events, including putative fusion and fission. Overall, we find that signaling endosomes do not remain as a singular endosomal subtype but instead exist in multiple populations that undergo dynamic endosomal trafficking events. These dynamic events might drive functional diversification of the signaling endosome.

## Introduction

The development of the sympathetic and sensory nervous systems requires the target derived neurotrophic factor, Nerve Growth Factor (NGF) (Levi-montalcini, 1987). The molecular and cellular mechanisms that enable target derived NGF to transmit signals long distances from distal axons to the cell body have been under intense investigation for several years. NGF binds to its receptor TrkA in axon terminals, and the NGF-TrkA complex is internalized into what is commonly referred to as the signaling endosome (SE) because of its capacity to maintain active signaling cascades such as MAPK, PI3K, and PLC-gamma (Delcroix et al., 2003; Harrington and Ginty, 2013; Harrington et al., 2011; Ye et al., 2003). The SE engages retrograde axonal transport machinery to traffic back to the cell body using the minus-end directed microtubule motor dynein (Heerssen et al., 2004). Once it reaches the soma, the SE is able to maintain signaling cascades for over 6 hours (Suo et al., 2013). These signaling pathways include activation of pro-survival transcription factors like CREB (Riccio, 1999) and suppression of apoptotic machinery like Bax (Tsuruta et al., 2002). There is mounting evidence that the SE mediates much more than just survival, including dendrite development, synapse formation, changes in metabolism, and acquisition of neurotransmitter phenotypes (Deckwerth, 1993; Lehigh et al., 2017; Schotzinger and Landis, 1988; Sharma et al., 2010). Ginty and colleagues previously showed that SEs are not confined to the soma, but can travel to dendrites and signal for postsynaptic clustering (Sharma et al., 2010). Dendritic SEs reside near nascent post-synaptic specializations and mediate post-synaptic density (PSD) clustering both *in vitro* and *in vivo* (Lehigh et al., 2017; Sharma et al., 2010). Indeed, axonally-derived TrkA SEs continue to signal in the dendrite, and inhibition of TrkA phosphorylation in the dendrite abolishes PSD clustering (Lehigh et al., 2017). In addition, we showed previously that SEs arriving in the soma can undergo exocytosis, and this pool of axonally-derived TrkA can subsequently re-endocytose (Suo et al., 2013). We refer to this trafficking pathway as “retrograde transcytosis”, and we refer to the axonally-derived TrkA endosomes that undergo retrograde transcytosis as “post-trancytotic signaling endosomes” (PTSEs, Supplementary Figure

1D-E). This nomenclature is used to distinguish naïve TrkA undergoing anterograde transcytosis to the axon (Ascano et al., 2009) from the pathway we are investigating in this study (Supplementary Figure 1E). Currently, nothing is known about PTSEs, including whether they are motile, whether they are found in dendrites, and what their endosomal identity is.

Much is known about endosomal pathways in other cell types, especially fibroblasts, and for other internalized receptors, such as transferrin receptor. From these non-neuronal model systems, it is well established that the fate of endocytosed cargos is regulated by several endosomal members of the Rab family of small GTPases, especially Rab5 (early endosome), Rab7 (late endosome), and Rab11 (recycling endosome) (Grosshans et al., 2006). Endocytosed cargos first arrive at the Rab5-positive early endosome (also marked by Early Endosomal Antigen 1, EEA1), from where they can sort into various arms of the endosomal pathway. From the EEA1+ early endosome, cargo can undergo distinct fates when sorted into segregated domains (reviewed in Jovic et al. 2010). These domains are then released through fission to create individual endosomes. For the purpose of this work, we will focus on sorting into the recycling and degradative arms of the endosomal pathway. Recycling-competent cargos are removed from the early endosome by sorting and fission events to generate endosomes positive for Rab11. In contrast, degradative cargos are typically sorted into Rab7-positive late endosomes which ultimately fuse with lysosomes. The TrkA SE also seems to follow these maturational rules. TrkA endocytosed at the distal tips of axons initially sorts into the early endosome (Delcroix et al., 2003). From there, TrkA signaling endosomes have been identified to travel retrogradely back to the soma in the early endosome, or in a late endosome for transit (Delcroix et al., 2003; Zhang et al., 2013).

We used a Flag-TrkA knockin mouse and a Flag antibody feeding assay in combination with sympathetic neurons cultured in microfluidic chambers to ask how SEs mature (Sharma et al., 2010; Suo et al., 2013).

We find that SEs exist in multiple endosomal populations beginning in the axon. Axonal SEs exist in equal populations of Rab11 (recycling) and Rab7 (late) endosomal compartments. Interestingly, dendritic SEs are frequently post-transcytotic and are also found in Rab11 (recycling) and Rab7 (late endosomal) pathways in roughly equal proportions. Live imaging of SEs in dendrites reveals frequent dynamic events, including presumptive fissions and fusions. Surprisingly, we also observe high levels of co-localization of re-endocytosed retrograde TrkA with simultaneously endocytosed transferrin, arguing that SEs do not sort into distinct pools of Rab11 endosomes, but mix extensively with other endocytosed cargos. In addition, we show that this endosomal diversification exists *in vivo*. We propose that two pathways contribute to SE diversification. First, TrkA in axons is found in both Rab11- and Rab7-positive endosomes. Second, axonally-derived TrkA can undergo retrograde transcytosis, and then sort equally into recycling (Rab11) and pre-degradative (Rab7) pathways. We propose that transcytotic resorting of internalized axonally-derived TrkA from early endosomes into Rab11- and Rab7-positive endosomal subpopulations results in SE molecular diversification.

## Materials and Methods

### Animals

All animal use was approved by the University of Virginia IACUC guidelines by protocol #3422 for the Winckler lab, and protocol #3795 for the Deppmann lab. All methods and experiments were performed in accordance within these guidelines and regulations. All animals were maintained in a C57BL/6 background. Both sexes were used. *TrkA*$^{FLAG/FLAG}$ animals were a gift from D. Ginty, and genotyping was performed as previously described (Sharma et al., 2010).

**Compartmentalized Sympathetic Neuronal Cultures**

Sympathetic neurons were dissected from the superior cervical ganglia of P1 mice as previously described. Dissociated neurons were plated in microfluidic devices as previously described(Park et al., 2006) and experiments were performed on DIV 10-15. Neurons were maintained in DMEM +10% FBS, penicillin/streptomycin, gentamycin, and 80-100 ng/mL NGF. For the first 5 days of culture, neurons were additionally maintained with 5 μM Ara-C to kill proliferating glia.

**Antibody feeding assays**

For fixed cells, anti-FLAG antibody feeding experiments were performed as previously described (Suo et al., 2013). Briefly, neurons were deprived of NGF overnight in the presence of a broad-pan caspase inhibitor BAF and anti-NGF antibody (Millipore Cat# AB1528SP, RRID:AB_90742) (1 μg/mL anti-NGF, 1 μg/mL BAF). Neurons were washed with DMEM +10% FBS, and NGF (100 ng/mL) and anti-FLAG antibody (M1, Sigma-Aldrich Cat# F3040, RRID:AB_439712) were applied exclusively to the distal axon chamber for 30 minutes. The distal axon chamber was then acid washed with pH 2.0 for 30 seconds to remove remaining surface-bound antibody followed by PBS and media washes. An Alexa-647 anti-mouse antibody (Life technologies A-31571, RRID: AB_2536181, 1:400) was applied to the cell body chamber after washes to label post-transcytotic signaling endosomes (PTSEs).

For transferrin-feed experiments, neurons were changed to DMEM with no FBS for 1 hour prior to the experiment. The anti-FLAG antibody M1 feeding was performed as above. In addition to the Alexa-647

anti-mouse antibody, a Cy3 Transferrin (Jackson ImmunoResearch Labs Cat# 015-160-050, RRID:AB_2337214) was added at a concentration of 40 ng/mL for 2 hours.

For live imaging, neurons were deprived of NGF overnight in the presence of a broad caspase inhibitor as described above. Neurons were washed with normal media, and NGF (100 ng/mL) was applied to the distal axon chamber. To identify retrograde SEs, a Cy3-coupled anti-FLAG antibody (M2-Cy3, Sigma-Aldrich Cat# A9594, RRID:AB_439700) was applied with NGF to the distal chamber. Neurons were washed with PBS after 30 minutes and imaged at the indicated times. For PTSE labelling, non-labelled anti-FLAG antibody (M1, Sigma) was added to the distal axon chamber with NGF as described above and washed off after 30 minutes. An Alexa-568 anti-mouse antibody (Life Technologies A10042, RRID: AB_2534017, 1:100) was then added to the cell body compartment for 4-6 hour, washed with DMEM +10% FBS and imaged.

**Immunocytochemistry**

Cells were fixed with 4% PFA for 15 min at room temperature. Cells were permeabilized and blocked with 1% BSA, 0.2% Triton X-100 for 10 minutes at room temperature. Primary antibodies were diluted in 1% BSA and applied overnight at 4°C. Secondary antibodies were diluted in 1% BSA and applied at room temperature for 1 hour. For Lambda Phosphatase treatment, after fixation and permeabilization, neurons were incubated with Lambda Phosphatase solution overnight at 4°C. Lambda Phosphatase solution: 1X PMP buffer (New England Biolabs, Cat# B0716), 1X $Mn^{+}$ buffer (New England Biolabs, Cat# B1761S), 2.4:100 dilution of Lambda Phosphatase (Santa Cruz, sc-200312). Neurons were washed and antibodies applied as described above.

**Antibodies**

Rab7 antibody (Cell Signaling Technology Cat# 2094, RRID:AB_2300652) was used at a dilution of 1:200 in 10% BSA. This antibody has been validated after siRNA-mediated knockdown on western blots (Jung et al., 2017). It has additionally been validated after knockdown and immunofluorescent staining (Ding et al., 2017). Validation after knockdown was also carried out in our lab for neuronal cultures (data not shown). Rab11 antibody (Cell signaling technology, Cat# 3539S, RRID: AB_2253210) was used at a dilution of 1:200. This antibody was validated by siRNA knockdown and western blot (Baumdick et al., 2015). It has additionally been validated through knockdown and immunofluorescent staining (Rauch et al., 2016). EEA1 antibody (Cell Signaling Technology Cat# 2411S, RRID:AB_2096814), was used at a dilution of 1:200. This antibody staining is consistent with early endosomes, co-localizing with Transferrin, but not Rab7 (Mu et al., 1995). In our hands, this antibody recognized overexpressed tagged EEA1 (Lasiecka et al., 2014), co-localizes with endocytosed Tfn at short but not long chase times, the staining does not co-localize with degradative cargos, and the staining disappears when early endosomes are disrupted by Rab5 interference (Yap et al., 2017).

**Rab Co-Localization**

Images of fixed cells were acquired on a Zeiss AxioZoom Oberserver.Z1 with Apotome 2.1 structured illumination and acquired with a 63x oil objective as Z-stacks. Imaris software was used for quantification. A 3 dimensional mask of the dendrites was made from the MAP2 channel. From the mask, each individual channel was isolated within the mask. Masked images were blinded, and thresholds for signal were set

against a t=30 minute time point, when there are very few retrograde signaling endosomes in dendrites. The Imaris spot function was used to identify endosomes within the MAP2 masked volume. Endosomes were considered co-localized if the intensity centers were within 0-0.4 μm apart (Figure 1A). This was chosen based on the pixel size of ~ 250 nm x 250 nm x 450 nm in the x-y-z dimensions in our imaging system, therefore all co-localization is within one voxel. All quantification is presented as the average ± standard deviation.

**Movie Analysis**

Movies were taken on a Zeiss-880 confocal microscope at a rate of 1 frame/sec. Instantaneous speeds were determined with the software Kymograph Clear and Kymograph Direct as previously described (Mangeol et al., 2016). Kymographs are a two-dimensional display of a movie whereby a line scan of each movie frame is sequentially assembled to show behavior of endosomes over time. The x-axis represents space along the process, with the cell body being towards distance "0", whereas the y-axis represent time. All quantification is presented as the average ± standard deviation. To determine directionality, color-coded kymographs from KymoClear (blue = stationary, red = anterograde, green = retrograde), were used. Line scans were drawn at 3 locations per kymograph and the number of peaks associated with retrograde, anterograde, and stationary events were quantified.

**Animal Injections**

Postnatal day 21 ± 2 days mice were initially anesthetized in a chamber with 4% isofluorane and subsequently maintained under anesthesia at 2% isofluorane with a hood. Eyes were coated in paralube

and the fur covering the throat and upper chest removed by Nair. After thorough washing with water, the area was amply coated in betadine and then washed with isopropanol. The right salivary gland was palpated out and held in place while 2 µl of material PBS or M2-Cy3, were injected in slowly over 30 seconds using a 30 and 1/2 gauge needle and a Hamilton syringe. The needle was held in place an additional 15 seconds to prevent backflow. The animals were given 25 mg/kg ketoprofen as a post-injection analgesic. The animals were maintained for 4-5 hours before euthanasia and the dissection of both SCGs.

**Immunohistochemistry**

SCGs of salivary gland injected animals were flash-frozen in 2-methylbutane. Cryosections of SCGs were cut at 20 µm thickness. Sections were warmed to RT for 30 minutes and submerged in 4% PFA for 15 minutes at RT. Sections were then subject to antigen retrieval and antibody staining as previously described (Barford et al., 2017b).

## Results

**Multiple pools of signaling endosomes exist in the dendrites**

Dendritic SEs were previously shown to carry out important signaling functions with respect to postsynaptic maturation (Lehigh et al., 2017). Additionally, at least a subset of the SEs that enter the dendrites have been shown to be actively signaling, as marked by the presence of phosphorylated TrkA (Lehigh et al., 2017). However, their molecular composition is not known. To investigate this, we took advantage of sympathetic

neurons isolated from FLAG-TrkA knock-in mice, $TrkA^{FLAG/FLAG}$, which have a FLAG tag knocked into the endogenous TrkA locus in frame with the extracellular domain of the protein (Sharma et al., 2010). These neurons were grown in microfluidic devices which allows for fluidic separation of axons and somas (Supplementary Figure 1A-B). SEs, decorated by feeding anti-FLAG antibody from the axon chamber, reach the soma starting at about 30 minutes after feeding and continue to accumulate for several hours. This system thus allows for assaying NGF-dependent transport of TrkA-SEs to the soma for signaling. To test specificity of the anti-FLAG antibody and integrity of the chambers, neurons from either $TrkA^{FLAG/FLAG}$ mice or WT mice (not containing FLAG-TrkA) were incubated with the NGF/anti-FLAG antibody at 37°C for 30 minutes. As previously described, no staining was observed in WT cultures, demonstrating that the antibody does not enter axons non-specifically (Supplementary Figure 1C) and can thus be used to faithfully track NGF-TrkA SEs. Additionally, we have developed advanced quantification techniques to analyze endosomal co-localization. Using Imaris software, we can mask single channels from Z-stacks within dendrites exclusively, and use object-based co-localization methodology using the "spot" function of Imaris to identify endosomes (Figure 1A).

To address the endosomal identity of dendritic SEs, neurons were grown for 10-15 days *in vitro* (DIV) and subjected to the FLAG-antibody feeding assay, fixed after a 2-6 hour chase, and co-stained for endosomal markers for early endosomes, recycling endosomes, and late endosomes (Figure 1B). To mark the early endosome, we utilized an EEA1 antibody as we were unable to find a reliable Rab5 antibody for our purposes. To mark the recycling endosome and late endosomal pools we used Rab11 and Rab7 antibodies respectively. Dendritic SEs showed co-localization with multiple endosomal subtypes, including early endosomes (EEA1), recycling endosomes (Rab11), and late endosomes (Rab7) (Figure 1C-E). The extent of co-localization was quantified as described in Materials and Methods (Figure 1A). Analysis revealed that dendritic SEs are overwhelmingly found in two distinct endosomal subtypes, namely Rab11-containing recycling endosomes (32% ± 8) and Rab7-containing late endosomes (41% ± 8) (Figure 1F), with a roughly

even distribution between the two pools. No differences between 2-6 hours were observed, and therefore results were not reported separately. There are two possible pathways for how dendritic signaling endosomes are diversified into Rab11- or Rab7-positive endosomal pools: FLAG-TrkA might already be transported in multiple compartments in the axon to arrive in the soma diversified, or FLAG-TrkA is sorted within the somatodendritic region into two distinct endosomal populations after undergoing retrograde transcytosis. These two pathways are not mutually exclusive and could both occur sequentially.

**Retrograde SE's are diversified before reaching the cell body**

Our finding that dendritic SEs exist in multiple endosomal populations prompts the question of whether they arrive in multiple distinct compartments, or if SEs diversify upon reaching the dendrite. It is now well established that SEs can travel in the axon in Rab7-positive compartments (Ye et al., 2018; Zhang et al., 2013). Late endosomes (Rab7) and, to a lesser extent, recycling endosomes (Rab11), are considered to be retrograde carriers for SEs in axons (Ye et al., 2018). To investigate whether Rab7 and/or Rab11 were associated with retrogradely transported signaling endosomes, neurons were subjected to the antibody feeding assay and fixed after 30 minutes when the vast majority of endosomal SE's are traveling retrogradely (Lehigh et al., 2017). Staining for Rab7, a marker of late endosomes, showed significant co-localization (50.14% +/- 5.28) (Figure 2A-C). However, approximately half of the signaling endosomes in the axon remained unlabeled (Figure 2A-C, Rab7). Interestingly, co-staining with Rab11 also showed high co-localization between axonal signaling endosomes and recycling endosomes (46.27% +/- 7.77) (Figure 2A-C). Axonal signaling endosomes thus show ~50% co-localization with both Rab7 and Rab11, suggesting that two molecularly distinct pools of endosomes are the predominant retrograde carriers for activated TrkA (Figure 2C). We were unable to find a reliable early endosome antibody for axons, but only a small subset of axonal SEs has been reported to co-localize with Rab5 (Ye et al., 2018). Therefore, in our

hands, retrograde SEs are not molecularly uniform, but are diversified prior to arriving in the soma into recycling and late endosomal pools.

