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Mechanisms of Morphogen Trafficking: Intraneuronal Transport of Shh

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Mechanisms of morphogen trafficking: Intraneuronal transport of Shh

By

Shawn Terrence Beug

A Thesis Submitted as a Partial Fulfillment of the Degree of Doctorate of Philosophy in
Biochemistry with Specialization in Human and Molecular Genetics

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ABSTRACT

The Sonic hedgehog (Shh) morphogen plays an important role in shaping central nervous system (CNS) development. While the function of Shh signalling in many tissues is well understood, the intracellular regulation of Shh trafficking during these processes is relatively unknown.

To examine the mechanisms of Shh trafficking in neurons, I generated cDNA constructs coding for Shh isoforms, transfected these into differentiated PC6 cells and primary hippocampal neurons, and monitored protein distribution by fluorescence microscopy and biochemistry. Shh is distributed along axons, dendrites and at the neurite terminals in neurons and is associated intra- and extracellularly with lipid raft markers. Intracellularly, wild type Shh associates with synaptic-like microvesicles and dense core granules (DCGs), and is transported with both fast and slow kinetics. Inhibiting Shh posttranslational modification, including cleavage and lipidation, resulted in the accumulation of Shh morphants with DCGs and lack of cell surface accumulation. Consistent with the enrichment of Shh morphants in DCGs, secretion of Shh could be induced under depolarizing conditions. Taken together, these observations suggest that long-range Shh transport and signalling in neurons involves trafficking to the regulated secretory pathway and cell surface accumulation of Shh on axons, and suggests a link between neuronal activity and Shh secretion.

The second aim involved a GST-pull down affinity purification method to identify potential Shh interacting proteins that could mediate Shh intraneuronal transport. Recombinant Shh-N or Shh-C fused to GST were used as bait and incubated with solubilised membrane fractions from the perinatal rat brain. Interacting proteins were resolved by SDS-

PAGE, and individual bands were excised and identified by mass spectrometry. From this screen, 22 proteins were identified to be potential Shh interacting factors. Several candidates specific for Shh-N or Shh-C were identified, and I selected three candidates, Sortilin (Sort1), Glypican 5 (Gpc5) and Low density lipoprotein receptor-related protein 1 (Lrp1), for validation by coimmunoprecipitation. These candidates demonstrate potential to be direct Shh interacting partners that are involved in Shh trafficking, extracellular transport and reception within CSN development.

Overall, these results indicate that Shh transport and release is tightly regulated and may be differentially secreted from the cell body or neurite, and rely on context- and tissue-specific protein:protein interactions for appropriate vesicular targeting, intraneuronal trafficking, extracellular transport and reception.

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LIST OF ABBREVIATIONS

C199A Shh	uncleaved Shh with palmitate modification
C25SC199 Shh	uncleaved Shh with no palmitic acid
C25S Shh	Shh with cholesterol modification
C25S ShhN	Shh with no cholesterol and palmitate modification
BDNF	brain-derived neurotrophic factor
CFP	cyan fluorescent protein
coIP	coimmunoprecipitation
CNS	central nervous system
CPE	carboxypeptidase E
Gpc	Glypican
CSP	constitutive secretory pathway
CSV	constitutive secretory vesicle
DCG	dense core granule
Disp	Dispatched
dlp	dally-like protein
dly	dally
DRM	detergent resistant membrane
Dhh	Desert Hedgehog
ER	endoplasmic reticulum
GPI	glycosylphosphatidylinositol
GST	Glutathione S-transferase
GST::ShhN	GST fusion protein with Shh-N
GST::ShhC	GST fusion protein with Shh-C
hh	<i>Drosophila</i> Hh
hhN	cholesterol-deficient hh-N with palmitate modification
hhNp	mature hh with cholesterol and palmitate modification
hh-C	carboxy-terminal domain of the hh protein
hh-N	amino-terminal domain of the hh protein
Hh	Hedgehog
HhN	cholesterol-deficient Hh with palmitate modification
HhNp	mature Hh with cholesterol and palmitate modification
Hhat	hedgehog acyltransferase
Hhip	hedgehog interacting protein
HPE	holoprosencephaly
HSPG	heparan sulphate proteoglycan
Ihh	Indian Hedgehog
ISG	immature secretory granule
Lrp	Low density lipoprotein receptor-related protein
OPC	oligodendrocyte precursor cell
Ptc	Patched
PTM	posttranslational modification
RAP	receptor associated protein
RGC	retinal ganglion cell
RPC	retinal progenitor cell

RSP	regulated secretory pathway
RSV	regulated secretory vesicle
Sort1	Sortilin
SG	secretory granule
Shh	Sonic Hedgehog
ShhC	processed Shh-C
Shh-C	carboxyl-terminal domain of the Shh protein
Shh-N	amino-terminal domain of the Shh protein
ShhN	cholesterol-deficient Shh-N with palmitate modification
ShhNp	mature Shh with cholesterol and palmitate modifications
ShhN::AP	alkaline phosphatase-conjugated ShhN
ShhN::Fc	ShhN tagged with the Fc portion of human IgG
Shh::YFP	Shh with YFP upstream of cleavage site
SLMV	synaptic-like microvesicles
SPShhC	processed Shh-C with an upstream signal peptide from the Shh protein
SV	synaptic vesicle
TGN	trans-Golgi network
YFP	yellow fluorescent protein

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1. INTRODUCTION

1.1 Hedgehog family members

Hedgehog (Hh) proteins are signalling molecules that control patterning and growth in a number of tissues in species ranging from *Drosophila* to humans. Hh was initially identified through a *Drosophila* mutagenesis screen as a segment polarity gene. Mutations in *hh* resulted in alterations in the denticle patterning of larval cuticle, yielding in an overall nonsegmented ‘hairy’ phenotype that resembled a hedgehog (1). Subsequent discovery and characterization of the mouse orthologues *Sonic*, *Desert* and *Indian Hedgehog* (*Shh*, *Dhh*, and *Ihh*, respectively) showed high conservation to *hh* and these orthologues also have important roles in embryonic development in vertebrates. In addition, downstream components of the Hh signalling pathway are critical for embryonic development, as mutations within these components leads to numerous abnormalities, including polydactyly, holoprosencephaly (HPE), coloboma, and tumour development in a number of tissues (2-5).