**SEs undergo dynamic endosomal processes in the dendrite**

It has been previously reported that SEs in dendrites have distinct movements from axonal SEs (Lehigh et al., 2017), however we find that axonal and dendritic SE pools exist in similar endosomal subtypes. To investigate the different movement profiles between axonal and dendritic SEs, we used a live imaging chamber and a fluorescent anti-FLAG antibody (M2-Cy3, Sigma) and determined the motility of endogenous retrograde SEs in both dendrites and axons. The labeled endosomes correspond to FLAG-TrkA SEs since only background labeling was observed when a control culture not expressing FLAG-TrkA (WT) was incubated with M2-Cy3 antibody. This assay is thus specific for $TrkA^{FLAG/FLAG}$ neurons (Supplementary Figure 2A), and importantly allows us to visualize endogenous protein trafficking of individual endosomes (Supp. Figure 2B, Movie 1). Similar to previous reports, we find that SEs in the dendrite take frequent pauses, change direction, and move at a slightly lower speed on average compared to axonal SEs (Figure 3A-E, Movie 2). Interestingly, we noticed incidences where multiple endosomes seemed to merge together into a stationary SE (Figure 3C, arrow). Dendritic endosomes carrying other cargos (such as transferrin) are capable of fusion and fission, leading to mixing (by fusion) or sorting (by fission) of cargos, but these types of dynamic events have not been reported for the SE. We thus asked if SEs in the dendrite might also undergo dynamic endosomal fission and fusion events. Movies acquired in dendrites were analyzed for putative fusion and fission events. Putative fusion and fission events were scored by the same criteria we have used previously (Lasiecka et al., 2014): putative fusions consist of a motile endosome merging with another punctum and not re-emerging on the other side (Movie 3). Putative fission events consist of a single punctum separating into more than one, which usually move away from each other (Movie 4). We

refer to these events as “putative” since light microscopy cannot resolve very close apposition of two endosomes from fusion into a single endosome. We find that putative fusion events between SEs in dendrites are observed frequently. Most dendrites (83.3% ± 30.5) displayed at least one putative fusion event and oftentimes more than one within the 7-8 minute movie. A representative kymograph shows two putative fusion events in a single dendrite (Figure 3F, arrows), however most SEs pass each other unimpeded (Figure 3F, arrowheads). This indicates that SEs are not a steric hindrance for each other, but that many SEs pass each other without stopping. One representative putative fusion event, corresponding to the arrow boxed in Figure 3F, is shown in single frames as Figure 3G.

In addition to putative fusion events, we were able to observe putative fission events where a single large endosome sits stationary for an extended period of time, but then suddenly generates multiple small motile vesicles (Movie 4) (Figure 3H). Representative kymograph shows small endosomes budding out of a large stationary carrier (Figure 3I). Intriguingly, these dynamic events suggest that there are multiple pools of signaling endosomes within the dendrite which have the capacity to attach to different motors, tether and fuse with different compartments, and bud out new single endosomes containing axonally-derived TrkA. These dynamic fission and fusion events were almost exclusively observed in the dendrites, and were rare in axons (Figure 3J).

**Post-Transcytotic Signaling Endosomes (PTSEs) are abundant in dendrites**

The observation that dendritic SEs co-localized to some degree (albeit low) with the early endosomal marker EEA1 in dendrites (7% ± 3) (Figure 1B-C) suggested that FLAG-TrkA-positive dendritic SEs underwent apparent “maturational reversion” back to an early endosome. We find that SEs are transported down the axon partially as Rab7 positive late endosomes (Figure 2), consistent with previous studies

(Bhattacharyya et al., 2002; Deinhardt et al., 2006; Ye et al., 2018; Zhang et al., 2013). However late endosomes are typically thought of as being on the path towards degradation, not towards reverting back to an early endosome. We additionally find that there is a large population of Rab11 positive recycling endosomes carrying retrograde TrkA in axons. However, recycling endosomes eventually fuse with the plasma membrane, as opposed to reverting back to early endosomes. One possible mechanism of such apparent "maturational reversion" is that dendritic SEs are derived from retrograde transcytosis. As mentioned previously, retrograde transcytosis is a pathway whereby axonally-derived TrkA is exocytosed onto the somatodendritic membrane and subsequently re-endocytosed (Suo et al., 2013). We refer to these re-endocytosed SEs as PTSEs (post-transcytotic SEs). The endosomal identities and dynamic behaviors of PTSEs are unknown. To examine if dendritic SEs are PTSEs, we utilized a recycling assay (see Materials and Methods) that specifically marks SEs that have undergone retrograde transcytosis (Figure 4A). Specifically, we used the distal anti-FLAG antibody feeding assay and, during the chase period, an Alexa 647 anti-mouse secondary antibody was added to the soma chamber. This antibody is capable of binding to the anti-FLAG antibody when it is externalized on the somatodendritic surface and is thus used as a way to track re-endocytosing TrkA which originated at the distal axon. Any naïve (biosynthetic) TrkA pool on the somatodendritic surface was never exposed to the anti-FLAG antibody and therefore would not be bound by the anti-mouse antibody, allowing specific tracking of PTSEs. There is no detectable signal of the Alexa 647-antibody in the $TrkA^{FLAG/FLAG}$ mouse unless anti-FLAG antibody is added to the distal axon chamber, showing specificity of the labeling procedure (Figure 4B). Using the recycling assay, we observed that PTSEs are abundant in dendrites (Figure 4C). Interestingly, PTSEs were rarely observed in axons.

To determine which endosomal subtype PTSEs correspond to, we co-stained for markers against the 3 major endosomal compartments of interest and found co-localization with each compartment (Figure 4D-G). Similar to total SEs examined above (Figure 1), PTSEs have low but consistent co-localization with EEA1 (7% ± 4), suggesting that they pass quickly through the early endosome after retrograde transcytosis.

They have much higher co-localization with Rab7 (42% ± 9) and Rab11 (36% ± 8), suggesting that they are sorted into both the recycling and degradative arms of the endosomal pathway (Figure 4H). This suggests that PTSEs exist in multiple endosomal pools within the dendrites. Simultaneous detection of FLAG-SEs and PTSEs in dendrites was carried out to determine to what extent dendritic SEs derive from retrograde transcytosis. The proportion was variable but often exceeded 50%. The highest observed proportions exceeded 80%, suggesting that a significant proportion of dendritic SEs undergo retrograde transcytosis. The high variability between different cells was likely due to technical limitations of detecting re-endocytosed pools above background on fainter labeled cells. Alternatively, it is possible that the extent of retrograde transcytosis is heterogeneous among different cells.

**PTSEs mix in with other endosomal cargo**

We observed high proportions of PTSEs to be recycling endosomes (Rab11) (Figure 4D). In addition, we saw frequent dynamic fusion events of SEs in dendrites (Figure 3J). These observations raised the possibility that re-endocytosing axonally-derived TrkA in dendrites does not stay segregated into a dedicated SE, but dynamically mixes with other cargos entering endosomes from the dendritic surface. We thus sought to determine whether re-endocytosed TrkA (PTSEs) co-localized with other cargos that traverse the dendritic recycling endosome. To this end, we utilized the canonical recycling cargo Transferrin (Tfn). After endocytosis, Tfn is quickly recycled back to the surface of the cell via the early endosome and the recycling endosome. To investigate if re-endocytosed TrkA intermixed with Tfn or stayed separate, we performed an antibody feeding assay where we pulsed NGF and anti-FLAG antibody for 30 minutes on the distal axon side, acid washed to remove remaining surface bound antibody, and incubated the cell body chamber with fluorescent Tfn for 2 hours (Figure 4I). Such a long incubation with Tfn will result in the filling of both early and recycling endosomes. Interestingly, we see approximately 37% ± 14 of PTSEs

positive for Tfn (Figure 4J). This matches closely with the percentage of PTSEs found in recycling endosomes (Figure 4H), suggesting that the re-endocytosed axonally-derived TrkA present in recycling endosomes mixes in with other cargos fated for the same destination.

In order to determine if TrkA was still signaling after retrograde transcytosis, we counterstained against two phosphorylation sites on TrkA which are associated with active signaling of TrkA, pY490 and pY785. Specificity of antibodies was tested by treating the cells with Lambda Phosphatase prior to immunostaining. We observed loss of both phosphorylated antibody signals (Figure 5A). Previous work reported that between 15 and 35% of dendritic SEs still had associated phosphostaining (Lehigh et al., 2017). Since a high proportion of dendritic SEs can arise via retrograde transcytosis, we expected to see co-labeling of PTSEs with anti pTrkA antibodies in a similar range. Indeed, some PTSEs are marked with antibodies against p-Y490 (5.56 ± 0.8%) or p-Y785 (10.13 ± 0.9%) (Figure 5C), suggesting that at least a subset of PTSEs are still signaling.

**PTSEs have distinct movement profiles**

We next determined the movement profiles of PTSEs. To this end, we utilized the same PTSE assay as for fixed cells (Figure 4A) and carried out live imaging with fluorescent secondary antibodies to capture re-endocytosing FLAG-antibody decorated TrkA in the somatodendritic domain. We observed many examples of fast persistent movements similar to that of dendritic SEs, showing that PTSEs are capable of long-range movement (Figure 6A). Interestingly, there is a large pool of stationary endosomes, pseudocolored as blue on kymographs (Figure 6A). The percentage of stationary endosomes is much larger for PTSEs than it is for axonal or dendritic SEs (Figure 6B). These differences are statistically significant. Additionally, the instantaneous speeds of PTSEs are slightly slower compared to SEs in the dendrites and

axons, both for the anterograde and retrograde direction (Figure 6C-E). It is possible that stationary PTSEs play a distinct signaling role within the dendrites, however we cannot rule out that the large stationary pool arises from technical differences in the two assays for PTSE and SE live imaging rather than biological differences. Visualization of PTSEs requires application of a secondary labeled antibody to the live cells. This extra antibody layer could cause less efficient internalization or reduced motility.

**SEs are diversified *in vivo***

Next, we asked whether or not SEs are diversified into Rab11 recycling endosomes and Rab7 late endosomes *in vivo* as they are *in vitro*. A previously published paper (Lehigh et al., 2017) labeled endosomes *in vivo* by injection of labeled WGA into the anterior eye, a target region for a subset of SCG axons. They show that WGA-containing endosomes travel retrogradely and label cell bodies within the SCG. Wheat germ agglutin is a lectin that binds to carbohydrate chains on many membrane proteins, and therefore the exact relationship between WGA-containing endosomes and TrkA-SEs is not known. In order to unambiguously label FLAG-TrkA SEs, we injected a fluorescent anti-FLAG antibody into the eye or the salivary gland, which is also innervated by the SCG (Figure 7A). We observed clear FLAG-antibody decorated SEs in the SCG of eye-injected FLAG-TrkA mice, but not in FLAG-TrkA mice eye-injected with PBS, showing that we are able to visualize TrkA-SEs *in vivo* (Figure 7B), similarly to what was previously shown (Lehigh et al., 2017). Additionally, injection into the eye labelled projections exclusively to the ipsilateral SCG, not the contralateral SCG, showing that the antibody is being contained within the eye and SCG and not spreading to the contralateral side of the body (Figure 7B). No label was observed when anti-FLAG antibody was injected into salivary glands of WT mice (another target of SCG axons) not expressing FLAG-TrkA, showing assay specificity (Figure 7C).

To ask if SEs *in vivo* are diversified, $TrkA^{FLAG/FLAG}$ animals received anti-FLAG antibody injections in the salivary gland, and their somata in the SCG were subsequently examined for Rab co-localization. Numerous labeled cells that successfully took up the antibody are clearly visible within the SCG (Figure 7C). Fixed sections were counter-stained with antibodies towards endosomal markers including early endosomes (EEA1), recycling endosomes (Rab11), and late endosomes (Rab7). Retrograde SEs partially co-localize with each of these markers *in vivo*, suggesting that retrograde SEs are diversified into recycling and late endosomal pools in the soma *in vivo* as well (Figure 7D-E).

## Discussion

NGF was the first neurotrophic factor discovered, and it has served as the archetypal ligand-receptor system in the nervous system to study the molecular underpinnings of nervous system development, maintenance, and pathology. How precisely the signal from target-derived NGF is transduced from distal tips of axons long distances back to the cell body has been of intense interest for over 20 years and has led to the important discovery that signaling takes place on endosomes after endocytosis of NGF-TrkA. The term "signaling endosome" was coined specifically based on the findings from the NGF-TrkA system (Grimes et al., 1996). It is now well established that the NGF-TrkA signaling endosome travels retrogradely from the axon terminal to the soma to mediate a multitude of developmental events in time and space (Howe and Mobley, 2005). We also know that retrograde SEs arriving at the soma from the axon can undergo additional trafficking events, including entering dendrites as well as undergoing rounds of exo- and endocytosis in the soma (termed retrograde transcytosis) (Suo et al., 2013). Very little is known, though, about these somatic and dendritic SE trafficking events. We show here that dendritic SEs undergo dynamic fusion and fission events, mix with other endocytosed cargos in recycling endosomes, and do not stay as a distinct endosomal entity in dendrites. In contrast, axonal SEs undergo few dynamic fusion events during their retrograde

transport even though they exist in the same endosomal sub-populations (Figure 3J). We thus propose a model whereby TrkA travels in the axon in distinct, non-intermixing SEs, but then undergoes additional molecular diversification into recycling and late endosomes by undergoing retrograde transcytosis in the somatodendritic domain. Dendritic SEs thus undergo dynamic trafficking events and intermix extensively with other endocytosing cargos.

After NGF-TrkA binding and internalization in the distal axon, we and others have determined that ~40-50% of retrograde SEs are Rab7-containing late endosomes (Zhang et al., 2013) (Figure 2). Rab7-containing late endosomes are typically thought of as a pre-degradative compartment (Huotari and Helenius, 2011). Additionally, late endosomes are typically multivesicular bodies (MVBs) with intraluminal vesicles (ILVs). It has been previously suggested that there are multiple morphologically distinct pools of SEs in the axon, including MVBs and single vesicles (Bhattacharyya et al., 2002). How neurotrophic receptors residing on ILVs can induce critical neurotrophic functions such as survival or PSD clustering remains unknown. Here, we report the first observed fusion and fission events of SEs (Figure 3), which is consistent with packing or unpacking SEs to and from MVBs into additional endosomal pools. Dynamic fusion and fission events are commonly observed in other cell types and with other cargos within the endosomal system. Fission and fusion are indicative of sorting events taking place. We propose a new model based on our findings that NGF-TrkA is able to dynamically sort away from degradation and recycle instead.

We previously identified NGF-TrkA in recycling endosomes in the soma, however the mechanism by which it arrived in the recycling endosome (Rab11) was not established (Suo et al., 2013). This created a conceptual challenge because late endosomes (Rab7) were considered the sole carrier of retrograde TrkA. Here we reconcile this by finding that that the SE travels retrogradely in roughly equal proportions of Rab7+

and Rab11+ carriers (Figure 2). Based on our data, we propose a model whereby upon internalization at the distal tips of axons, NGF-TrkA rapidly moves through an early endosome compartment and diversifies into 2 major retrograde carriers: recycling endosomes and late endosomes. While this is the first evidence that a substantial pool of SEs can travel retrogradely in a recycling endosome, others have found that anterogradely transporting TrkA and post-endocytic TrkA in the cell body can co-localize with Rab11 (Ascano et al., 2009; Suo et al., 2013).

Rab11 endosomes recycle cargo to the surface of the cell, whether in a similar location or somewhere far away. Therefore, it was an intriguing possibility that recycling endosomes may be fated towards retrograde transcytosis. This retrograde transcytosis may then allow re-endocytosed NGF-TrkA to enter Rab11 carriers. We indeed find that re-endocytosed retrograde TrkA sorts quickly out of the early endosome equally into the recycling (Rab11) and degradative (Rab7) arms of the endosomal pathway, providing a novel model of diversification for TrkA signaling endosomes.

What roles do distinct pools of NGF-TrkA endosomes play in the development of a neuron? An intriguing possibility is that the function of the SE is partitioned into molecularly discrete pools. The SE is able to initiate many different signaling cascades, each with distinct functional outcomes. In the axon, SEs are able to signal locally to enhance axon growth and branching (Harrington et al., 2011; Suo et al., 2015). Additionally, they can signal through PI3K to promote internalization and retrograde transport (Kuruvilla et al., 2000). In the soma, they are able to signal through calcium and MAPK to promote CREB phosphorylation and large transcriptional changes (Deppmann et al., 2008; Lonze and Ginty, 2002). How is the signaling endosome able to initiate so many unique signaling cascades? One possibility is that endosomal diversity could lead to functional diversity between endosomes. For example, recruitment of Coronin-1a in the cell body endows the SE with the ability to induce calcium release, avoid lysosomal

fusion, and undergo retrograde transcytosis (Suo et al., 2013). Interestingly, Coronin1a also plays a role in axon outgrowth by affecting PI3K signaling, but does not affect MAPK signaling (Suo et al., 2015). Because of their distinct maturational and trafficking capacities, an intriguing possibility is that these pools have distinct signaling capacities. Indeed, the trafficking and signaling of cargo, including TrkA, are often intertwined (Barford et al., 2017a). Therefore, the multiple trafficking patterns (late and recycling endosomal carriers) could influence endosomal maturation and signaling function. Much more work will need to be done to elucidate these potential signaling patterns.

Another interesting possibility for the role of multiple endosomal populations within the dendrite is the development of post-synaptic densities. TrkB and BDNF signaling play a critical role in synaptic development in neurons within the central nervous system, specifically with regard to glutamatergic synapses (Figurov et al., 1996; Huang and Reichardt, 2003; Lu et al., 2014). As opposed to NGF and TrkA's canonical role as retrograde carriers for neurotrophic signaling, it has been shown that BDNF can be released locally and act in a paracrine manner within the dendrites during long-term potentiation (Hartmann et al., 2001). Interestingly, during long term potentiation (LTP), BDNF-dependent TrkB endocytosis results in the recycling of TrkB to the surface via the Rab11 recycling endosome. This recycling via Rab11 is critical for sustained TrkB signaling, but unnecessary for transient signaling (Huang et al., 2013). Interestingly, internalized BDNF also gets recycled back to the surface in activity-dependent secretion. This recycled BDNF is then available to act again locally at the dendrite. This recycled pool of BDNF is critical for maintaining LTP (Santi et al., 2006). This draws many parallels to the retrograde transcytosis pathway in sympathetic neurons, and begs the question: Is NGF being released by SEs that undergo retrograde transcytosis? Indeed, only a subset of PTSEs retain phosphorylated TrkA, indicating that they may have lost the bound NGF. The fate of NGF, the importance of TrkA recycling back to the surface, and the role retrograde transcytosis plays in the development of dendritic specializations in sympathetic neurons will be an intriguing question for the field.