Hh genes encode secreted proteins that regulate diverse developmental events through morphogen-mediated regulation of downstream mitogenic and patterning genes. Hh proteins can directly regulate cell proliferation and cell fate specification in a spatiotemporal fashion, acting at a distance from the production source in a concentration-dependent manner. For example, *Shh* induces different cell fates along a dorsoventral axis in the developing spinal cord through a concentration-dependent regulation of transcription factor expression (6-11). The Hh orthologues regulate diverse developmental processes, which is dependent on tissue-specific expression of the Hh genes. *Shh* is widely expressed in multiple systems in the developing embryo, including midline tissues (node, notochord, floor plate), zone of polarizing activity of the limb bud and in most epithelial tissues, while *Ihh* is

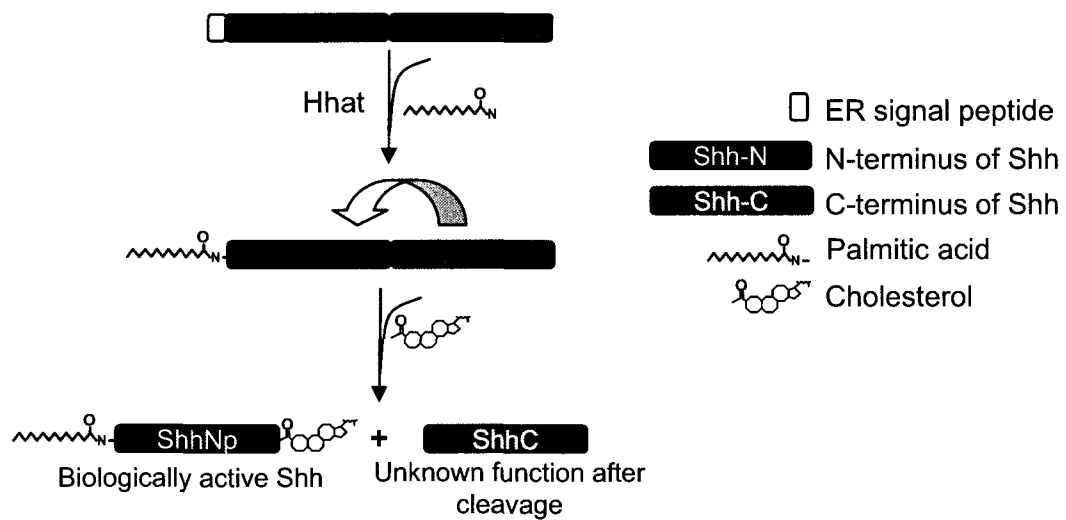
localized to the primitive endoderm, gut and developing bones, and Dhh expression is restricted to sertoli, granulosa and Schwann cells (12-27). Despite different expression patterns of the Hh orthologues, Hh proteins mediate their effects on patterning and growth through extracellular signalling and activate the same Hh signal transduction pathway. Shh is the most widely researched of the Hh proteins, and numerous studies in animal models have highlighted the importance of Shh posttranslational modifications (PTMs) that govern Shh signalling pathways and function (5, 28, 29).

1.2 Hedgehog processing

Shh undergoes multiple PTMs that are required for the generation and secretion of the mature protein. Shh is translated as a full-length precursor protein and the nascent polypeptide is targeted to the secretory pathway by the presence of an endoplasmic reticulum (ER) signal sequence (30). Additionally, Shh undergoes proteolysis at a conserved three amino acid motif (GlyCysPhe) that is catalyzed by residues in the carboxyl terminal domain of the protein (Shh-C) to generate an amino terminal fragment (Shh-N; Figure 1.1; 31, 32, 33). All of the biological activity of Shh described to date is mediated by Shh-N. Shh-N is further processed by two lipid modifications: cholesteroylation at the carboxyl terminus and *N*-palmitoylation on an N-terminal cysteine to yield processed ShhNp (34, 35).

Palmitoylation is catalyzed by the action of Hedgehog acyltransferase (Hhat), although it is unknown whether this reaction is carried out in the ER or Golgi (36-38). *In vivo*, the majority of Shh appears to be cleaved and dually lipid-modified (31, 34, 37). Cholesterol modification is necessary for membrane tethering and multimerization, presumably by association with lipid rafts, and for long-range signalling (35, 39-41). Interestingly, cholesterol-deficient Shh (ShhN) diffuses over longer distances compared to ShhNp *in vivo* (29, 42, 43). Additionally,

Figure 1.1. Posttranslational modification of Shh. Newly translated Shh is processed by removal of the endoplasmic reticulum (ER) signal peptide and cleavage into amino- and carboxyl-terminal fragments (Shh-N and Shh-C, respectively) that is mediated by residues in Shh-C. Shh-N is further modified by palmitoylation mediated by Hh acyltransferase (Hhat) at the N-terminal and cholesteroylation at the C-terminal to yield mature Shh (ShhNp). All of the biological activity of Shh described to date is mediated by the Shh-N while a role for Shh-C beyond proteolysis is unknown.



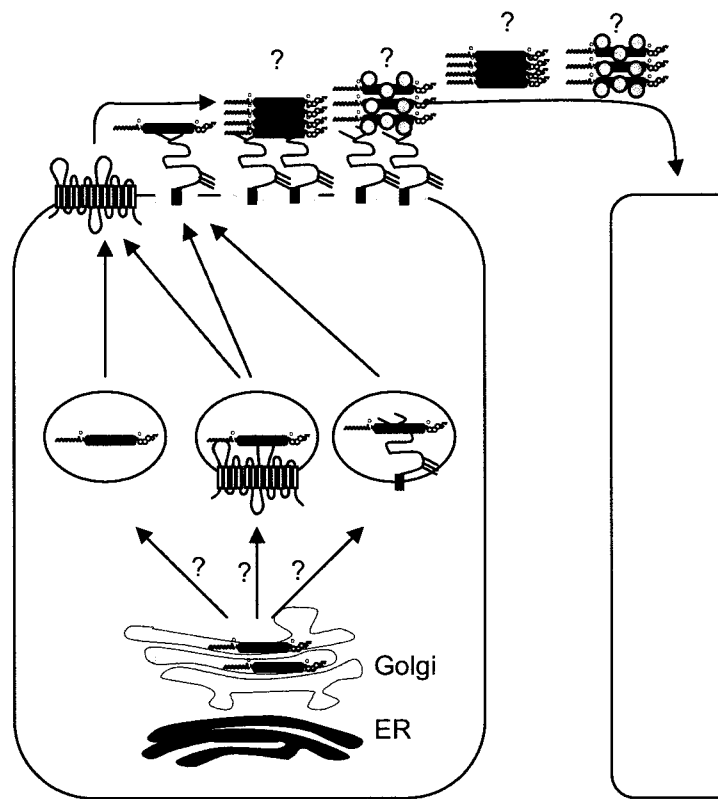
cholesterol is believed to retain Shh in producing cells, as cholesterol-deficient Shh (ShhN) is secreted at a higher level *in vitro* in nonneuronal cell lines (34, 37, 40, 41, 44). The addition of palmitate is necessary for maximal activity, multimer formation, membrane association and is required for long-range patterning (34, 37, 40, 41, 44).

The carboxyl domain of Shh has been demonstrated to be essential for Shh-N maturation and intracellular trafficking. Shh processing is mediated by autoproteolysis arising from specific residues in the Shh-C domain (32, 45). Cleavage results in the formation of a thioester intermediate that allows for the covalent attachment of cholesterol (35). Expression of a cDNA coding for Shh-N produces a protein that lacks cholesterol, suggesting that Shh-C possesses cholesteroltransferase activity or permits for a cholesteroltransferase protein to add cholesterol to the amino terminal of Shh-N (35). Interestingly, inhibiting Shh proteolysis results in a Shh protein that has the potential to be palmitoylated at the N-terminal, but this isoform is retained in the pre-medial Golgi compartment (46), suggesting that cleavage is required for trafficking to the secretory pathway. In contrast, an uncleaved hh transgene expressed in *Drosophila* photoreceptors is sorted to secretory vesicles (47), indicating that the requirements for PTMs may be context- or species-specific. Furthermore, recent evidence suggests that the hh-C fragment may also modulate hh-N trafficking in fly photoreceptor axons (47).

1.3 Hedgehog extracellular transport

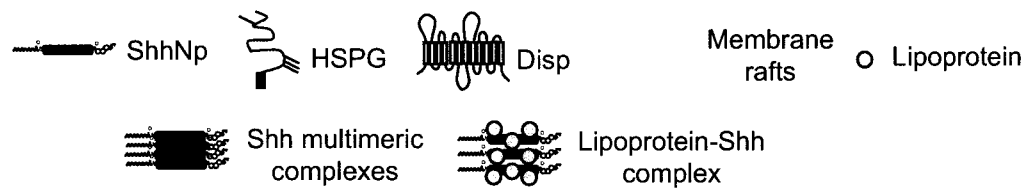
Multiple factors are required for Shh secretion and extracellular transport (Figure 1.2). Secretion of cholesterol-modified ShhNp requires the activity of Dispatched (Disp), a 12-domain transmembrane protein (48-53). Disp contains a sterol-sensing domain that is known to be involved in cholesterol transport in other proteins (51). The requirement for

Figure 1.2. Shh processing and secretion in producing cells. Shh biogenesis and posttranslational modifications occurs within the ER-Golgi and results in mature Shh (ShhNp) trafficking to the secretory pathway and deposition of ShhNp on the cell surface. Dispatched (Disp) is required for release of cholesterol-modified ShhNp; however it is unknown whether Disp interacts with Shh in the secretory pathway or at the cell surface. Movement of Shh from its site of production may be mediated by association with heparan sulphate proteoglycans (HSPG) including glypicans. It is possible that glypicans interact with Shh within the ER-Golgi and glypicans and Shh are cotransported to the cell surface in secretory vesicles. At the cell surface, Shh may associate with membrane raft microdomains which facilitate clustering of ShhNp into large multimeric complexes that solely consists of ShhNp oligomers or in complexes containing lipoproteins and HSPGs.



Shh producing cell

Shh receiving cell



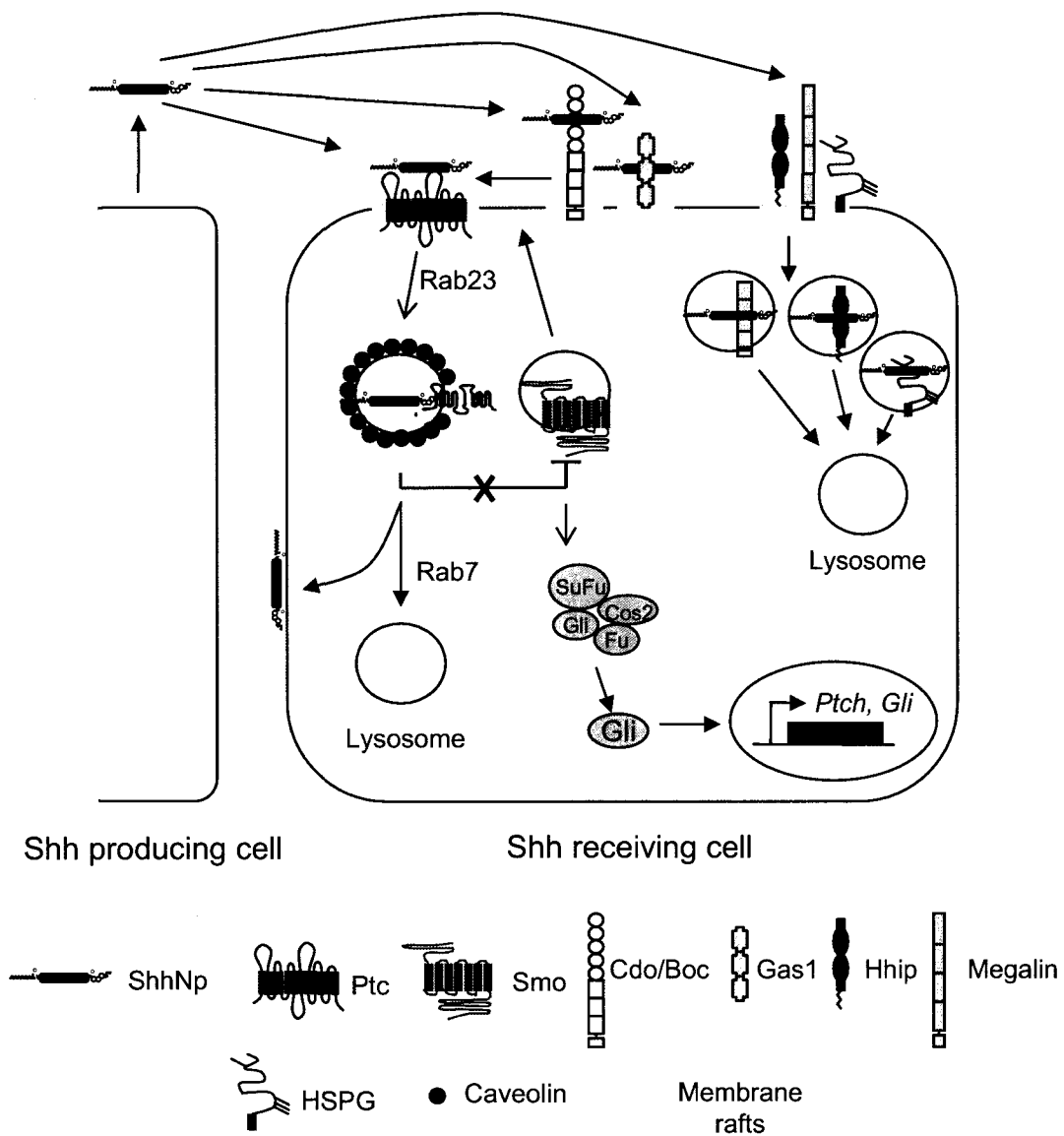
Disp in Hh patterning is highly conserved, as inactivation of Disp in flies and mice results in Hh accumulation in producing cells and perturbation of Hh patterning (48-50, 54). However, the mechanisms of Disp function are currently unknown. Several models for the role of Disp in Shh secretion have been proposed: the sterol-sensing domain of Disp may promote multimerization of lipidated Shh with other factors, such as lipoproteins, and regulate release of lipidated Shh from the cell surface or Disp may have a role in the intracellular trafficking of Shh to regulate Shh transport from the trans-Golgi network (TGN) to the plasma membrane to facilitate Shh secretion (48-54).