In addition to retrograde transcytosis, Kuruvilla and co-workers have discovered an additional transcytotic pathway which is distinct from the retrograde transcytosis pathway described by us (Ascano et al., 2009; Yamashita et al., 2017). Their work shows that naïve TrkA travels anterogradely by transcytosis from the soma surface to the axon. Interestingly, this is dependent on signaling from retrogradely arriving NGF-TrkA. In their recent work, they propose an intriguing model by which the NGF-TrkA SE is exocytosed on the soma, trans-phosphorylates and stimulates the endocytosis of unliganded naïve TrkA through interaction of the naive receptor with the axonally-derived TrkA. The naive receptor is then able to undergo endocytosis. After endocytosis, the naïve receptor is de-phosphorylated by PTP-1B and is trafficked anterogradely into the axon (Yamashita et al., 2017). This creates a feedforward loop whereby recycled NGF-TrkA induces anterograde trafficking of newly synthesized naïve TrkA. Intriguingly, this anterogradely transported naïve TrkA pool also travels in Rab11 recycling endosomes (Ascano et al., 2009). However, it remained unclear as to what happened to the axonally-derived TrkA that interacts with naive TrkA. Our work specifically follows axonally-derived TrkA, and we interestingly do not see PTSEs traveling anterogradely into axons but instead find them throughout dendrites. Even after 6 hours, we see few to no PTSEs in axons. How the naïve TrkA pool which re-endocytoses together with axonally-derived TrkA from the somatodendritic surface sorts away into a distinct axon-bound Rab11 endosome is completely unknown at this point, but will be of great importance to discover in the future.

In summary, our findings provide the first evidence of molecular diversification of NGF-TrkA SEs. We find that there are two mechanisms of diversification: initial diversification of axonal retrogradely transporting SEs, and subsequent diversification through retrograde transcytosis. The SE is additionally able to undergo dynamic fusion events, as well as mixing with other somatodendritically endocytosed cargos, showing that it is much more dynamic than has been previously appreciated. Our results suggest

the hypothesis that functional diversification of NGF signaling might be achieved by molecular diversification of SEs, a hypothesis we will test in future experiments.

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**Author Contributions**

The authors take full responsibility for the integrity of the data. Study concept and design: KB, CCY, AK, BW, CD. Analysis: KB, LM, KM. Interpretation of data: KB, CCY, AK, CD, BW. Drafting of manuscript: KB, AK, BW, CD.

**Additional Information**

Authors have no conflicts of interest.

## Figures

**Figure 1.** Dendritic SEs exist in multiple different endosomal populations. (A) Illustration of Imaris spot co-localization. The endosome on the bottom left of the inset is co-localized (arrow), while the other two SEs are not. (B) SEs were fixed after 2-6 hours of distal anti-FLAG antibody feeding and co-stained for endosomal markers EEA1, Rab7, and Rab11 as indicated. Arrows are co-localized, arrowheads are not. Individual channels and merged images shown. Dendrites are outlined with dotted lines. (C-E) Representative line scans with corresponding images showing intensity peaks of EEA1 (C), Rab11 (D), and Rab7 (E) in green and TrkA SEs in red. Co-extensive peaks indicate co-localization. (F) Quantification of dendritic SE co-localization with endosomal marker (n=3-4 independent experiments, 15 fields of view per experiment).

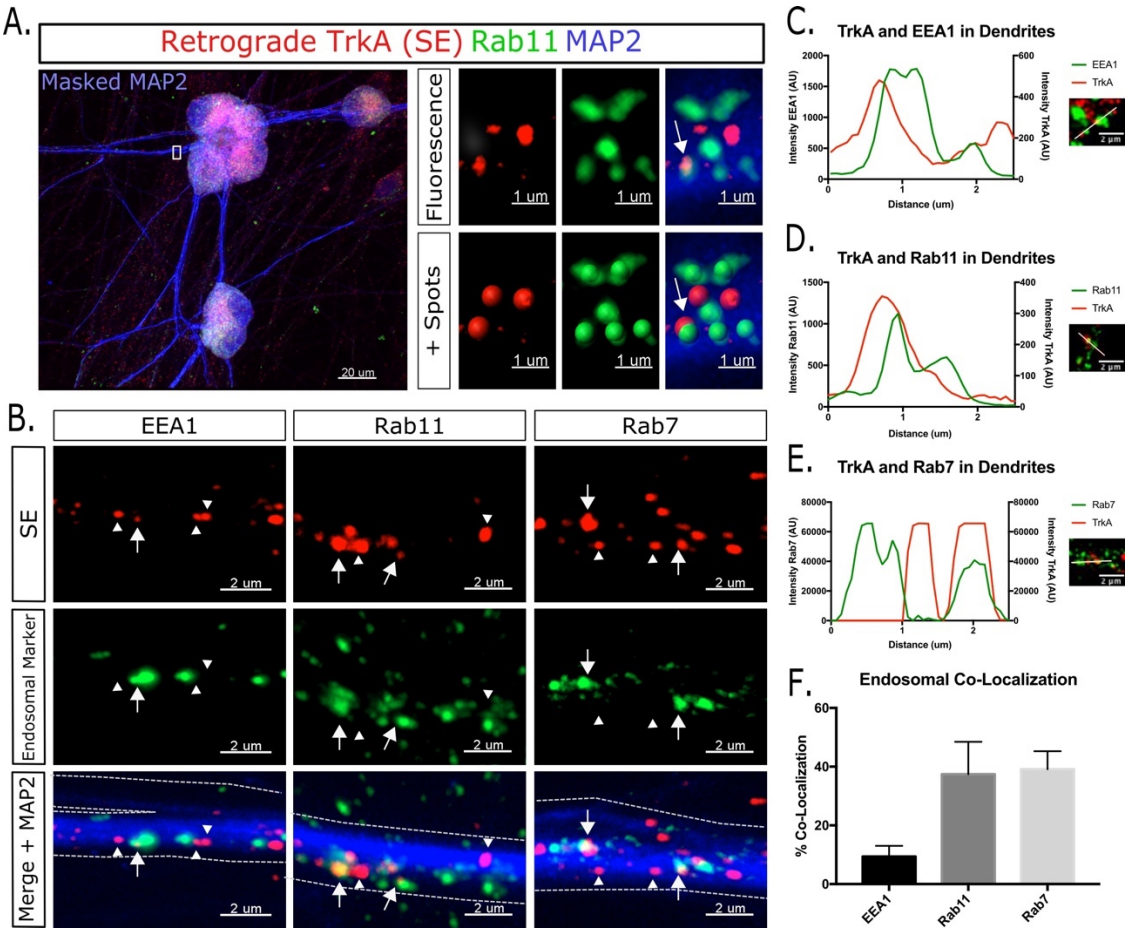


**Figure 2.** Axonal SEs transport retrogradely in late endosomes and recycling endosomes. (A) Retrograde SEs show co-localization with late endosomes (Rab7) and recycling endosomes (Rab11). Arrows are co-localized, arrowheads are not. (B) Imaris spot co-localization was used to determine co-localization. (C) Quantification of axonal SE co-localization with endosomal marker (n=3-4 independent experiments, 15 fields of view per experiment).

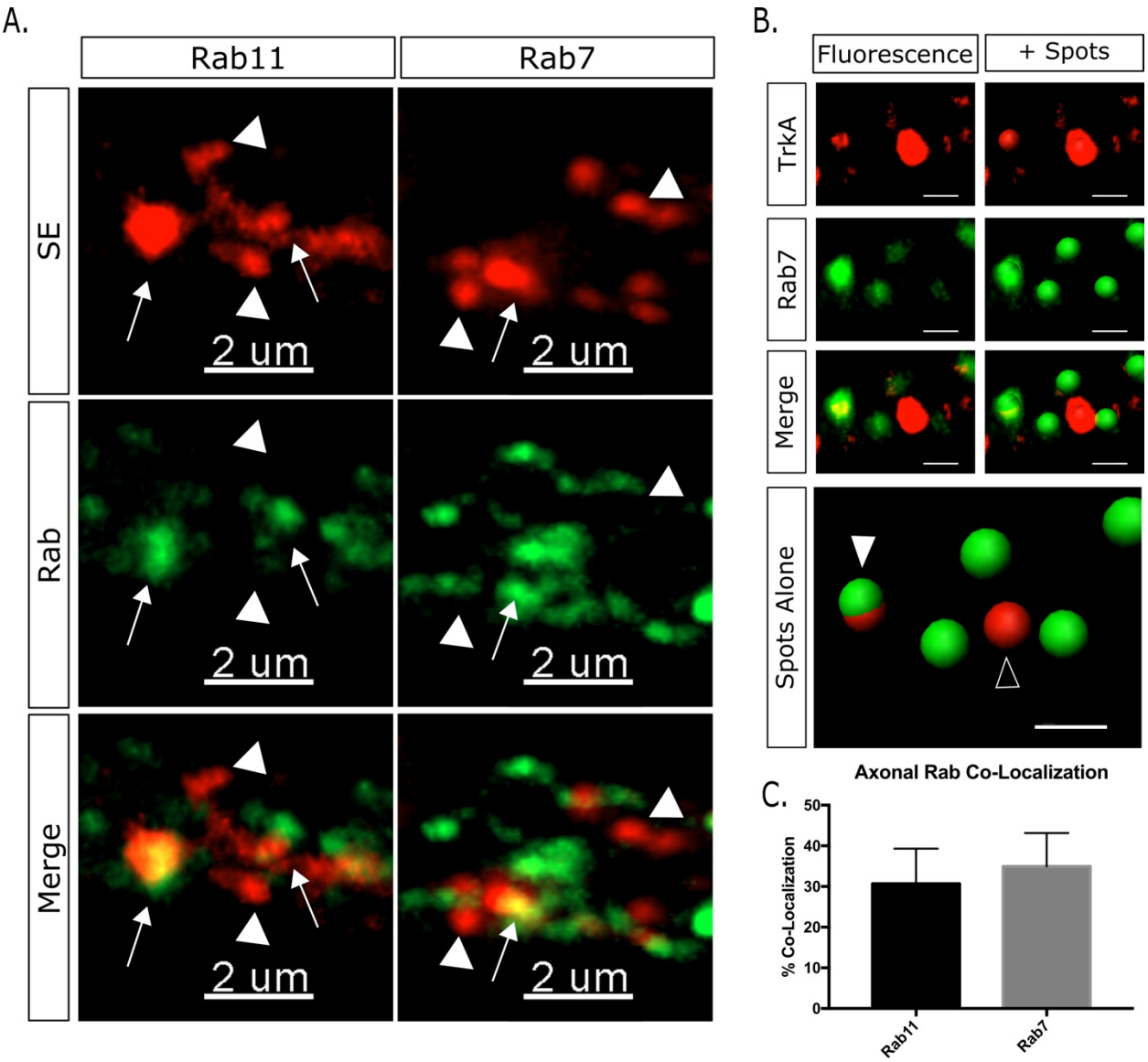


**Figure 3.** SE movement in dendrites. (A) Representative kymograph of retrograde axonal movement of signaling endosomes (visualized with Cy3-anti-FLAG antibody fed in distal chamber). Images were taken at a rate of 1 frame/sec. Kymographs were generated by KymoClear in which stationary events are coded in blue, retrograde events in green, and anterograde events in red. (B) Axonal retrograde speeds were calculated from 34 of movies from 3 independent experiments and show an average speed of 0.99 μm/s. (C) Representative kymograph of SE movements in a dendrite. Arrow indicates multiple endosomes coming together in a putative fusion event. (D) Histogram of anterograde dendritic speeds. Speeds were calculated from 3 independent experiments and show an average speed of 0.71 ± 0.28 μm/s. (E) Histogram of retrograde dendritic speeds. Speeds were calculated from 3 independent experiments and show an average speed of 0.67 ± 0.26 μm/s. (F) Representative kymograph showing multiple putative fusion events in a single dendrite. Arrows indicate putative fusion between SEs, arrowheads point to an SE that passes with no fusion. Box indicates position of still images in (G). (G) Stills from dendritic movie showing putative fusion event. Green arrows point at position of putative fusion, and yellow arrows indicate individual endosomes dynamically merging and splitting. (H,I) A stationary SE undergoing multiple fission events is shown as stills from a dendritic movie (H) and as a kymograph (I). (J) Quantification of putative fission/fusion events in axons vs dendrites. n=15 cells for axons; 13 cells for dendrites; 3 independent experiments each.

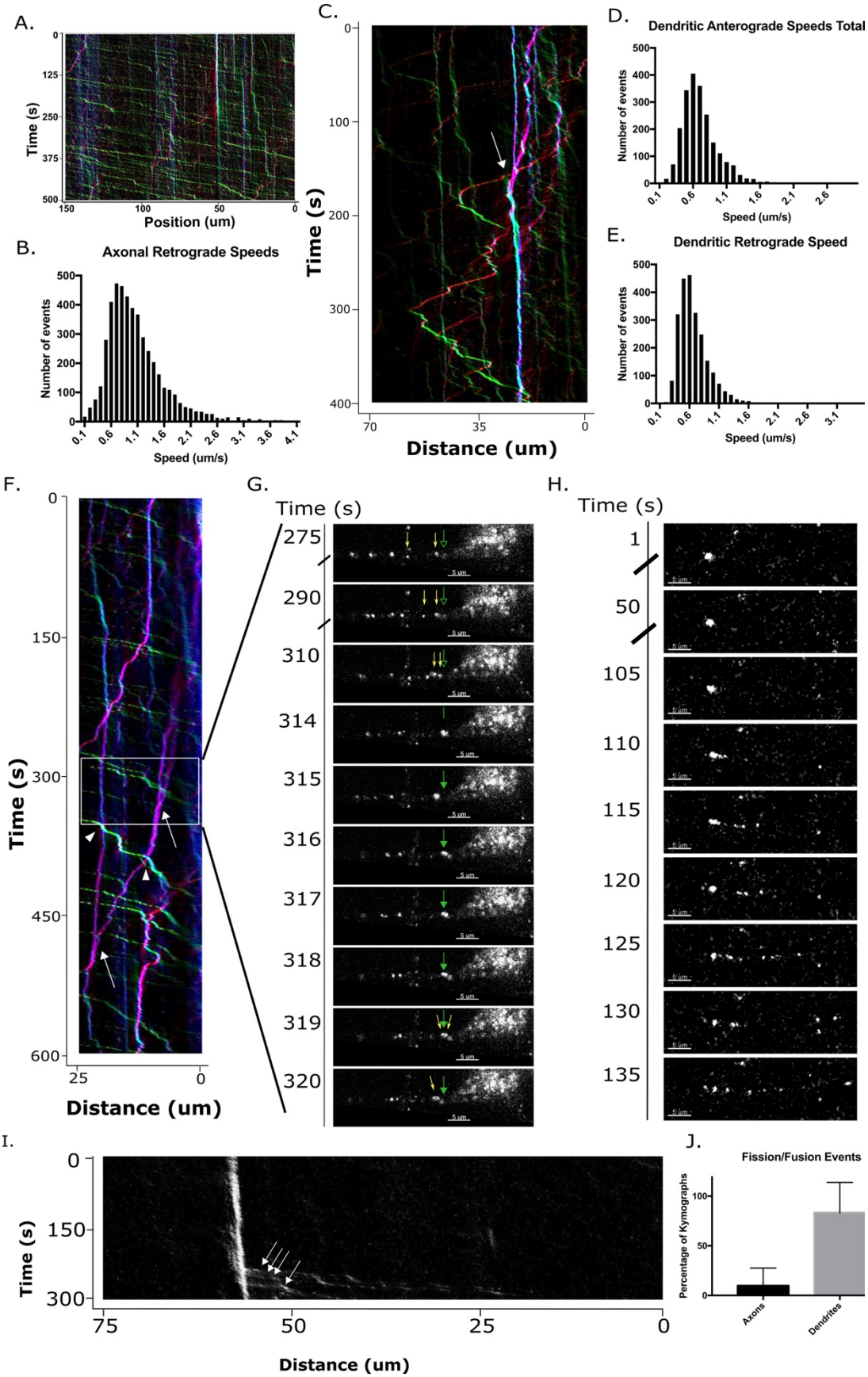

A.
Time (s)
Position (um)
B.
Axonal Retrograde Speeds
Number of events
Speed (um/s)
C.
Time (s)
Distance (um)
D.
Dendritic Anterograde Speeds Total
Number of events
Speed (um/s)
E.
Dendritic Retrograde Speed
Number of events
Speed (um/s)
F.
Time (s)
Distance (um)
G.
Time (s)
H.
Time (s)
I.
Time (s)
Distance (um)
J.
Fission/Fusion Events
Percentage of Kymographs
Axons
Dendrites

**Figure 4.** Post-transcytotic signaling endosomes (PTSEs) are abundant in dendrites. (A) Antibody feeding assay to label PTSEs as detailed in Materials and Methods. (B) PTSE labelling assay is specific for anti-FLAG antibody. No label is detected with the 647 secondary antibody without prior feeding of the anti-FLAG antibody (647 alone). (C) Many SEs are labelled by the 647 anti-mouse antibody, indicating that they have undergone retrograde transcytosis. PTSEs are thus abundant in dendrites. Dendrites are outlined with dotted lines. (D) After PTSE labelling, PTSEs co-localize with EEA1, Rab11, and Rab7 in dendrites (MAP2 indicated in blue). Arrows indicate co-localized SEs, arrowheads indicate non co-localized SEs. Dendrites are outlined with dotted lines. (E-G) Representative line scans of intensities showing intensity peaks of EEA1 (E), Rab11 (F), and Rab7 (G) in green and PTSEs in pink. (H) Quantification of co-localization of PTSEs with endosomal markers (n=3-4 independent experiments, 15 fields of view per experiment). (I) After 2 hours of dual PTSE and fluorescent Tfn feeding, Tfn (green) substantially colocalizes with PTSEs (purple) in dendrite (MAP2 in blue) (filled arrowheads). Non co-localizing endosomes (empty arrowheads) are also observed. (J) Quantification of co-localization between PTSEs and Transferrin (n=10 fields of view).