Heparan sulphate proteoglycan (HSPG) biosynthesis and function in *Drosophila* and mouse are required for short- and long-range Hh activity (Figure 1.2, 55, 56-63). HSPGs are extracellular matrix-associated proteins that are composed of long branched sugar chains (64). In *Drosophila*, extracellular transport of hh depends on the presence of HSPG biosynthetic enzymes of the exostosin (EXT) family (56, 58, 62). HSPG core proteins dally (dly) and dally-like protein (dlp) are EXT family substrates that have been shown to stabilize hh and regulate hh extracellular dispersal from both producing and receiving cells in a dynamin-independent process (55, 57, 65). Interestingly, the distribution of cholesterol-deficient hhN is unaffected by the loss of HSPGs, suggesting that cholesterol may mediate the interaction between hh and HSPGs (39). Although hh-N clusters on the cell surface colocalizes with dlp, hh-N aggregation is not dependent on the presence of HSPGs (66). However, the mechanism behind HSPGs in regulating short and long-range hh movement is not clear. For instance, HSPGs may control hh distribution by direct interaction that protects hh from degradation by extracellular surface proteases, facilitating clustering of hh-N to form high molecular weight aggregates, controlling interaction with receptors, or by modulating endocytosis that results in hh transcytosis or targeting for degradation.

As mature Hh is dually lipid modified, there are mechanisms to prevent HhNp from being restricted to producing cells (Figure 1.2). *Drosophila* hh has been shown to copurify with lipoproteins and these lipoproteins are necessary for long-range hh activity in wing imaginal discs (67, 68). These lipoproteins complexes are composed of a phospholipid monolayer surrounding a core of esterified cholesterol and triglyceride and contain proteins such as apolipoproteins and hormones. Interestingly, these lipoprotein complexes interact with dly and dlp and are dependent on cleavage of the glycosylphosphatidylinositol (GPI) anchor of these glypicans for secretion (67). hh-receiving cells exhibit a punctate pattern of hh staining (67-69), which may reflect assembly of hh into large lipoprotein complexes. In *C. elegans*, hh endocytosed from the cell surface is sorted to multivesicular bodies for apical secretion as exosomes (70). However, it is unlikely that *Drosophila* hh is internalized and trafficked to this recycling pathway, as hh does not colocalize with Rab11 (69). Overall, it is possible that Hh proteins are secreted and transported in tissues as lipoprotein-associated multimeric complexes whereby the hydrophobic lipid moieties are tucked inside the lipoprotein aggregates (67, 68, 71, 72). Currently, it is unclear whether the majority of mammalian Hh proteins are transported as individual molecules or as aggregates, or whether such packaging is context-specific.

Hh proteins can also bind other plasma membrane-associated proteins, resulting in either positive or negative feedback of Hh signalling (Figure 1.3). The transmembrane proteins Cdo and Boc (iHog and boi in *Drosophila*) increase binding affinity of Hh to its transmembrane receptor Patched (Ptc), resulting in amplification of the Hh signal (73-77). Similarly, Gas1 has recently been shown to be a positive regulator of Shh signalling (78-81). In contrast, Megalin (Lrp2/gp330), a member of the low density lipoprotein receptor family, Hh interacting protein (Hhip) and Ptc negatively regulate Hh signalling by mediating

Figure 1.3. Activation of the Shh signal transduction cascade in receiving tissues. Shh binds to its cognate receptor, Patched (Ptc) and Shh-Ptc complexes are endocytosed under the control of Rab23 in a dynamin-dependent mechanism. Internalization leads to phosphorylation and mobilization of Smoothened (Smo) to the plasma membrane. Stabilization of Smo at the plasma membrane leads to inhibition of Gli cleavage and initiates transcription of Shh target genes. The Ptc-Shh complex is subsequently targeted to lysosomes for degradation by Rab7 or is recycled to the cell surface. Shh binding to coreceptors Cdo, Boc or Gas1 results in augmentation of the Shh pathway by facilitating Shh presentation to Ptc. Alternatively, Shh can be sequestered on the cell surface, endocytosed and targeted for degradation by binding to various proteins including glypicans, Hh interacting protein (Hhip) and Megalin.



endocytosis of Hh proteins that target Hh for degradation or transcytosis, thus reducing the long-range movement of Hh proteins (65, 82-87). Lastly, Shifted (Shf), a *Drosophila* homologue of Wnt inhibitory factor-1, is a secreted protein that interacts with Shh, and Shf mutations result in reduced long-range Shh signalling (67, 68).

1.4 Hedgehog signal transduction

Activation of Hh signalling requires several factors that are involved in both negative and positive feedback mechanisms (Figure 1.3; 88, 89). In the absence of Hh protein, Ptc, a 12-transmembrane protein, antagonizes the activity of Smoothened (Smo), a 7-transmembrane protein that is the obligate signal transduction component of the Hh pathway (90-94). Smo controls the activity of the cubitus interruptus (ci in *Drosophila*) Zinc finger domain transcription factors that regulate target gene expression downstream of Smo. In mammals, Hh target gene induction is controlled by 3 ci homologues, Gli1, Gli2 and Gli3 where Gli2 and Gli3 possess both activator and repressor activities while Gli1 acts solely as a transcriptional activator and serves to amplify the Hh signal (88, 95). When the pathway is inactive, ci/Gli is inactivated by phosphorylation and in the case of ci and Gli3, by cleavage to shorter transcriptional repressor forms of the proteins. Hh binding to Ptc derepresses Smo activity, which leads to phosphorylation and stabilization of Smo at the plasma membrane, thereby activating cytoplasmic downstream effectors that ultimately induce target gene expression (96-98). Smo activation promotes Gli-mediated Hh target gene activation by repressing phosphorylation and cleavage and promoting nuclear translocation of transcriptional activator forms of Gli, in part by inducing Gli dissociation from *Suppressor of Fused* (*Su(Fu)*). Moreover, both Hh and Ptc colocalize in endocytic vesicles that are triggered by a dynamin-dependent process involving caveolae-1 proteins, resulting in

transport of these vesicles away from the plasma membrane (65, 85, 99, 100). Rab23 is colocalized with Ptc, affecting Shh signalling presumably by regulating Ptc-Shh endocytosis from the plasma membrane or by regulating its endocytotic path to lysosomes (101, 102). Ultimately, Gli induces expression of Hh target genes such as *Ptc* and *Gli*, with *Ptc* involved in a negative feedback signal. Lastly, recent evidence has indicated that primary cilia are required for Hh signalling. Many tissues that respond to Hh contain most of the Hh signal transduction factors in the primary cilia (103-110). In these cells, a functional cilium is required for the Ptc-mediated regulation of Smo trafficking and subsequent Gli processing.