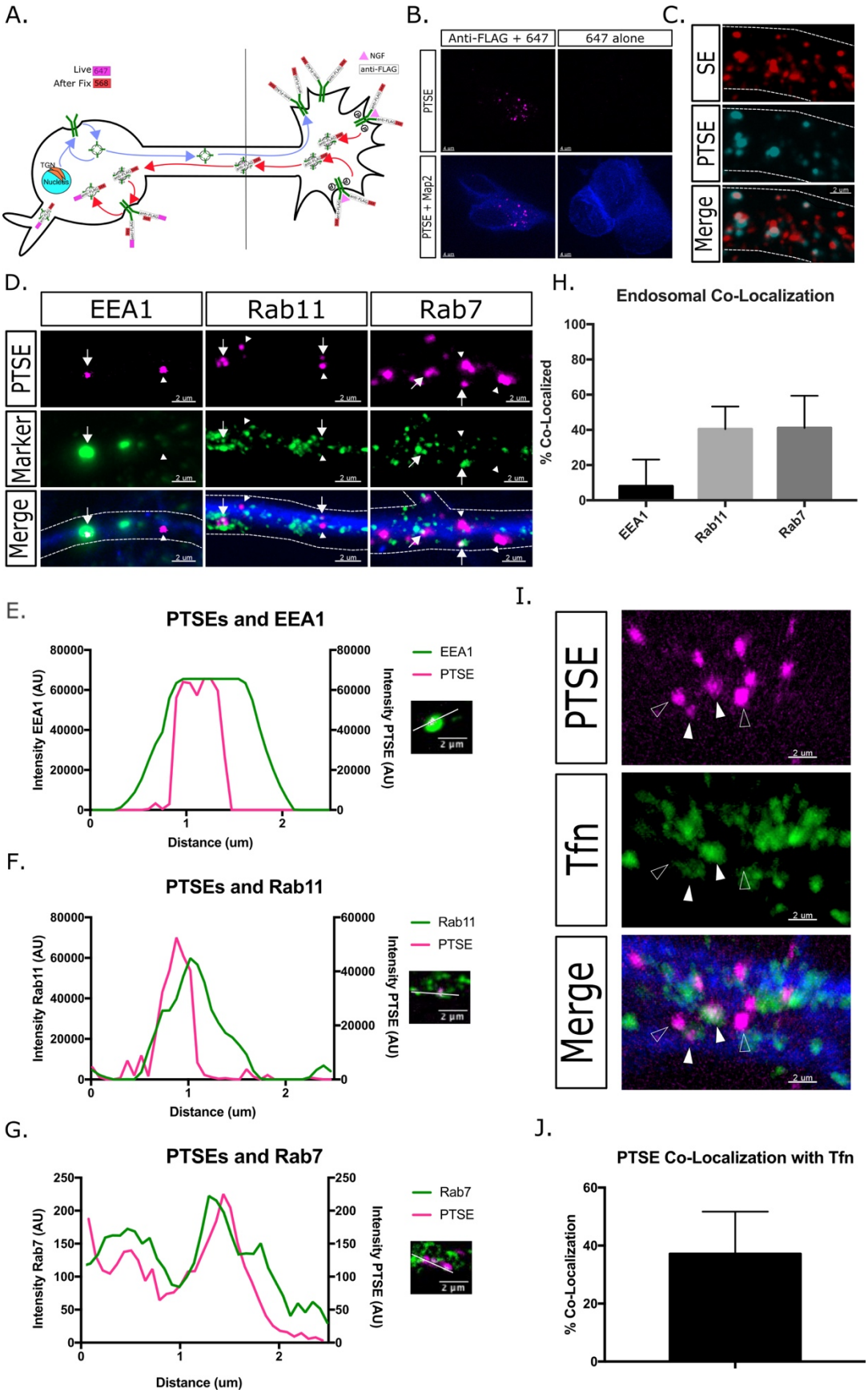

A.
Live 647
After Fix 568
NGF
anti-FLAG
TGN
Nucleus
B.
Anti-FLAG + 647
647 alone
PTSE
PTSE + Map2
C.
SE
PTSE
Merge
D.
EEA1
Rab11
Rab7
PTSE
Marker
Merge
H.
Endosomal Co-Localization
% Co-Localized
EEA1
Rab11
Rab7
E.
PTSEs and EEA1
Intensity EEA1 (AU)
Intensity PTSE (AU)
Distance (um)
EEA1
PTSE
F.
PTSEs and Rab11
Intensity Rab11 (AU)
Intensity PTSE (AU)
Distance (um)
Rab11
PTSE
G.
PTSEs and Rab7
Intensity Rab7 (AU)
Intensity PTSE (AU)
Distance (um)
Rab7
PTSE
I.
PTSE
Tfn
Merge
J.
PTSE Co-Localization with Tfn
% Co-Localization

**Figure 5.** A subset of PTSEs contain activated TrkA. (A) Antibodies against phosphorylated TrkA (p-Y490 and p-Y785) show high signal in dendrites (as shown by MAP2) after 6 hours of NGF feeding, but signal is abolished when neurons are treated post-fixation with Lambda Phosphatase. Dendrites are outlined with dotted lines. (B) PTSEs partially co-localize with p-Y490 and p-Y785. Arrows are co-localized, arrowheads are not co-localized. Dendrites are outlined with dotted lines. (C) Quantification of co-localization between PTSEs and P-TrkA (n=2-3 independent experiments, 15 fields of view per experiment).

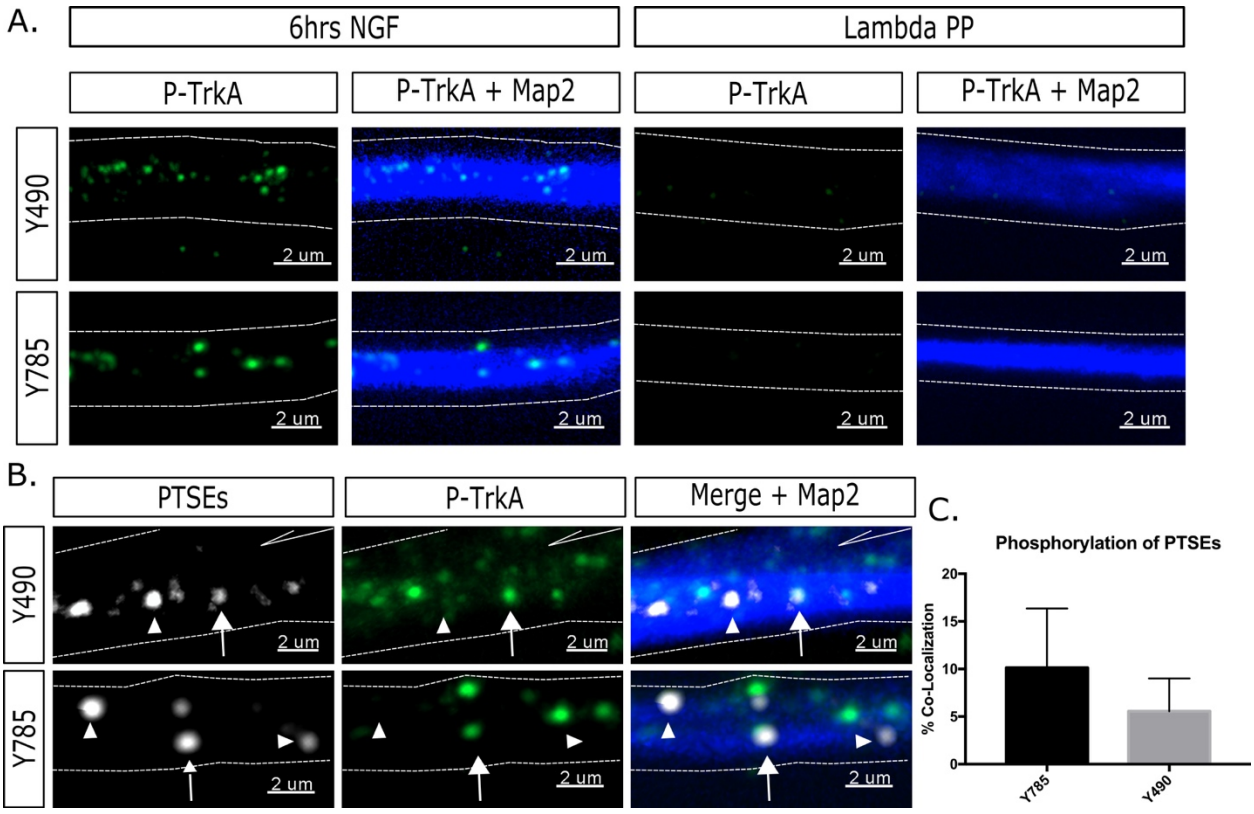
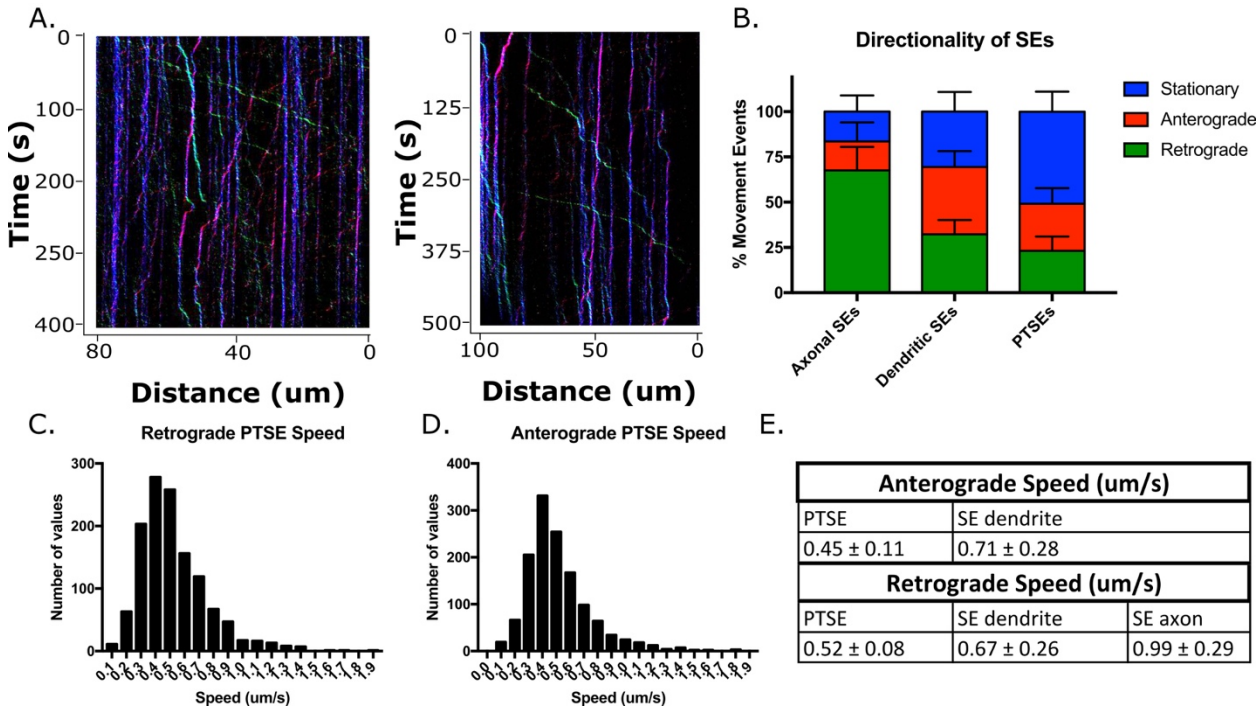


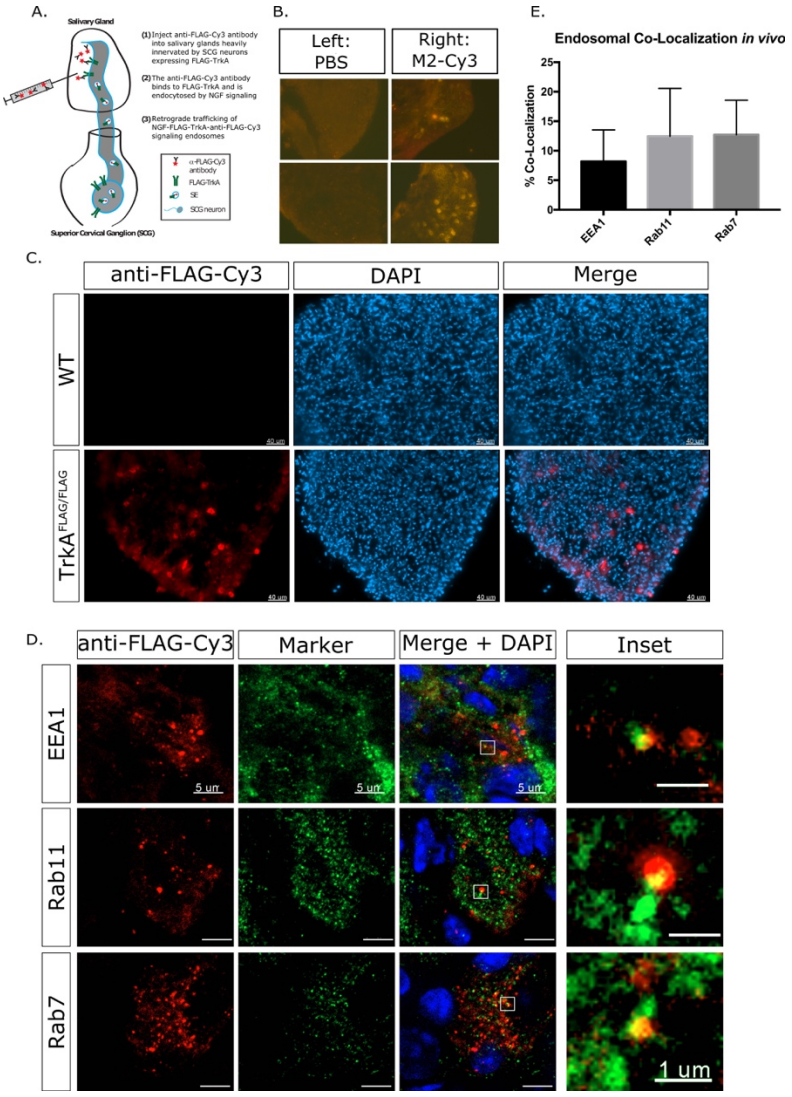
**Figure 6.** PTSE movement in dendrites. (A) Representative kymographs showing many stationary endosomes (blue) and long-range movements (red = anterograde and green = retrograde). (B) Quantification of directionality (n= 15-30 kymographs from 4 independent experiments). Each direction for the three population were significantly different from each other (i.e. percent anterograde was significant between axons and dendrites, axons and PTSEs, and dendrites and PTSEs) at $p<0.0001$, except retrograde movement between dendritic SEs and PTSEs, which was significant at $p=0.0014$ as determined by ANOVA. (C) Dendritic anterograde PTSE speeds were calculated from 24 movies from 4 independent experiments. (D) Dendritic retrograde PTSE speeds were calculated from 24 movies from 4 independent experiments. (E) Quantification of PTSE speeds using KymoClear and KymoDirect comparing axonal SEs, dendritic SEs and dendritic PTSEs.



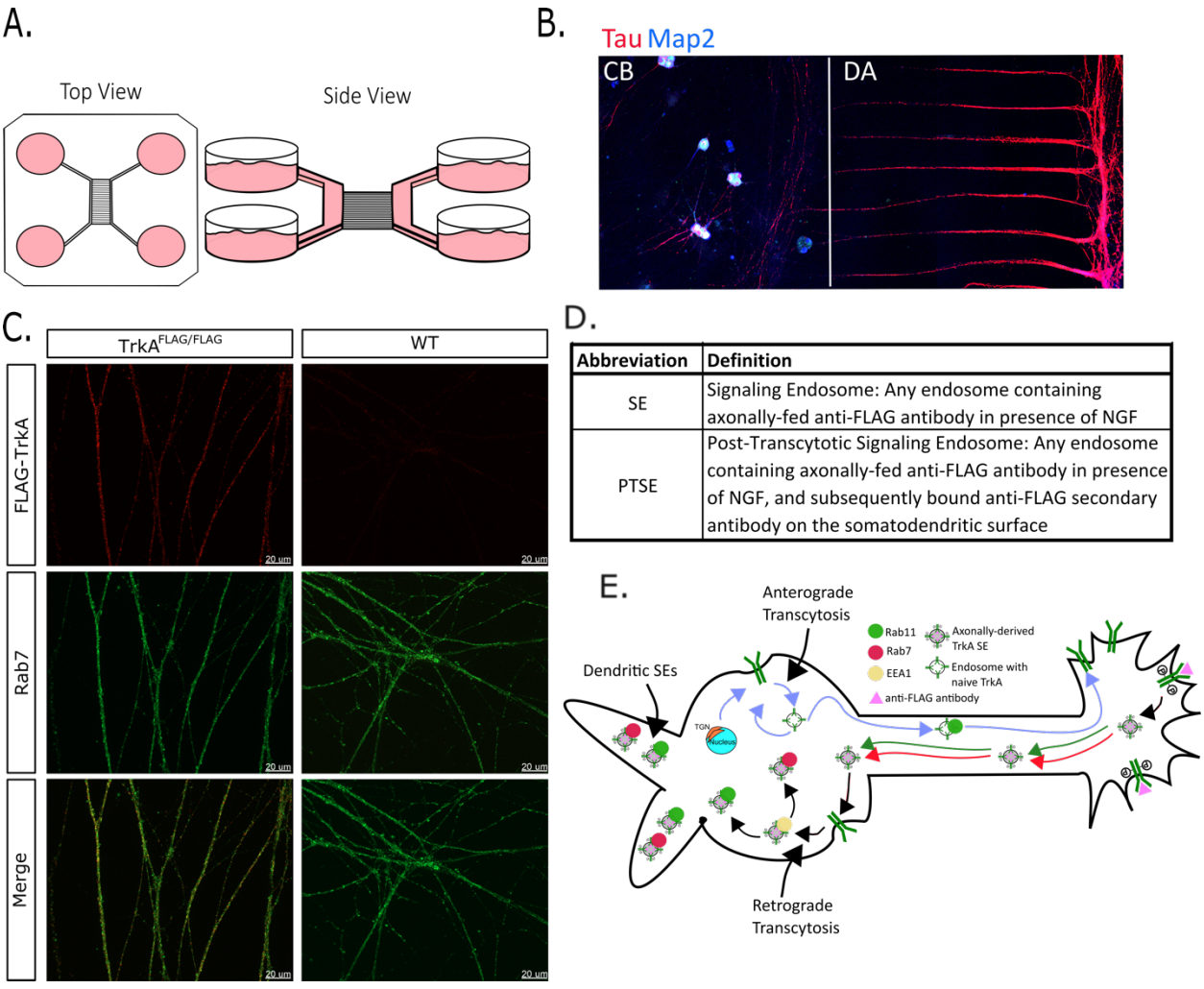

E.

| Anterograde Speed (um/s) | | |
|---|---|---|
| PTSE | SE dendrite | |
| 0.45 ± 0.11 | 0.71 ± 0.28 | |
| **Retrograde Speed (um/s)** | | |
| PTSE | SE dendrite | SE axon |
| 0.52 ± 0.08 | 0.67 ± 0.26 | 0.99 ± 0.29 |

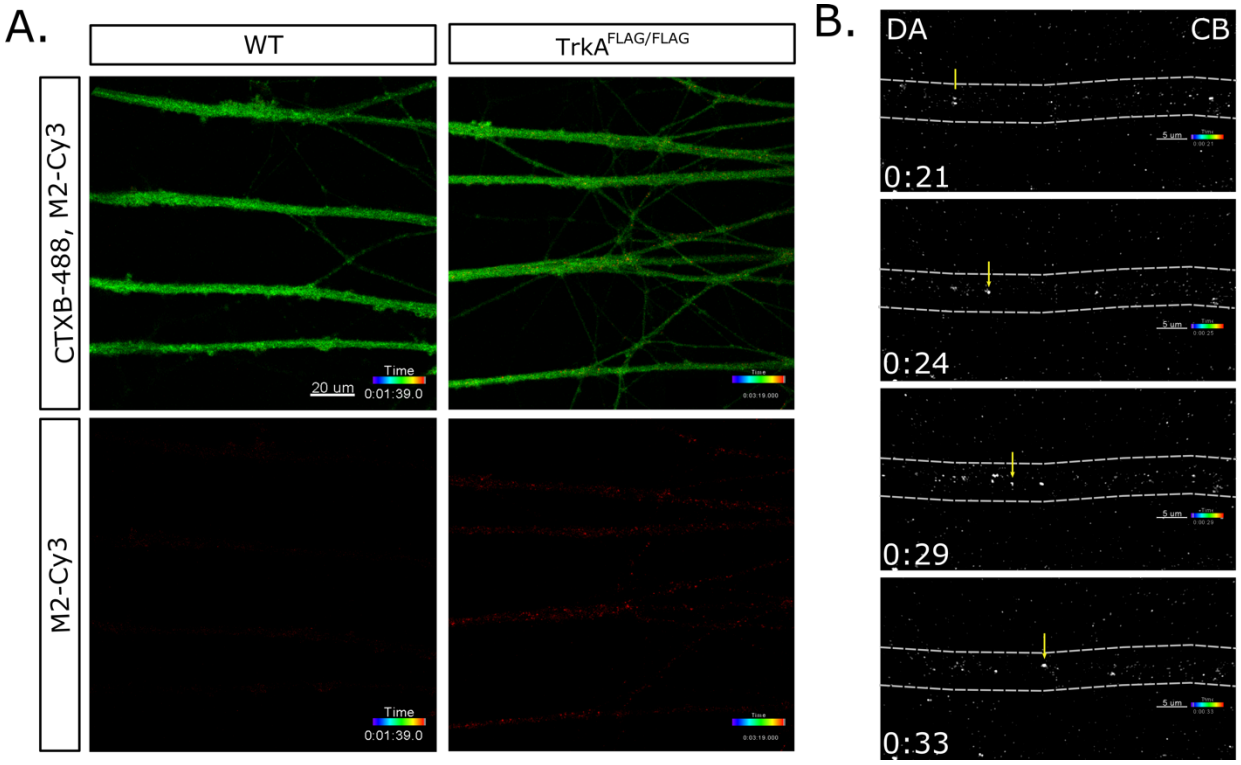
**Figure 7.** SEs are diversified *in vivo*. (A) SEs can be labelled *in vivo* by injection into the salivary glands, shown diagrammatically. (B) Specificity of SE labelling. Injection of M2-Cy3 into the right eye and PBS into the left eye shows exclusive signal in the ipsilateral (right) SCG but not the contralateral (left) SCG. (C) Bright antibody labelling is detected when M2-Cy3 is injected into the salivary gland of *TrkA*$^{FLAG/FLAG}$ animals, but no label is observed when M2-Cy3 is injected into WT (non-FLAG) animals. (D) Close up imaging of single cells shows partial co-localization of *in vivo* SEs in the SCG with EEA1, Rab11, and Rab7. Scale bar 5 µm, inset scale bar 1 µm. (E) Quantification of SE co-localization with Rab proteins (n=30-40 cells from 2 independent experiments).



**Supplementary Figure 1.** Microfluidic devices were used to isolate cell bodies (CB) from distal axons (DA). (A) Microfluidic devices. (B) Cell bodies and dendrites, as marked by MAP2, were separated from distal axons, as marked by Tau, by microgrooves. (C) Anti-FLAG antibody application for 30 minutes shows staining exclusively in *TrkA*$^{FLAG/FLAG}$ neurons and not WT neurons. (D) Defining terms. (E) Summary diagram of anterograde transcytosis (blue arrows) versus retrograde transcytosis (black arrows). The two axonal pools of retrograde SEs are indicated by red arrows (Rab7) and green arrows (Rab11).



**Supplementary Figure 2.** Specificity of live imaging paradigm. (A) anti-FLAG antibody feeding assay is specific for *TrkA*$^{FLAG/FLAG}$ mice compared to WT mice. Axons shown with CTXB-488. (B) Individual tracking of endosomes.



# Chapter IV: Conclusions and Discussion

Some of the ideas presented in this paper were adapted from "The neurotrophin receptor signaling endosome: where trafficking meets signaling". **Barford K**, Winckler B, Deppmann C. *Developmental Neurobiology* 2016; p 1-26.

**Abstract**

Neurons are the largest cells in the body and form subcellular compartments such as axons and dendrites. During both development and adulthood building blocks must be continually trafficked long distances to maintain the different regions of the neuron. To transport these building blocks, neurons have adapted unique endosomal machinery to properly maintain their functional domains. Beyond building blocks, signaling complexes are also transported, allowing for example, axons to communicate with the soma. The critical roles of signaling via ligand-receptor complexes is perhaps best illustrated in the context of development, where they are known to regulate polarization, survival, axon outgrowth, dendrite development, and synapse formation. However, knowing 'when' and 'how much' signaling is occurring does not provide the complete story. The location of signaling has a significant impact on the functional outcomes. There are therefore complex and functionally important trafficking mechanisms in place to control the precise spatial and temporal aspects of many signal transduction events. In turn, many of these signaling events affect trafficking mechanisms, setting up an intricate connection between trafficking and signaling.

## NEEP21 and regulation of the neuronal endosomal system

### Regulation of NEEP21 expression developmentally and by hippocampal and Purkinje neurons

NEEP21 might act as a regulator of the endosomal system, but unlike the Rabs is only expressed in neurons (Steiner et al., 2002). Even more interesting, NEEP21 is not expressed in every neuron equally, arguing that different cell types can regulate the endosomal system so that it works efficiently for that cell's particular needs (Barford et al., 2017b).

In Barford et al 2017, we show that NEEP21 expression is maintained in only a subset of neurons within the brain. Specifically, Purkinje cells of the cerebellum, Layer V cortical neurons, and hippocampal pyramidal neurons. Why would a regulatory endosomal protein be retained in only a subset of neurons? One possibility is that NEEP21 itself is a cargo, and not a marker of a specific endosomal compartment. As mentioned in the Introduction, our laboratory recently showed that NEEP21 itself passes rapidly through the endosomal system towards degradation (Yap et al., 2017). This type of flux through the endosomal pathway is much more reminiscent of a receptor than an endosomal regulator, such as a Rab protein. Indeed, receptors are often localized to specific neuronal cell types within the brain whereas endosomal regulators are often ubiquitously expressed. It is well appreciated that the brain is made up of many different subtypes of neurons, each with their unique proteomic profile (Molyneaux et al., 2007). This diverse expression of proteins is important for many functions including development, synapse formation, maintenance, and firing patterns. However, no ligand for NEEP21 has been identified. Additionally, binding partners have become confusing as we no longer know whether NEEP21 is exclusively a Type 2 membrane protein.

It is now well understood that protein expression and cell fate are intimately linked to the progenitor pool from which the cell is born (Leone et al., 2008). Within the cortex, neurons are born within the neurogenic region of the ventricular zone (VZ) and subventricular zone (SVZ). Neurons born at the same time migrate

to the same layer, filling the cortex in an inside-out fashion. In this way, the first born neurons migrate to Layer 6, followed by Layer 5 and so on. Interestingly, NEEP21 is present within the SVZ during embryonic development. Therefore, NEEP21 is presumably important for the early development of cortical neurons. Endosomal regulation of cell fate and determination has been shown for a number of endosomal proteins, including Numb and SARA. Numb, an endosomal adaptor, has been linked to cell division whereby disruption in protein levels leads to dysregulation of the cell cycle (Mestres and Sung, 2017). The same is true for the endosomal marker SARA. Does NEEP21 have any influence on the cell cycle? This is currently not known, however, our laboratory has shown (unpublished data) that the brains of NEEP21 knockout mice are the same size at WT mouse brains, suggesting that NEEP21 may not be a critical regulator of cell division and cell fate.

In addition to the SVZ, NEEP21 is also expressed embryonically in neurons of all layers of the cortical plate. Therefore, it can be ruled out that NEEP21 is a marker of a certain cell fate, such as CTIP2 or Satb2 for subcortical projections and callosal projections respectively. Why, then, would NEEP21 remain enriched only in a certain subset of cells (namely, cortical Layer V and cerebellar Purkinje neurons)? One possibility is that NEEP21 is exceptionally important for cells with long processes. Subcortical projections can have very long axons, and Purkinje and hippocampal neurons have elaborate dendritic trees. With this immense size comes additional strain on the endosomal system to correctly transport membrane proteins and receptors to their correct locations, regulate signaling, and efficiently degrade waste. While this is an intriguing hypothesis, it cannot completely explain the distribution of NEEP21 in the brain. In additional to neurons with long projections, NEEP21 is also expressed in certain interneurons, which have much shorter processes and typically connect cells within one hemisphere of the neocortex. The NEEP21 expressing interneurons include Parvalbumin positive neurons in the cortex. The same is true in the spinal cord, where NEEP21 is highly expressed throughout the spinal cord rather than being restricted to a subset of projection neurons (**Appendix I, Figure 1**). While it is unclear currently how the expression pattern of NEEP21 is correlated with its protein function, it will be an intriguing question for the future.

**Spatial Regulation of NEEP21: Exclusion from the Axon**

In addition to cell-specific expression, NEEP21 is also spatially regulated within the cell. Our lab and others have shown that NEEP21 is present in the somatodendritic region but not in axons (Steiner et al., 2002). Here, I show that that this expression is maintained *in vivo* and in the sympathetic nervous system (Barford et al., 2017b, Appendix I). How are endosomes permitted into or excluded from the axon? This question has many potential answers, and there have been a number of excellent studies on it to date, most of which focus on the axon initial segment (AIS). It has been shown that there exists a diffusion barrier within the AIS whereby there is markedly less lateral mobility in the AIS as compared to other areas of the neurons (Winckler et al., 1999). Additionally, there may be preferential transport of certain organelles into the axon through attachment to specific motor proteins. For example, Myosin V recruitment in the AIS will halt movement (Janssen et al., 2017). Yet other motors are able to selectively enrich cargo in axons (Huang and Banker, 2012). An additional mechanism through which cargo is transported into the axon is through transcytosis whereby a protein is deposited on the somatodendritic domain, endocytosed, and targeted in an endosome to the axon. This is known to happen for cargo such as TrkA (Ascano et al., 2009) and L1/NgCAM (Yap et al., 2008). While NEEP21 is able to regulate the spatial distribution of L1/NgCAM, how NEEP21 itself gets distributed is still unclear.

What are the implications for this spatial, temporal, and intracellular regulation of NEEP21 expression? A protein whose expression is so tightly regulated points towards NEEP21 being an essential member of the neuronal endosomal system. However, despite careful analysis of TrkA signaling and behavioral tests, I found that the NEEP21 knockout mouse shows little to no phenotype in the PNS (Appendix I). One potential explanation is functional redundancy. Indeed, NEEP21 is a member of the neural-specific gene family (Nsg) of proteins which consists of NEEP21, P19, and Calcyon (Muthusamy et al., 2009). Calcyon has been implicated in internalization of Tfn and recycling of AMPA receptors (Muthusamy et al., 2015).

However, the functional role of P19 is still unclear. P19 is more closely related to NEEP21 than Calcyon, however P19 levels are unchanged in the NEEP21 knockout mouse (CCY, LM unpublished). Additionally, P19 and NEEP21 are not expressed in the all the same tissues. NEEP21 is expressed in the adult Purkinje neurons, but P19 expression in the cerebellum is restricted to early developmental time points (Barford et al., 2017b). In a tissue such as this, where P19 would be unable to compensate for NEEP21 loss, one might expect the NEPE21 knockout mouse to have functional deficits. However, the NEEP21 knockout mice show no deficits on the rotarod test, a measure of cerebellar function (Appendix I). Whether or not NEEP21 is needed in older mice to prevent neurodegeneration, however, is an open question. Taken together, these results suggest that there is either full compensation for the loss of NEEP21, or that the knockout mice hae a phenotype that requires more sophisticated assays to detect.

## TrkA signaling endosome: trafficking regulates signaling and signaling regulates trafficking

### Trafficking affects signaling: How NGF signaling transmitted from the distal axon to the soma?

Neurotrophin binding causes dimerization, autophosphorylation, and internalization of Trk receptors. Phosphorylated TrkA is active and initiates canonical receptor tyrosine kinase (RTK) signaling including activation of the PI3K/Akt, Ras/ERK, and PLCγ pathways. These signaling pathways have been extensively studied (Huang and Reichardt, 2003; Kaplan and Stephens, 1994). NGF-mediated signals must travel retrogradely back to the soma to exert many of their effects during development, *i.e*. trafficking regulates signaling. Indeed, when NGF is added exclusively to neuronal cell bodies but not axons, the neurons survive but retract their axons (Campenot, 1977). Additionally, dynein transport back from the distal axon is needed to ensure cell survival (Heerssen et al., 2004). Therefore, internalization of NGF-TrkA in the distal axon and retrograde transport are both necessary features for signaling cascades that allow a neuron to survive and develop correctly. Correct trafficking back to the soma is integral for survival signaling and dendrite

development, and much work has been done to understand how TrkA signaling endosomes travel retrogradely down the axon (Heerssen et al., 2004; Sharma et al., 2010; Zhou et al., 2012).

After internalization, Trk receptors are endocytosed into a signaling competent compartment. NGF-TrkA complexes have been shown to enter a Rab5-positive early endosome upon endocytosis in the distal axon (Deinhardt et al., 2006; Philippidou et al., 2011). Whether or not the signaling endosome is trafficked retrogradely in a Rab5-containing compartment was unclear until recently. Contrary to initial studies, the majority of Rab5-positive carriers containing TrkA-NGF actually remain stationary (Ye et al., 2018). This correlates well with studies that show activated TrkA in endosomes is able to recruit and activate RabGAP5, a GTPase activating protein (GAP) that inactivates Rab5 (Bucci et al., 2014; Liu et al., 2007). However, the authors actually find that inactivation of Rab5 prolongs signaling in PC12 cells and neurite outgrowth, arguing that signal duration is controlled by the residence time in the maturing endosome. In this case, delay in maturation prolongs signaling. The activation of RabGAP5 by activated TrkA is thus a clear example of how the cargo itself (in this case TrkA) takes charge of its own endosomal fate by regulating the activity of Rab5, i.e. signaling regulates trafficking.

On the other hand, there is evidence that Rab7 escorts the signaling endosome back to the soma (Saxena, 2005; Zhang et al., 2013) where it continues to signal. In apparent contradiction, interference with Rab7 function via a dominant-negative Rab7 also enhances NGF signaling, again suggesting that slowing progression of endosomes towards the late endosome increases signal strength. Again, this work was performed in PC12 cells and local and long distance signaling endosomes could not be distinguished from each other. Further work will be needed to determine whether conversion to Rab7 endosomes is necessary for some NGF-mediated signaling events or whether conversion shortens signaling.

For TrkB, a Rab5 to Rab7 early-to-late endosome conversion event in the distal axon has been shown to result in TrkB trafficking retrogradely back to the soma in a Rab7-containing late endosome (Deinhardt et

al., 2006). Until recently, it was thus an open question if there is only one homogeneous retrograde signaling endosome, or if multiple distinct classes of signaling endosomes (Rab5 and Rab7-positive) reach the soma from the axon.

In this work, I show that there are two major retrograde carriers of the signaling endosomes in axons: Rab11 (recycling) and Rab7 (late) (Barford et al., 2018). However, another recently published paper suggested that the majority of retrograde SE carriers are multivesicular bodies (MVBs) (Ye et al., 2018). Ye et al show by electron microscopy that the majority of TrkA carriers in the axons are MVBs, and that these progress into the single vesicles in the soma with time. This is contradictory to my recent work showing that approximately 40% of signaling endosomes are Rab11 carriers, with an additional 40% being Rab7. What could be the basis for the discrepancies be these two findings? One potential caveat could be the use of a different antibody. The Ginty laboratory used a different Rab11 antibody from the one used in our laboratory, and Rab antibodies are traditionally very difficult to visualize by immunostaining. Additionally, it is unclear how the Ginty group assesses co-localization. For my work, I've utilized Imaris software to determine the distance between intensity centers of endosomes for a precise measurement of their percent co-localization. It's unclear how the Ginty laboratory assessed this, or if they are simply reporting the Pearson's coefficient as opposed to the percent co-localization. However, even without this contradiction, our work does support theirs in that we see a significant amount of co-localization of retrograde SEs with Rab7 in late endosomes.

**What is the significance of multiple retrograde carriers?**

The significance of different Rab proteins as carriers of endosomes is described above in regards to signaling. However, in this case it may be important for signaling to even occur, let alone through what cascade. If the Rab7 carriers that I visualize are the same MVB's that are seen in Ye et. al, how are they able to signal? It was not shown whether the retrograde TrkA in MVB's is on the limiting membrane or in

an ILV, however if they are present in ILV's they would be unable to signal. In that case, there would either need to be escape from the MBV within the axon or an additional pool of single vesicles carriers. In my work, I show that there are no fission events occurring during retrograde transport of SEs in the axon, but instead those are reserved for dendritic SEs. Therefore, the possibility of fission out of the MVB to a signaling-competent carrier in the axon would be extremely unlikely. That leaves the possibility of there being two potential carriers for retrograde SEs: MVBs, which can be Rab7 positive, and single vesicles, which may be the Rab11 endosomes I observe.