1.5 Shh in the Central Nervous System

Numerous studies have demonstrated that Shh is essential in the establishment of patterning and growth of a diverse set of tissues during embryogenesis including the central nervous system (CNS). Dysregulation of the Shh signalling pathway leads to severe defects in CNS development. Specifically, loss of Shh in humans and mice results in embryonic lethality and a phenotype consistent with defects in establishment of midline structures: absence of ventral cell types within the neural tube, and formation of HPE and cyclopia as a result of the failure of the forebrain hemispheres to separate (111, 112).

1.5.1 Shh in neural tube development

Dorsoventral patterning of the neural tube is one of the key forerunners for organization of the CNS. The developing neural tube outlines the distinct regions of the CNS; prosencephalon (telencephalon [forebrain/cerebrum] and diencephalon [optic vesicles and hypothalamus]), mesencephalon (midbrain) and rhomencephalon (pons and cerebellum). The neural tube is patterned along its dorsal-ventral axis by signals from the dorsal

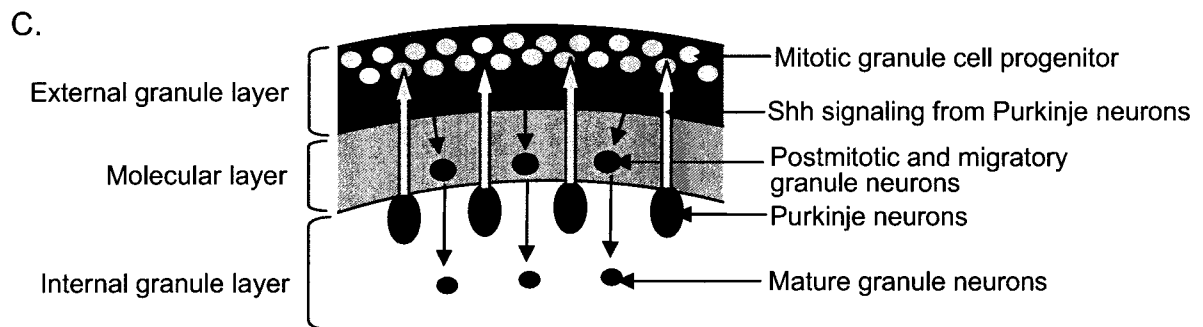
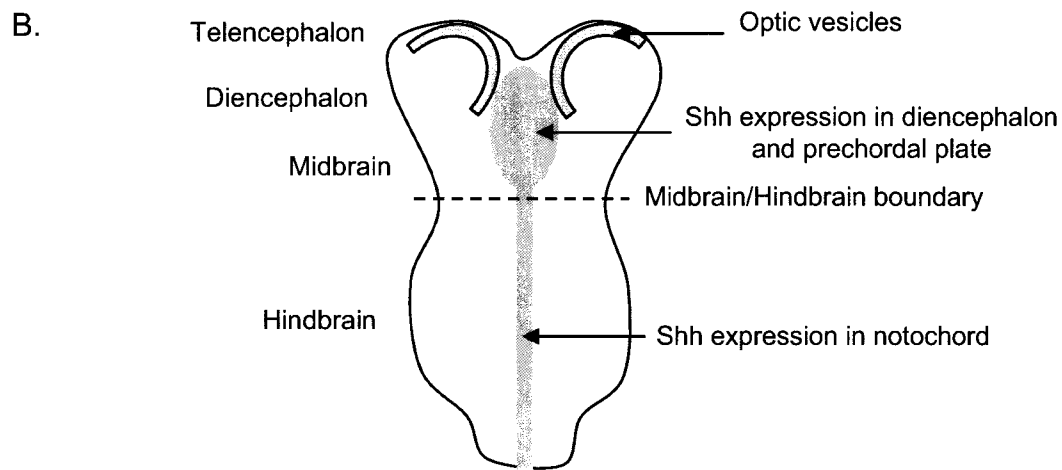
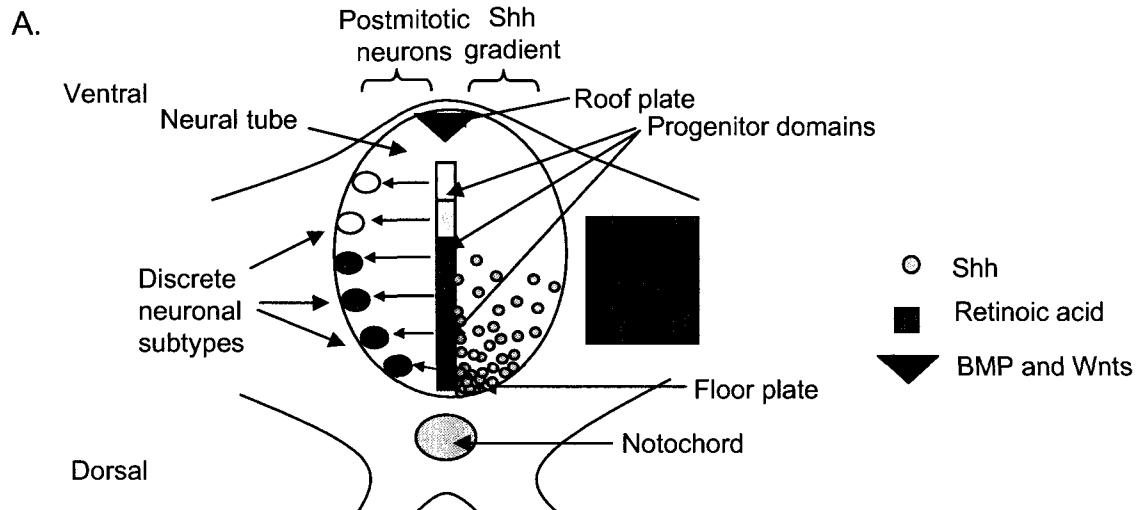
nonneuronal ectoderm and the ventral notochord and floor plate. Shh is expressed in the axial mesoderm (ventral notochord and prechordal plate) and ventral midline (floor plate) of the neural tube, and is expressed at the times where the neural tube is ventralized (20, 113-115). Shh functions as a morphogen in the presumptive spinal cord by directing the pattern of neurogenesis through conferring spatiotemporal positional information to the ventral neural tube progenitors (Figure 1.4A; 116). In combination with other inductive signals such as bone morphogenic proteins (BMPs), Shh regulates the expression pattern of different transcription factors that results in the proper segregation of each neuronal subtype along the dorsal-ventral axis (116). In addition to its inductive role in neural tube specification, Shh promotes progenitor cell proliferation, and consequently growth of neural tube and brain structures (117-122). Formation of the Shh concentration gradient in the neural tube depends on Shh production and secretion and likely the degradation and removal of Shh from receiving cells, all of which is tightly regulated and involves several Shh interacting factors, including Boc/Cdo, Hhip and Megalin (73, 76, 123, 124).