Why would a single cargo be transported in two separate types of endosomes? One explanation is an evolutionary one. TrkA SEs are critical for axon branching, target innervation, cell survival, synapse formation, and the creation and maintenance of the sympathetic nervous system as a whole. With such vast demands places on a single signaling system, it would make sense for there to be redundancy set in place to be sure that an organism could survive, even if there is a mutation in a critical gene. This is exemplified by Rab7 mutants and knockouts. In a mutation of Rab7 that causes Charcot-Marie Tooth Disease Type 2B (CMT2B), the sympathetic nervous system is placed under extreme stress by the hyperactive mutation (Zhang et al., 2013). Indeed, these mutations cause premature degradation of TrkA and inhibit the duration of signaling. However, if Rab7 were the sole carrier of TrkA, and TrkA was all being degraded too soon, the developmental dysfunction of the nervous system would be much more severe. Interestingly, *in vitro* studies in N2A and PC12 cells have shown that these mutants inhibit neurite outgrowth (Cogli et al., 2010). However, the main phenotype in human patients is axonal degeneration, pointing towards more of a role in maintenance than development. Indeed, symptoms of CMT2B typically begin in the teenage years (Houlden et al., 2004). It's possible that Rab11 compensates for the mutant Rab7 during development, allowing patient sympathetic systems to develop without extreme cell death during the period of neuronal pruning.

A similar trend is seen in the Rab7 knockout mouse. Ye et al utilize a $Rab7^{fl/fl}$ mouse crossed to a $TH^{CRE}$ mouse to selectively remove Rab7 from neurons that express the enzyme Tyrosine Hydroxylase, which

includes sympathetic neurons. Interestingly, even with homozygous loss of the gene, only 70% of sympathetic neurons are lost due to cell death (Ye et al., 2018). That leaves 30% of surviving neurons. However, if 100% of SEs were transported in Rab7-compartments, there would be no survival signaling and subsequently much more cell death. This is supported by the complete loss of sympathetic neurons in $NGF^{-/-}$ and $TrkA^{-/-}$ mice. This again points to a potential need for a secondary transport system for the SE to ensure that the sympathetic nervous system develops correctly.

**What happens when the signaling endosome reaches the soma?**

The trafficking mechanisms that the signaling endosome undergoes upon reaching the soma are not yet well understood. It is known that TrkA maintains persistent signaling to induce transcriptional changes after reaching the soma. In sympathetic neurons grown in compartmentalized cultures, TrkA signaling endosomes accumulate for 6 hours before disappearing, but a pool TrkA signaling endosomes can survive for up to 25 hours in the soma without being degraded (Suo et al., 2013). Somatic signaling endosomes thus need to escape fusion with lysosomes in order to continue signaling. How the fate of somatic TrkA signaling endosomes is regulated has only begun to be addressed, but regulation of trafficking is likely a major factor.

**Trafficking affects signaling: Trk receptor signaling to dendrites**

BDNF and TrkB have long been known to be necessary for correct dendrite development in many neurons in the central nervous system (Kuczewski et al., 2010; Leal et al., 2015; Lu, 2003; Miller and Kaplan, 2003). In addition to local signaling, BDNF and TrkB retrograde signaling is critical for the correct formation of dendrites (Zhou et al., 2012). TrkB is transported retrogradely by attaching to dynein through the adaptor protein snapin. When snapin is lost in the knockout animal, TrkB is not adequately transported retrogradely from the axon, and when BDNF is added in the distal axon of compartmentalized cultures it is insufficient

to phosphorylate CREB in the cell body. Additionally, knockout of snapin reduces dendrite arborization, however whether or not this is a result of retrogradely transported TrkB signaling endosomes was not directly tested (Zhou et al., 2012). TrkA retrograde signaling has also been linked to deficits in synapse development. A subset of retrogradely derived TrkA signaling endosomes are transported into dendrites to support synapse development (Sharma et al., 2010). These endosomes continue to signal, and their signaling is critical for clustering of post-synaptic densities both *in vitro* and *in vivo*. However, until recently, the endosomal identities of these dendritic SEs was unknown. Here, I show that these exist at multiple populations split between recycling endosomes (Rab11) and late endosomes (Rab7).

What are the implications for having multiple pools of signaling endosomes within the dendrites? One possibility is that each pool is fated towards a different paths. Rab7 positive endosomes are typically fated towards degradation, whereas Rab11 endosomes are typically recycled back to the surface. It's possible that Rab7 SEs have lost their signaling capabilities and are en route to degradation whereas Rab11 SEs are fated towards retrograde transcytosis. However, it is still unclear as to the functional implications that multiple pools of SEs have on the dendrites.

**Retrograde Transcytosis: intertwining trafficking and signaling**

TrkA signaling endosomes must survive for a long time in the soma. Indeed, it has been shown that TrkA signaling endosomes can survive in the soma for up to 25 hours (Suo et al., 2013). Recent experiments to better understand what happens to signaling endosomes after they reach the soma have uncovered an unexpected pathway: local recycling in the soma, a process we refer to as "retrograde transcytosis" (**Introduction, Figure 2**). Upon reaching the soma, the TrkA signaling endosome is exocytosed on the soma membrane and then re-internalized (Suo et al., 2013). What is the purpose of this recycling, and is it induced by a TrkA signaling cascade? One possible function of this pathway is that retrograde transcytosis

allows for some signaling endosomes to switch compartment identity: a signaling endosome arriving retrogradely risks degradation, especially when associated with Rab7. Exocytosis and subsequent endocytosis would allow for re-partitioning into Rab11-positive recycling endosomes. Consistent with this hypothesis, signaling endosomes are found associated with Rab11 in the soma (Barford et al., 2018; Suo et al., 2013).

As discussed above, retrograde SEs are a mixture of Rab11 recycling endosomes and Rab7 late endosomes. Rab11 recycling endosomes are canonically thought of as recycling to the cell surface, thus providing an easy route for signaling transcytosis to occur. However, could the Rab7-population of retrograde SEs also undergo retrograde transcytosis? Retrogradely trafficked SEs that are Rab7-positive late endosomes would have a canonical fate of degradation. In this case, it would be unlikely for SEs to survive for 25 hours in the soma (Suo et al., 2013). Indeed, to the best of our knowledge there is no current model that shows Rab7-positive late endosomes progressing into Rab11-positive recycling endosomes. However, there are data supporting the idea that Rab7 late endosomes can be exocytosed onto the soma membrane. In a carcinoma cell line, $\alpha5\beta1$ integrin is co-localized with Rab7 and LAMP1, which typically mark late endosomes/lysosomes. Instead of degrading, however, $\alpha5\beta1$ integrin is re-directed from a degradative fate to the plasma membrane. Rab25 targets $\alpha5\beta1$ integrin to the lysosome, where an interaction with CLIC3 re-directs its fate from degradation to recycling on the plasma membrane (Dozynkiewicz et al., 2012). Therefore, it is possible that the Rab7-containing population of retrograde signaling endosomes can be deposited on the soma membrane, re-internalized, and sorted into a recycling endosome to maintain its lifetime. Another example of this is the lysosomal secretion pathway that is regulated by Rab27 and Rab3. Rab27 and Rab3 are involved in the maturation and function of secretory lysosomes, which are deposited on the plasma membrane to secrete their contents. Both of these are potentially pathways that TrkA could take to re-direct its fate from degradative to recycling, and reconcile the findings that retrograde TrkA

signaling endosomes are present in both Rab7 and Rab11 endosomes in the soma. Regulating the trafficking of TrkA in the soma could thus profoundly affect the duration of its associated signaling.

It is possible that the role of retrograde transcytosis is to increase the signaling endosome's lifetime, thus trafficking affects signaling. Yet, what prompts the signaling endosome to undergo retrograde transcytosis, as opposed to degrading or not fusing with the plasma membrane, is not known. Currently, only one protein has been identified: signaling transcytosis is facilitated by the actin-binding protein Coronin1a (Coro1a, aka Coronin 1, Appendix 2) (Suo et al., 2013).

Coronin1a is a well described actin binding protein. In addition, Coronin1a was shown to associate with endosomes in macrophages, where it acts to shield pathogenic mycobacteria from lysosomal fusion. Interestingly, protection from lysosomal fusion via Coronin1a occurs through mediation of calcium release and induction of calcineurin activity (Jayachandran and Pieters, 2015; Jayachandran et al., 2007). Coronin1a plays a similar role for the signaling endosome in sympathetic neurons. The loss of Coronin1a in *Coro1a*$^{-/-}$ mice results in decreased survival of SCGs due to a decrease in NGF-TrkA survival signaling. Surprisingly, loss of Coronin1A causes a dramatic reduction of signaling transcytosis in sympathetic neurons. Additionally, *Coro1a*$^{-/-}$ sympathetic neurons showed accelerated fusion of TrkA signaling endosomes with lysosomes (Suo et al., 2013). However, the exact mechanism by which Coronin1a protects the signaling endosome is not clear. It is likely that, similar to its role in mycobacteria, Coronin1a induces calcium release and calcineurin activity, and this signaling is sufficient to evade lysosomal fusion (Jayachandran et al., 2007). Indeed, Coronin1a has been shown to induce these same signaling cascades in sympathetic neurons (Suo et al., 2013). Interestingly, the premature fusion of SEs with lysosomes can be mimicked in wild-type neurons when the cell bodies are treated with a calcineurin inhibitor, potentially pointing toward a role for Coronin1a in the soma in relation to calcium regulation (Suo et al., 2013). At this time Coronin1a is the only known effector of signaling transcytosis, and because loss of lysosomal avoidance and loss of signaling transcytosis both occur in the Coronin1a KO (Suo et al., 2013), it's

impossible to uncouple signaling from trafficking and determine the sequence and importance of both events.

## Conclusion and Future Directions

Neuronal endosomes are complex in terms of their signaling and trafficking capacities. They are able to influence a cell's survival, axon extension, dendrite formation, and synapse maintenance through complex connections and feedback loops between signaling and trafficking. Disruption of endosomal trafficking, including neurotrophin-dependent processes, have been long attributed to signaling deficits. In this work, I have described the localization of the neuron-enriched protein NEEP21 (Chapter 2), how the loss of NEEP21 ($Nsg1^{-/-}$ mouse) does not affect development of the PNS or trafficking of TrkA (Appendix 1), the interactions between NEEP21 and the actin-binding family of Coronin proteins (Appendix 2), the trafficking of TrkA in the sympathetic nervous system (Chapter 3), and the effect of distally-derived TrkA on PSD clustering (Appendix 3).

How does the loss of a neuron-specific endosomal protein affect the brain? Unfortunately, whether loss of NEEP21 has a more prominent effect on the CNS has not been widely studied. Unpublished data from CCY suggests that there lacks an obvious phenotype. However, given the importance of L1/NgCAM for axon growth and development, a detailed study of the $Nsg1^{-/-}$ mouse axon tracts within the brain could be worthwile. Additionally, whether the loss of NEEP21 affects older mice and neurodegeneration is still unknown. Given that NEEP21 associates with APP (Norstrom et al., 2010) and that older $Nsg1^{-/-}$ mice have eye problems more frequently than WT mice, studying loss of NEEP21 in aged mice could provide insight into the function of this protein.

Recently, the neurotrophin field has begun to recognize the importance of trafficking in shaping Trk receptors' signaling capabilities and vice versa. There are many remaining questions about Trk receptor trafficking including switching from constitutive endocytosis to anterograde transport, the type of endosome that carries Trk back from the axon, how the signaling endosome is able to evade lysosomal fusion for an elongated period of time, and how it is able to be transported into the dendrites. Given that NEEP21 co-localizes with distally-derived TrkA (Appendix 1), it could be an additional regulator. Currently, the only known regulator of distally-derived TrkA is Coronin1a. Interestingly, NEEP21 does not associate with Coronin1a, but instead associates with its family member, Coronin1c (Appendix 2). How these three proteins coordinate the trafficking and signaling of TrkA could lead to further insights into the development of the PNS. Intriguingly, I found that loss of Coronin1a (Coro1a$^{-/-}$) leads to an increase in PSD clustering *in vitro* (Appendix 3), and this is supported with *in vivo* data as well (AK, unpublished). How NEEP21, Coronin1a, and Coronin1c work together to influence dendrite development is also an important question for the field. These are questions common to many other neuronal RTKs, even though each receptor's action and transport may be different. Therefore, understanding the specifics of Trk receptor trafficking will yield important insights into the receptor tyrosine kinase trafficking field as a whole.

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# Appendix I: The role of NEEP21 (Nsg1) in nervous tissue outside of the brain

## Introduction

Here, we investigate the role of NEEP21 (Nsg1) in the peripheral nervous system and its potential interaction with TrkA. Specifically, we examined the expression of NEEP21 in the spinal cord, sympathetic neurons, dorsal root ganglia, and the functional consequences of its absence in the *Nsg1*$^{-/-}$ mouse. We find that NEEP21 is highly expressed in sympathetic, sensory, and spinal cord neurons. Additionally, we find that NEEP21 co-localizes with TrkA after NGF feeding both from the cell body and from the distal axon, and that NEEP21 also co-localizes with post-retrograde transcytosis signaling endosomes. However, we find that there is very little phenotypically wrong with the *Nsg1*$^{-/-}$ mouse. This raises questions about the necessity of different endosomal effectors and the possibility of functional redundancy.

## Materials and Methods

### Cryosectioning and Immunohistochemistry

Spinal Cord and DRGs were removed from P0 mice and fixed in 4% PFA overnight at 4° C. Samples were then placed in placed in 30% sucrose at 4°C for at least 24 hours before sectioning at -20° C. For SCG sections, P0 animals were flash frozen in OCT and sectioned at a thickness of 10 μm. Anti-NEEP21 and NeuN antibody information can be found in Materials and Methods of Chapter II. Tyrosine Hydroxylase antibody was used at a dilution of 1:400 (Sheep anti-Tyrosine Hydroxylase, Millipore Cat# AB1542, RRID: AB_90755). CC3 staining was performed by Irene Cheng of the Deppmann Lab (Cleaved Caspase-3, Cell Signaling Technologies Cat# 9661, RRID: AB_2341188).

### Cell Culture and *in vitro* Experiments

FLAG antibody feeding assay, FLAG antibody information, Cy5 (DαMs 647) antibody information, and MAP2 antibody information can be found in Materials and Methods of Chapters II and III. Neurite length was assessed by β3-tubulin staining and measuring neurite length in ImageJ. The β3-tubulin antibody was raised in mouse, generously provided by the Deppmann lab, and used at a dilution of 1:500. PSD clustering was assessed by anti-MAGUK antibody staining (pan-MAGUK 1:400, NeuroMab Cat# 73-029, RRID: AB_10698179) and anti-MAP2 antibody staining to identify dendrites. Images were acquired on Zeiss AxioZoom Observer.Z1 with Apotome 3.1 structured illumination on 63x magnification in z-stacks. Images

were masked in Imaris and the spot function was used to identify clusters blindly (see Chapter III Materials and Methods). Clusters were counted by hand.

**SCG Cell Counting**

P0 animals were flash frozen and sectioned as described above at a 10 μm thickness and were processed with Nissl staining. Every 5$^{th}$ section was acquired and imaged on the a Zeiss Observed Inverted Widefield microscope located in the Deppmann Lab. Images were counted using ImageJ by measuring area per section and counting three sections per slide. Nuclei stained with Nissl were counted as neurons (Chen et al., 2005). For CC3 cell counts, P1 SCGs were dissected, flash frozen, sectioned at 10 μm and stained with anti-CC3 antibody. Images were taken on Zeiss AxioZoom Observer.Z1 with Apotome 3.1 structured illumination on 20x lens and analyzed in ImageJ. Section area and the number of CC3-positive cells were blindly counted.

**Behavioral Testing**

For behavior, mice were kept in consistent cages (no cage changes by vivarium staff) during testing week, and mice were tested between 6-8 weeks of age. No observed difference between sexes, so both sexes were tested. Accelerating rotarod was performed as previously described (Andre et al., 2017), and was located in the Deppmann Lab behavior space. Briefly, the maximum duration of the accelerating rotarod test was 9.00 minutes. Start speed was set to 04, and end speed was set to 70. Defect delay was set to 00. Trials were performed in the dark cycle, with 2 trials per night for 3 nights, culminating in 6 trials. The hot plate test was performed as previously described (Wheeler et al., 2014). Briefly, mice were placed on hot plate

(Columbus Instruments Hotplate analgesia meter) set to a consistent temperature. Movement was restricted by placement of a 1L beaker over the mouse. Latency to groom/lick hindpaw was recorded.

## Results

### NEEP21 is strongly expressed in the peripheral nervous system and spinal cord

We previously showed that NEEP21 expression is high during development, but falls off during the juvenile years in the mouse brain (Chapter II). Interestingly, it remains highly expressed in neurons with very long processes, such as Layer V neurons in the cortex and Purkinje cells (Barford et al., 2017b). To investigate if NEEP21 was expressed in other neuronal tissues, we first examined the spinal cord. We found that NEEP21 was highly expressed in the spinal cord in neurons (NeuN-expressing), but not non-neuronal cells (DAPI-expressing only) (Figure 1A). Indeed, NEEP21 shows the canonical staining pattern of high expression in the Trans-Golgi Network (TGN) (Figure 1A, panel 2). To examine if NEEP21 was also expressed in the sensory nervous system, we looked at the Dorsal Root Ganglia (DRG). We find that NEEP21 is highly expressed in a subset of neurons in the DRG (Figure 1B). Interestingly, NEEP21 are highly expressed in a subset of large-diameter neurons and small-diameter neurons, but there is a population of both that remains unlabelled. To examine if other neurons that express TrkA also had NEEP21 expression, we examined the Superior Cervical Ganglia (SCG). Indeed, NEEP21 is highly expressed in the SCG (Figure 1C). These data show that NEEP21 expression is not restricted to the brain, but is highly expressed in other sections of the nervous system.