1.5.2 Shh in eye development

Shh is involved in eye patterning at several distinct stages and regions during early development. Specifically, Shh is critical for the ventral diencephalon to form the optic vesicles, specifies the optic stalk and retinal tissues, and regulates retinal development.

The first appearance of eye development begins as bilateral evaginations of the neural tube at the level of the ventral diencephalon to form the optic vesicles. The optic vesicles grow laterally, contact the surface ectoderm, and invaginate resulting in the formation of a proximal optic stalk and distal bilayered cup. The developing eyecup remains attached to the rest of the brain via the optic stalk, which will become the optic nerve once it is invaded by

Figure 1.4. Shh in early CNS development. (A) Schematic of a transverse section of a mammalian embryo showing the somites, neural tube and notochord. Within the spinal cord, distinct neuronal subtypes are generated in a spatially segregated manner in response to signals emanating from the neural tube and surrounding tissue including Shh from the notochord and floor plate, retinoic acid from somites and bone morphogenic proteins (BMP) and Wnts from the dorsal roof plate. Shh forms a concentration gradient from the ventral floor plate which controls positional identity of neuronal subtypes by regulating transcription factors. Each progenitor domain is defined by its combinatorial transcription factor code and generates different neuron subtypes. Adapted from Dessaud et al. (116). (B) Dorsal view of the neural plate prior to neural tube closure at E7.5. Expression of Shh in the diencephalon and prechordal plate is essential for bifurcation of the optic vesicles. Adapted from Fuccillo et al.(126). (C) Sagittal section of the cerebellum at P0. Shh is expressed by Purkinje cells (red) and stimulates gene expression and proliferation of granule neuron progenitors located in the external granule layer (yellow). Currently, it is unknown how Shh is trafficked to the external granule layer. Mature granule cells migrate inward to the internal granule layer (brown and black). Pink arrows denote Shh signalling from Purkinje neurons to granule precursor cells. Adapted from Fuccillo et al. (126).



blood vessels and the axons of retinal ganglion cells (RGCs), the projection neurons of the retina. The infolding of the optic vesicles and progressive development of the retina and stalk is dependent on Shh signalling (Figure 1.4B).

Shh signalling from the axial mesoderm induces the ventral midline of the neural tube along the rostrocaudal axis. The ventral midline that underlies the developing neural tube is required for the induction of midline structures along the rostral-caudal axis of the CNS. In mice deficient for positive activators of the Shh signalling pathway, the ventral midline fails to specified, leading to the loss of midline structures, including cyclopia where the ventral diencephalon precursor cells fail to bifurcate into a bilateral eye field, generating a single, centrally located optic vesicle (50, 112, 127-129). In addition to its role in the establishment of the bilateral eye field, midline-derived Shh signals are also important for patterning the optic vesicle along the proximodistal axis. Perturbation of signalling from axial tissues in the diencephalon results in conversion of the optic stalk to neural retina (112, 130). The boundary between the presumptive optic stalk and neural retina is established through reciprocal transcriptional repression between homeodomain transcription factors (112, 130-138). One of these transcription factors, Pax6, has essential roles in retinal cell fate specification and its expression is largely excluded from the optic stalk (139, 140). The optic stalk specific homeodomain transcription factor genes Pax2 and Vax2 are sensitive to Shh dosage and are restricted to the posterior-ventral half of the optic vesicle.

Multiple experiments have revealed a requirement for Shh in optic cup morphogenesis. Perturbing the Shh pathway in mice and chick leads to absence of the optic stalk marker *Pax2* and expansion of the optic cup (112, 134, 135). On the other hand, overexpression of *Shh* expands the *Pax2* and *Vax1* expression domains with a concomitant reduction of *Pax6* expression in the optic cup, resulting in expansion of the ventral optic

stalk tissue at the expense of the retina (130, 131, 135-137). Moreover, optic stalk fates are induced adjacent to regions of highest Shh activity. Specifically, the proximodistal fate is specified by transcriptional repression between *Pax6* and *Vax2*, which is regulated by Shh-dependent subcellular localization of *Vax2* whereby *Vax2*⁺ cells close to the midline source of Shh remains in the astroglial cell fate (135, 136, 138, 141, 142). Lastly, maintenance of dorsoventral components of the optic vesicle is regulated by the antagonistic interaction between ventrally derived Shh and dorsally derived BMP4 (135, 143-145). Taken together, these results indicate that specification of optic stalk and retinal tissue is regulated through interaction between the BMP and Shh pathways coupled with temporal and spatial regulation of Shh-dependent *Vax2* subcellular localization, leading to demarkation of the *Pax6* territory whereby cells exposed to Shh specify the optic stalk and the rest of the optic vesicle develops into the neural retina.

During retinal development, Shh expression is coupled to RGC differentiation and retinal cell proliferation (146). *Shh* is initially expressed in RGCs, the first-born neurons of the retina, and influences retinal precursor cell (RPC) development. Interestingly, Shh appears to function as a factor that prevents RPCs from adopting the RGC fate (143, 147). Thus, Shh secreted from RGCs contributes to negative feedback inhibition for further RGC genesis. In addition to regulating RGC differentiation, Shh signalling from RGCs stimulates local RPC proliferation (147-149).

1.5.3 Hh is anterogradely transported in RGC axons

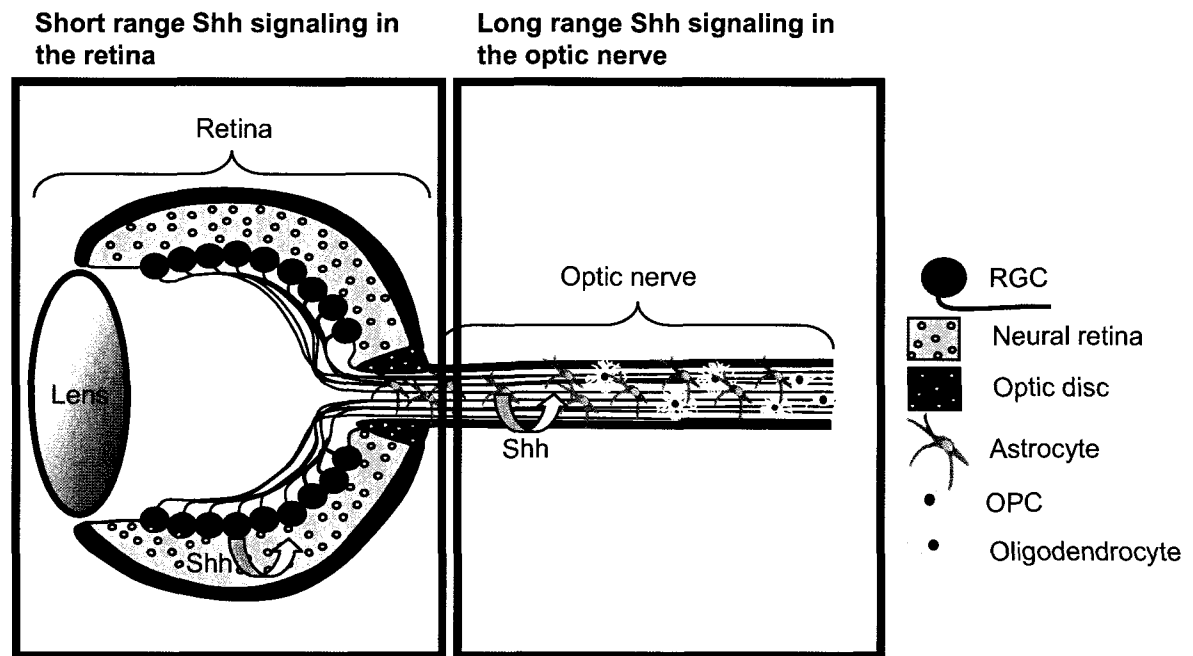
RGC axon-derived signalling regulates glial cell development in the optic nerve. RGC axons are the only neuronal component of the optic nerve and it is well-established that signals from RGC axons are important for the development of the two glial cell populations

in the optic nerve: astrocytes derived from neuroepithelial cells that line the optic stalk and oligodendrocytes that migrate into the optic nerve from the optic chiasm (150, 151). Astrocyte lineage cell development from neuroepithelial cells and their subsequent proliferation depends on innervation of RGC axons from the retina (150, 152-158). For instance, transection of the optic nerve is associated with reduced proliferation rate of astrocytes, and conversely, increasing the number of RGC axons is associated with an expanded astrocyte population in the optic nerve (150, 154). Additionally, astrocyte proliferation is dependent on microtubule-based axoplasmic transport but not electrical activity (150). Thus, astrocyte proliferation depends on anterograde transport of factors from the retina to the optic nerve. Oligodendrocyte precursor cell (OPC) proliferation and survival requires functional RGC axons, as well as astrocyte-derived signals that stimulate OPC migration from the optic chiasm and promotes the subsequent maturation of OPCs into oligodendrocytes (151, 159-166). Thus, signals originating from RGCs influences astrocyte proliferation which in turn regulates oligodendrocyte development and subsequent myelination of the optic nerve.

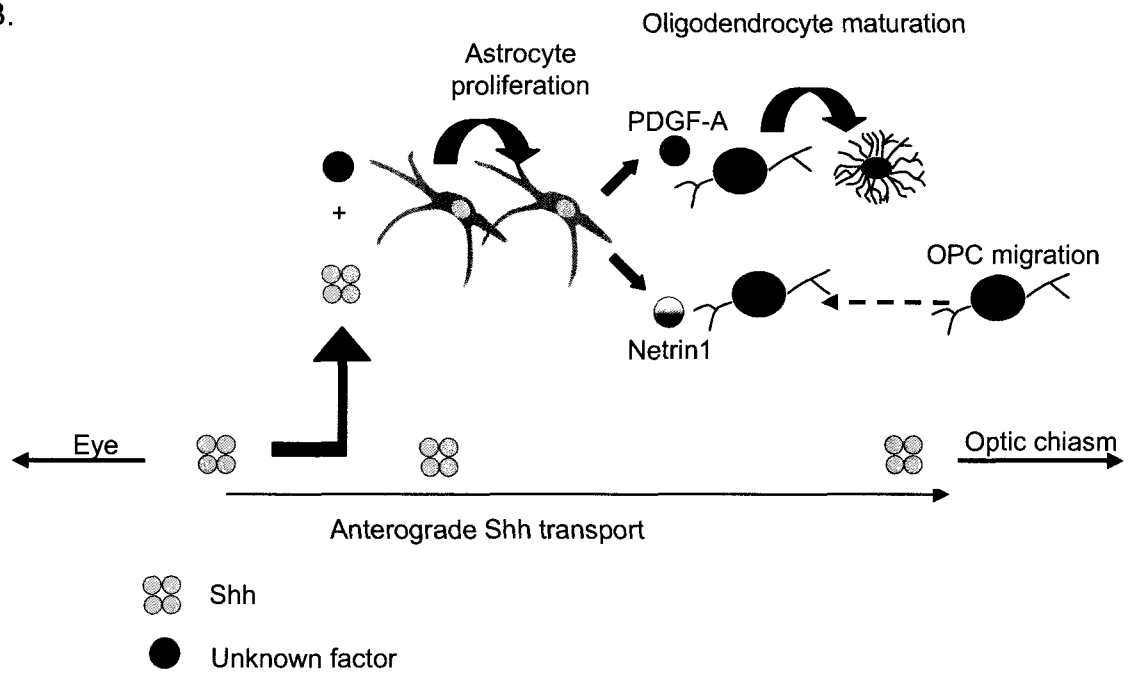
RGC-axon derived Shh has been demonstrated to have an essential role in optic nerve gliogenesis (Figure 1.5; 167, 168). Astrocytes in the perinatal optic nerve express Hh target genes, indicating that astrocytes are responding to an Hh signal, and the maintenance of Hh target gene expression and astrocyte proliferation in the optic nerve is Hh-dependent and requires intact axons (167, 169). Specifically, axotomy-induced RGC axon degeneration inhibits Hh signalling and astrocyte proliferation in the neonatal optic nerve (167) and inactivation of *Shh* in RGCs during embryogenesis results in a failure of astrocyte development in the nerve (169). In this context, Shh is required for the induction of astrocytes at the optic disc (in part by maintaining expression of Pax2) and for astrocyte

Figure 1.5. Shh is anterogradely transported in RGC axons. (A) Shh from retinal ganglion cells (RGCs) signals at short range to drive *Gli* expression and proliferation of progenitor cells in the neural retina. Shh is also transported in an anterograde direction from RGC bodies within axons in the optic nerve where it signals to astrocytes (B). Model of Shh intercellular interactions in optic nerve gliogenesis. The combination of Shh and an unknown factor stimulates astrocyte proliferation. In turn, astrocytes secrete factors such as Netrin-1 and Pdgf-A to stimulate oligodendrocyte cell precursor (OPC) migration from the optic chiasm and differentiation of OPCs to mature oligodendrocytes. Shh protein is released from axon terminals at targets in the brain including the superior colliculus; however, its functions in this context are unknown.