### NEEP21 co-localizes with TrkA

Given that NEEP21 is expressed in tissues that are dependent on the endosomal trafficking of TrkA for survival, we next asked if NEEP21 co-localized with TrkA, which elicits major signaling cascades from within an endosome. To do this, we utilized the FLAG-TrkA knock-in mouse which has a FLAG tag endogenously knocked-in to the extracellular domain of the protein (*TrkA*$^{FLAG/FLAG}$, Chapter III). Sympathetic neurons from the *TrkA*$^{FLAG/FLAG}$ mouse were grown for 7 days *in vitro* (DIV), and were pulsed with NGF and an anti-FLAG antibody for 30 minutes. Co-staining revealed co-localization between NEEP21 and internalized TrkA (Figure 2B).

TrkA exerts most of its signaling in a retrograde fashion, by which is internalized with NGF in the distal axon and transports retrogradely to the soma where it can signal to the nucleus and to post-synaptic densities. However, in CNS neurons NEEP21 is not present in axons. To determine the expression pattern of NEEP21 in sympathetic neurons, neurons were grown in microfluidic devices (Chapter III, Figure 1A) for 7 DIV and stained with an antibody against NEEP21. Similarly to CNS neurons, we find that NEEP21 is excluded from the axon, but is retained in the somatodendritic region (Figure 2A). If NEEP21 is only present in the somatodendritic region, but TrkA exerts its effects after retrograde transport from the axon, how would the two proteins interact? To interrogate this question, we great neurons from FLAG-TrkA knock-in mice (*TrkA*$^{FLAG/FLAG}$) in microfluidic devices and performed an antibody feeding assay (Chapter III, materials and methods). After feeding NGF and an anti-FLAG antibody from the distal axon chamber, we observed co-localization between retrograde TrkA and NEEP21 (Figure 2C). This data shows that NEEP21 must associate with the signaling endosome upon its arrival in the soma, because there is no NEEP21 expression in the axons.

NEEP21 is a transmembrane protein, therefore, for it to interact with TrkA after arriving from the distal axon, there would either have to be a fusion event between a TrkA-positive endosome and a NEEP21-postiive endosome, or NEEP21 could endocytose together with post-transcytotic TrkA (PTSE). TrkA is known to undergo a transcytotic event known as retrograde transcytosis (RT) whereby the signaling endosome is exocytosed onto the somatic surface and TrkA is subsequently re-endocytosed (Chapter I, Figure 2). To investigate how NEEP21 could co-localize with retrograde TrkA, we performed the PTSE labelling assay (Chapter III, materials and methods), and were indeed able to observe co-localization between NEEP21 and PTSEs (Figure 2D). Unfortunately, we are unable to determine what percentage of TrkA SE's undergo RT. Therefore, it remains unclear whether NEEP21 associates with SEs both before and after RT, or if all NEEP21-positive SEs have undergone RT but did not pick up the secondary antibody.

**NEEP21 KO sympathetic neurons have no phenotype *in vitro***

Given the importance of TrkA in sympathetic neuronal function, we next asked whether loss of NEEP21 through genetic knockout would impair sympathetic neurons *in vitro*. TrkA is critical for numerous aspects of neuronal survival and functions. The robustness of TrkA signaling governs if a cell survives, the amount of neurite branching it will have, and the number of post-synaptic density clusters present. To investigate whether the amount of retrograde TrkA arriving in the soma was different between $TrkA^{FLAG/FLAG}$ (WT) and $TrkA^{FLAG/FLAG}Nsg1^{-/-}$ ($Nsg1^{-/-}$) neurons, we grew neurons for 7 DIV in microfluidic devices and subjected them to the antibody feeding assay. The number of retrograde SEs were counted using Imaris software (Chapter III, Figure 1), and we observed no difference between WT and $Nsg1^{-/-}$ neurons (Figure 3A). Given that NEEP21 co-localizes with TrkA after it has undergone RT, we next asked whether the amount of RT taking place was the same between WT and $Nsg1^{-/-}$ neurons. Interestingly, although NEEP21 co-localizes, its loss does not affect the percentage of SEs that undergo RT (Figure 3B). We next asked whether or not

there were any functional deficits in TrkA signaling *Nsg1*$^{-/-}$ neurons *in vitro*. First, we examined neurite length, a known indicator of TrkA activity. We did not find any significant difference after 24 hrs *in vitro* between the length of WT neurites and the length of *Nsg1*$^{-/-}$ neurites (Figure 3B). To ask whether loss of NEEP21 might affect a process later in development, we asked whether there were the same number of post-synaptic density clusters (PSDs) after 14 DIV. Interestingly, we did not observe any difference between the number of PSDs between WT neurons and *Nsg1*$^{-/-}$ neurons.

**NEEP21 knockout mice have small deficits**

While we did not observe any phenotype *in vitro*, we do see expression of NEEP21 and TrkA in the same tissues *in vivo*, which prompted us to ask whether or not the *Nsg1*$^{-/-}$ mice have any phenotypes *in vivo*. First, we examined the number of neurons in the SCG at P0, a time after the majority of cell death. We did not observe any significant difference between WT and *Nsg1*$^{-/-}$ animals. We next asked whether or not death was simply delayed, and if we could see an increase in cleaved caspase-3 (CC3) staining in the SCG at P0. We did not observe any significant difference between WT and *Nsg1*$^{-/-}$ animals. NEEP21 is expressed both in the SCG and in the DRG. In the DRG, it is expressed by a subset large-diameter nociceptive neurons (Figure 1). To determine if *Nsg1*$^{-/-}$ animals had nociceptive deficits, we performed the hot plate assay. Indeed, *Nsg1*$^{-/-}$ animals had a small but slightly significant increase in the amount of time they were able to stay on the 47.5°C hotplate before grooming. Finally, given the high expression of NEEP21 in Purkinje neurons, we asked whether or not the *Nsg1*$^{-/-}$ mice had any deficits in the rotarod test. We did not observe any significant difference between WT and *Nsg1*$^{-/-}$ animals.

## Figures

**Figure 1.** Neep21 expression (A) Expression of NEEP21 in the spinal cord at P0. Bottom panel is the inset from the top panel merged image. Top panel scale bar: 100 µm, Bottom panel scale par: 10 µm. In the bottom panel, staining that resembles the TGN can be observed. (B) Expression of NEEP21 in the DRG at P0. Scale bar: 100 µm. (c) Expression of NEEP21 in the SCG at P0. Scale bar: 50 µm.

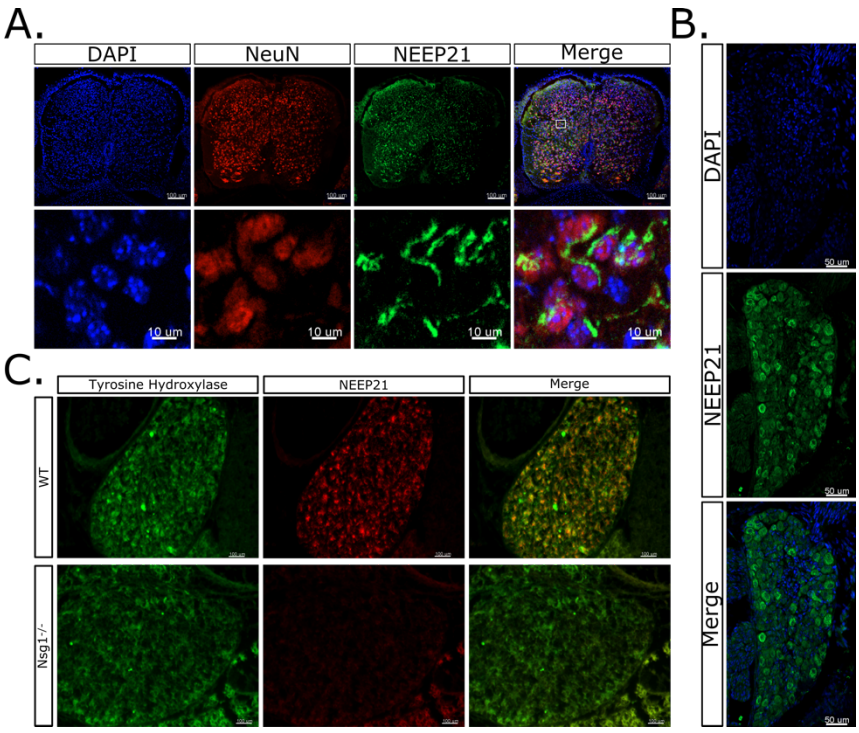


**Figure 2.** NEEP21 co-localizes with TrkA in culture. (A) NEEP21 is contained in the somatodendritic region in neurons. (A) Sympathetic neurons grown in microfluidic devices for 7 DIV. Green inset shows Tau-positive axons lacking NEEP21 staining. Pink inset shows MAP2-positive cell bodies showing strong NEEP21 expression. (B) NEEP21 co-lcoalizes with TrkA after feeding NGF from the CB compartment at 7DIV. (C) NEEP21 co-localizes with TrkA after feeding NGF from the DA in 7 DIV neurons. (D) NEEP21 co-localizes with PTSEs in 7 DIV neurons.

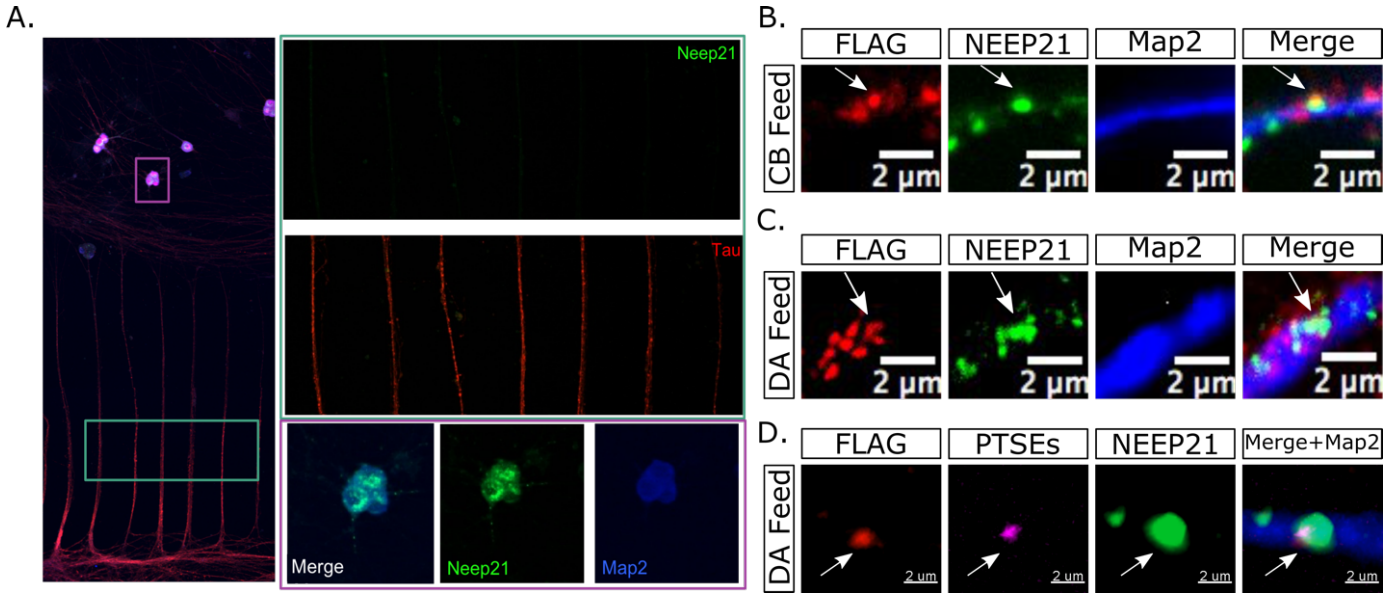


**Figure 3.** *Nsg1*$^{-/-}$ sympathetic neurons have no phenotype *in vitro*. (A) *Nsg1*$^{-/-}$ neurons have the same number of retrograde SE's as WT neurons after 7 DIV. n=4-5 independent experiment, 15 fields of view per experiment. (B) *Nsg1*$^{-/-}$ neurons have the same percentage of PTSEs compared to SE's as WT neurons after 7 DIV. n=2 independent experiments (C) *Nsg1*$^{-/-}$ neurons have the same length of neurites after 24 hours *in vitro*. n=1 experiment, 34-36 neurons. (D) *Nsg1*$^{-/-}$ neurons have the same number of retrograde PSD cluster after 14 DIV. n=2 independent experiments, 15 fields of view per experiment.

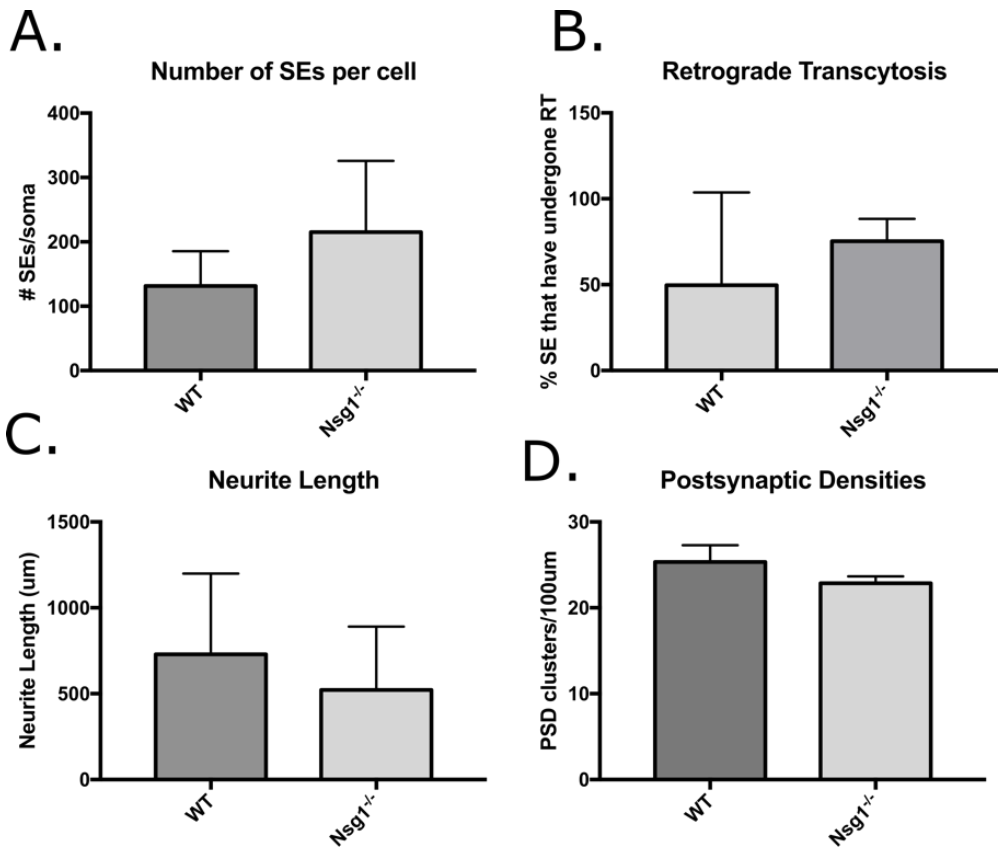
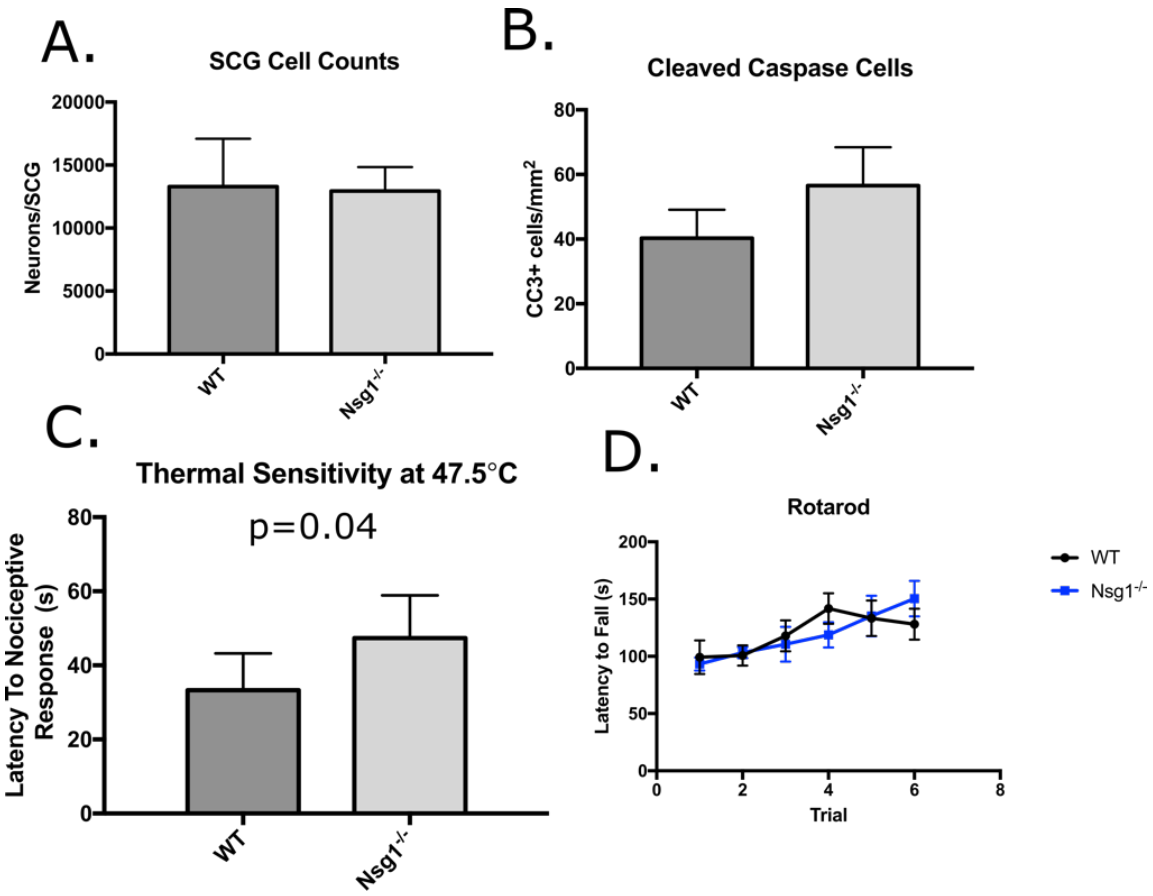


**Figure 4.** Neep21 knockout mice have slight deficits. (A) *Nsg1*$^{-/-}$ mice have no change in SCG number *in vivo* compared to WT animals at P0. n=3 independent experiments, 3 animals per experiment per genotype. (B) *Nsg1*$^{-/-}$ mice have no change in cell death *in vivo* at P1. n=3 animals per genotype, 5-12 sections per animal. (C) *Nsg1*$^{-/-}$ mice have small nociception deficits compared to WT neurons. n=4 independent experiments, total 26 WT 13 KO. (D) *Nsg1*$^{-/-}$ mice have no deficits in the rotarod test. n=2 independent experiments, 20 animals/genotype.



# Appendix II: NEEP21 interactions with Coronin proteins

## Introduction

Here, we investigate the interaction of NEEP21 with Coronin1a and Coronin1c. Given that NEEP21 co-localizes with TrkA (Appendix I, Figure 2), we asked if NEEP21 interacted with the other known somatic effector of TrkA, Coronin1a (Suo et al., 2013). The family of Coronin proteins are known as actin-binding proteins, however their involvement in neuronal signaling has recently become appreciated (Martorella et al., 2017). Indeed, in a screen for NEEP21 interacting partners, Coronin1c was identified as an interacting partner of NEEP21 in a whole-brain pull down and mass spec from anti-NEEP21 antibody (CCY, unpublished). To probe the potential interaction between the Coronin proteins and NEEP21, we performed immunoprecipitations from transfected HEK293 cells. Interestingly, NEEP21 does not interact with Coronin1a, however it does interact with Coronin1c. Further, all fragments of NEEP21 (cytosolic and luminal) pull down with Coronin1c.

## Materials and Methods

### Cell Culture and Transfection

HEK293T cells were maintained in DMEM +10% FBS. For a 10 cm dish of cells, 7.5 μg of DNA and 18.5 μL of lipofectamine 2000 were incubated for 20 min at RT. The DNA/lipofectamine mixture was then added to cells and allowed to incubate for 48 hours in the 37° C incubator. DNA fragments: NEEP21-Emerald, NEEP21 1-82-Emerald, NEEP21 1-111 Emerald, NEEP21 104-185 Emerald, NEEP21 77-185 Emerald, Coronin1c-myc-FLAG (courtesy of the Deppmann Lab), empty HA, NEEP21-cherry, Coronin1a-GFP (courtesy of the Deppmann Lab), empty Cherry, empty GFP.

**Cell Lysis and Immunoprecipitation**

Cells were lysed in lysis buffer (20 mM Hepes pH 7.4,  mM EDTA, 2 mM EGTA, 100 mM NaCl, 10 mM NaF, 1 mM PMSF, 1% Triton X-100, 1 tablet Roche Protease inhibitor, 5% glycerol) on ice for 40 minutes. Cells were then centrifuged at 14K at 4° C for 20 min and pre-cleared with protein A/G Sepharose beads. Lysates were added to the beads and pre-cleared for 3 hrs at 4° C or overnight. Supernatant was then added to conjugated beads for IP and rotated overnight at 4° C. Samples were then washed 2 x 10 min with low salt buffer (200 mM Hepes pH 7.4, 100 mM NaCl, 1% TritonX-100) followed by 2 x 10 min with high salt buffer (200 mM Hepes pH 7.4, 250 mM NaCl, 1% Triton X-100). Sample buffer was then added and samples were boiled for 5 min at 95° C. Samples were run on 10-15% gels and transferred to membrane. Western blot antibodies: Coronin1a (Chicken anti-Coronin1a, Abcam Cat# ab53395, RRID: AB_869226), Coronin1c (anti-Coronin3, Abcam Cat# 153954, RRID: AB_2617135), NEEP21 (see Chapter II).

**Results**

**Coronin1a and NEEP21 do not interact**

Given that Coronin1a and NEEP21 are the only known effectors of the SE once it arrives in the soma, we asked whether it was possible that they interacted. To that end, we transfected HEK293T cells with either NEEP-Cherry (Np-Cherry) or Coronin1a-GFP (Coro1a-GFP). Interestingly, NEEP21 and Coronin1a did not pull down together, indicating that they do not interact directly, nor are they in a complex (Figure 1).

**Coronin1c and NEEP21 interact**

Given that Coronin1a did not interact with NEEP21, but its family member, Coronin1c was identified in a screen for NEEP21-interacting proteins, we next asked whether NEEP21 interacted with Coronin1c. To this end, we transfected HEK293T cells with either Np-Cherry or Coronin1c-myc-FLAG (Coro1cmycFLAG) constructs. Interestingly, NEEP21 and Coronin1c pulled down together in both directions, indicating that they are minimally in a complex (Figure 2).

**Coronin1c interacts with the cytoplasmic and luminal tails of NEEP21**

Given that NEEP21 interacts with Coronin1c in some fashion, we wanted to probe this interaction further. NEEP21 was published to be a Type-2 membrane protein with its C-terminal tail in the cytosol (Steiner et al., 2002). However our lab has data suggesting that it may exist in two forms (CCY, unpublished data). To this end, we sought to determine whether the N- or C-terminus of NEEP21 was accessible to interact with Coronin1c. Interestingly, we find that Coronin1c pulls down both the cytoplasmic and luminal domains of NEEP21 (Figure 3).

**Figures**

**Figure 1.** NEEP21 does not interact with Coronin1a. (A) NEEP21-cherry does not pull down Coronin1a-GFP in HEK293T Cells. Transfection/lanes: (1) Lysate Coronin1a-GFP, Cherry, (2) Lysate Coronin1a-GFP, NEEP-Cherry, (3) IP Coronin1a-GFP, Cherry, (4) IP Coronin1a-GFP, NEEP-Cherry. (B) Coronin1a-GFP does not pull down NEEP21-cherry in HEK293T Cells. Transfection/lanes: (1) Lysate Coronin1a-GFP, Cherry, (2) Lysate Coronin1a-GFP, NEEP-Cherry, (3) IP Coronin1a-GFP, Cherry, (4) IP Coronin1a-GFP, NEEP-Cherry.

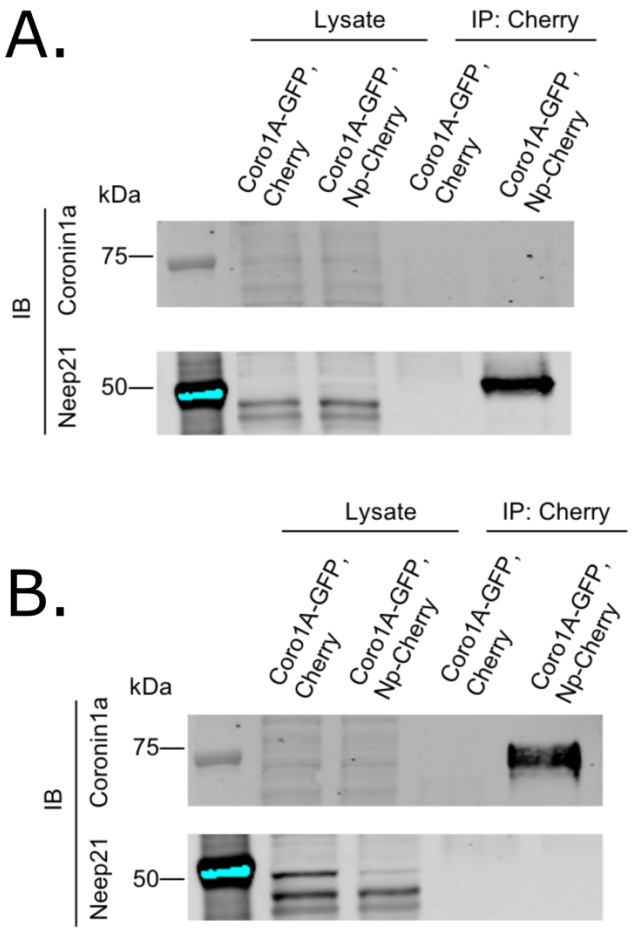


**Figure 2.** NEEP21 interacts with Coronin1c. (A) Coronin1a-myc-FLAG pulls down NEEP21-cherry in HEK293T cells. Transfection/lanes: (1) Empty FLAG vector does not pull down NEEP21-Cherry, (2) Coronin1a-myc-FLAG pulls down NEEP21-Cherry. (B) NEEP21-Cherry pulls down Coronin1c in HK293T cells. Transfections/lanes: (1) Empty mCherry vector does not pull down Coronin1c-myc-FLAG, (2) NEEP21-Cherry pulls down Coronin1c-myc-FLAG.

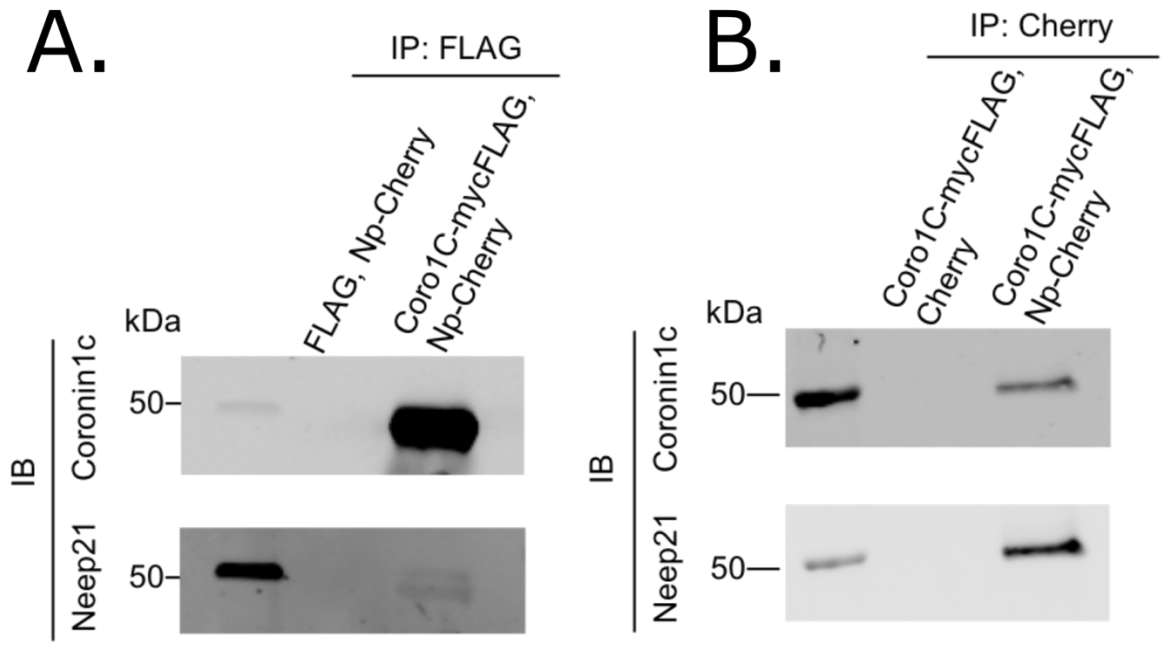
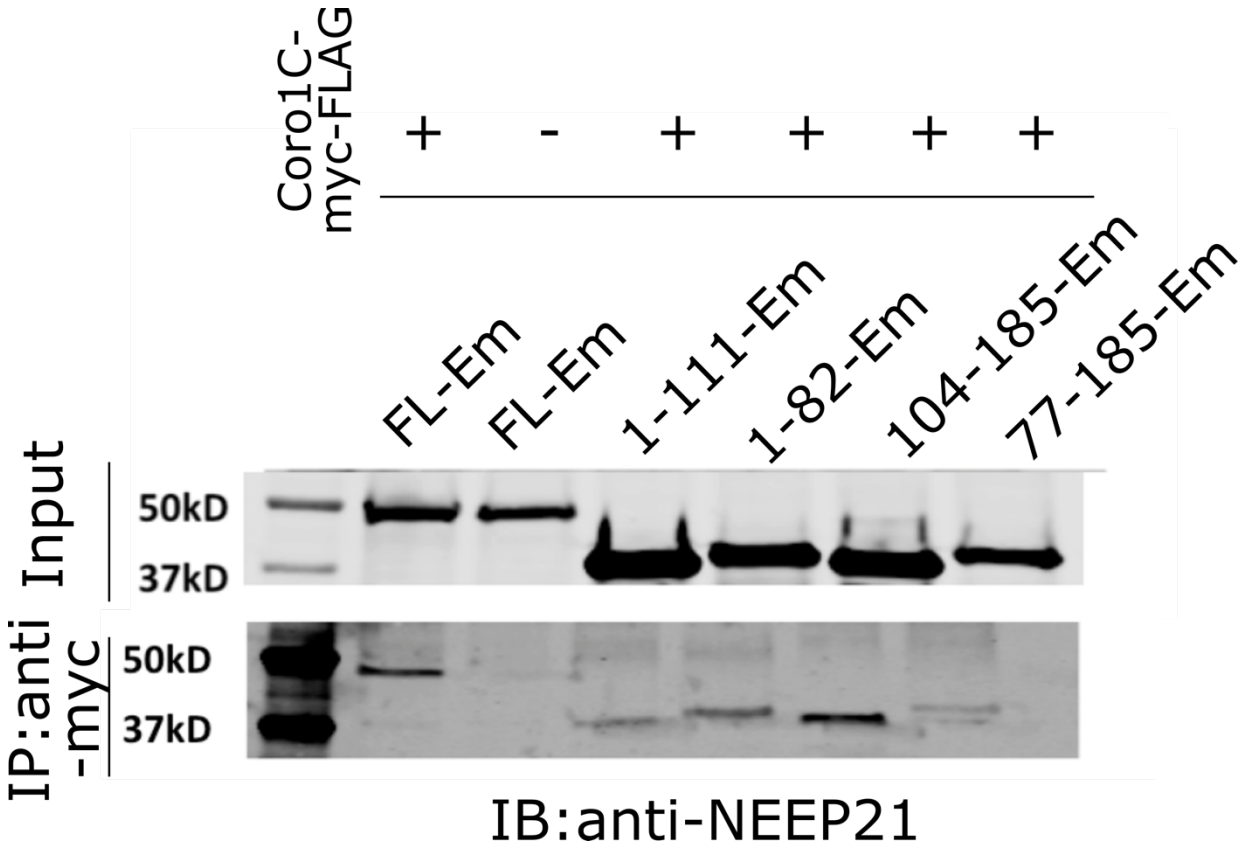


**Figure 3**. Coronin1c pulls down both the N- and C-terminal fragments of NEEP21. Either full-length (FL) NEEP21 tagged with Emerald (Em), or fragments of NEEP21 tagged with Ememerald, were co-transfected into HEK293T cells wither either Coronin1c-myc-FLAG or empty vector myc. Pull down of Coronin1C shows that it is associated with both the N- an C-terminal fragments of the NEEP21.



# Appendix III: Post-Synaptic Density Clustering in Sympathetic Neurons *in vitro*

## Introduction

Here, we investigate the role that TrkA/NGF signaling plays in post-synaptic density (PSD) clustering. It has been shown that retrograde TrkA passes through the soma to arrive in the dendrites (Sharma et al., 2010). These dendritic SEs have been shown to maintain their signaling capabilities, and their signaling is necessary for PSD cluster formation (Lehigh et al., 2017). Given that Coronin1a (Suo et al., 2013) has been shown to associate with the SE upon arrival in the soma, we asked whether the loss of Coronin1a in the Coronin1a knockout neurons (*Coro1a*$^{-/-}$) would affect the clustering of dendritic PSDs. Intriguingly, we found that Coronin1a knockout mice exhibit more PSD clusters after 14 DIV than WT.

## Materials and Methods

See Appendix I for cell culture and immunofluorescence methods. Chicken anti-Coronin1a antibody used for immunostaining (anti-Coronin1a, Abcam Cat# ab53395, RRID: AB_869226, 1:200) as previously described (Appendix I).

## Results

### PSD clusters did not disappear after NGF withdrawal

Sharma et al. showed that 12 hours NGF withdrawal leads to a loss of PSD clusters in 10-14 DIV sympathetic neurons. Interestingly, after just 2 hours of re-application of NGF to the distal axons the PSD clusters could be recovered (Sharma et al., 2010). To test the role of NEEP21 (Appendix I) and Coronin1a in the re-appearance of PSD clusters, we first tried to replicate this data in our own hands. Interestingly, we were unable to see a reduction in PSD clusters at 12, 24, or 26 hours of NGF withdrawal (with the addition of the apoptosis inhibitor BAF).

### *Coro1a*$^{-/-}$ mice have increased PSD clustering

Although we are unable to assess reappearance of PSD clusters after NGF application, we were still able to assess PSD clustering at baseline. Interestingly, *Coro1a*$^{-/-}$ neurons have an increase in the number of PSD clusters *in vitro*. This correlates with *in vivo* data showing increased synapse formation in the SCG (AK, unpublished data).

**Figure 1**. PSD clusters persist after NGF withdrawl. 10-15 DIV SCG cultures were treated with 1 µg/mL anti-NGF, 1 µg/mL BAF for the indicated time frame, and analyzed for PSD cluster formation through pan-MAGUK antibody staining. Each data point indicates an independent experiment.

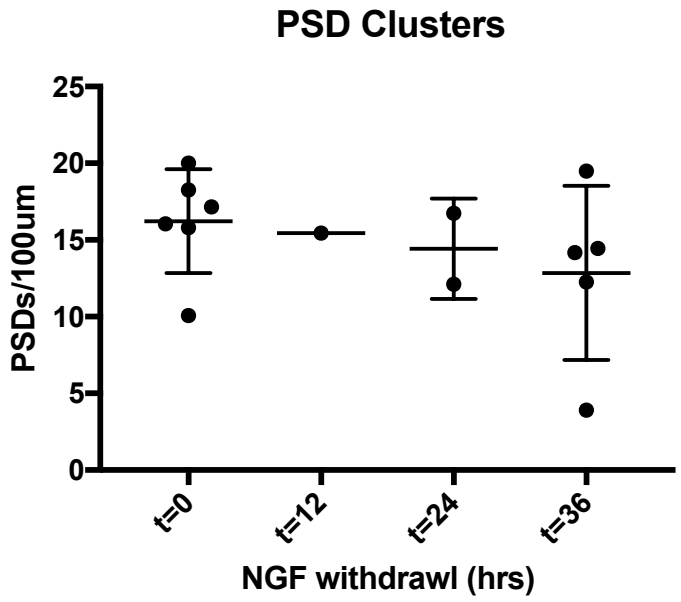


**Figure 2.** Coronin1a neurons may have an increase in PSD clustering. 14 DIV cultures were assessed for PSD clustering through staining with a pan-MAGUK antibody.