A.



B.



proliferation in the optic stalk (135, 169-171). Oligodendrocyte development also appears to depend on Shh signalling, as blockade of Shh signalling in the optic chiasm in chick abrogates OPC invasion into the optic nerve from the chiasm (172). However, the effects of Hh signalling on OPCs are likely to be indirect, as OPCs do not express Hh target genes (167). Lastly, downregulating Shh expression in RGCs reduces proliferation and the size of the astrocyte population in the optic nerve, and is also associated with a reduction of oligodendrocytes and Wallerian degeneration of axons in the optic nerve (168). The reduction of the oligodendrocyte population in the optic nerve is likely a result of a decrease of astrocyte-derived factors, such as Pdgf-A and Netrin-1, which control OPC migration and differentiation (168). However, Shh is unlikely to be the sole growth factor necessary for stimulating astrocyte proliferation, as activation of the Hh pathway fails to increase astrocyte proliferation *in vitro* (167, 168). Overall, these results indicate that axon-derived Shh signalling plays a key role in optic nerve development by regulating the development of both glial cell populations.

Shh is anterogradely transported from the cell body to axon terminals in both mammalian and *Drosophila* retinal neurons. Shh protein, but not mRNA, is present in the optic nerve (167, 173). As well, transport of radiolabelled peptides corresponding to Shh protein from the eye to the superior colliculus, a target of RGC axons in the brain, has been detected in the rodent CNS (173). These findings suggest a model in which Shh protein is transported from the RGC body in an anterograde direction in axons and is released within the optic nerve to modulate astrocyte proliferation (Figure 1.5B). Similarly, in *Drosophila*, cholesterol-modified hh is transported from the eye to the brain along retinal axons to regulate neurogenesis of progenitor cells in the lamina (47, 174-176). It appears that several

residues in hh-C are required for hh-N transport to the axon terminals (47). Moreover, hh elicits different cellular responses in epithelial tissues when hh protein is presented apically or basolaterally (39, 69). However, it is unknown if there are divergent consequences of Shh presented from the apical (axons) versus basolateral (dendrites) sides of mammalian neurons. Overall, these studies indicate that Shh from RGCs is released basally in the retina to pattern the retina and is targeted apically in axons to pattern the optic nerve and synaptic targets in the CNS.

1.5.4 Shh in axon guidance

During retinal development, RGCs migrate towards their final position in the neural retina and the RGC axons extend along the vitreal surface of the retina and grow towards the optic disc where the axons project through the optic nerve (Figure 1.5A; 177, 178). Precise RGC axon pathfinding within the optic cup is shaped by both attractive and repulsive guidance cues secreted by the neural retina and the optic disc. A role for Shh in axon pathfinding was first described in zebrafish where Shh mutants exhibited defects in retinotectal projections (179-181). Shh signalling in the retina also promotes normal RGC axon guidance, in part by promoting the development of the optic disc astrocytes which secrete chemoattractant cues, such as Netrin (169). Moreover, Shh mediates concentration-dependent effects on RGC axon pathfinding, functioning as a chemorepellent at high concentrations and as a chemoattractant at low concentrations (182, 183). Thus, the concentration-dependent effect of Shh on axon outgrowth might be a mechanism that prevents invasion of RGC axons into the neural retina where the concentration of Shh is higher, promoting the guidance of these axons along the nerve fibre layer where Shh expression is reduced. Moreover, it appears that Shh-dependent axon guidance also functions

as an autocrine signal as blocking the ability of RGCs to respond to a Hh signal leads to defasciculation and aberrant navigation of these projecting axons (184).

Shh also has a role in the formation of the retinotopic map of RGC projections to the optic tectum. After RGC axons exit the eye and project towards the optic chiasm, the astroglial cells located at the optic chiasm regulate ipsilateral and contralateral axonal projections by differentially guiding RGC axons at the optic chiasm into the ipsilateral or contralateral trajectories (185, 186). Perturbation of Shh signalling at the optic chiasm results in axon projection errors (182, 187). Shh functions indirectly in this context, by exerting a concentration-dependent effect on the expression of Netrin and Slit molecules at the chiasm (188-191). Astroglial cells exposed to higher levels of Shh express the chemorepellent Slit2/3, whereas astroglial cells that are exposed to low levels express the chemoattractant Netrin1 (190). As contralateral RGCs express the Slit receptor, roundabout (Robo), these projecting axons are funnelled into a contralateral trajectory due to repulsion by the Slit2/3⁺ astroglial cells. On the other hand, ipsilateral RGC axons do not express Robo and, thus, are insensitive to the chemorepellents Slit2/3. It is unknown whether this Shh concentration-dependent effect on astroglial cells is a temporal phenomenon such that early exposure to Shh leads to expression of chemoattractant genes and later exposure of cumulative Shh results in expression of chemorepulsive cues. These results indicate that Shh expression at the optic chiasm guides RGC axon pathfinding by affecting cell fate specification of the astroglial population close to the midline source of Shh.

While midline-derived Shh influences formation of the retinotopic map, Shh expression along the midline also mediates axon pathfinding of commissural neurons in the developing neural tube. Removing the ability of the commissural axons to respond to midline-derived Shh reduces axon turning to the floor plate (192). The chemoattractant

mechanism requires the combinatorial effect of both Netrin-1 and Shh, where Shh acts directly as a secreted chemoattractant. Moreover, guidance of these commissural axons to the floor plate is dependent on binding of Shh to Boc, leading to activation of the Src family kinase members which mediates alterations in the growth cone cytoskeleton (76, 193). After commissural axons have crossed the floor plate and grow along the longitudinal axis, Shh also acts as a chemorepellent signal through binding of Shh to Hhip (125). Thus, Shh has a role as a chemoattractant for commissural axon guidance towards the floor plate during early neural tube development and a role as a chemorepulsive cue late in commissural axon growth, and Shh function in this context depends on the activity of Shh coreceptors.

1.5.5 Shh drives proliferation of neural progenitor cells in the brain

In the adult brain, the Shh pathway is critical for regulation of progenitor cell proliferation. Although the expression of Hh signalling components are downregulated as embryonic development progresses, the Hh signalling components are still present at discrete regions in the brain, including neurons and progenitor cells (8, 194-196). For instance, Shh is a neuron-derived signal that drives proliferation in the cerebellum, subgranular zone (SGZ), subventricular zone (SVZ) and retinal neuron targets in the brain (117, 121, 122, 197-204). As well, Shh proteins have been localized to other postmitotic neurons within the CNS (8, 205). Whereas the spatial localization of Shh and Hh target genes are usually adjacent during embryonic development, Shh and Hh target genes are not coincident in the adult CNS and, in some instances, Shh protein distribution reflects the pattern of innervation (8, 173, 194-196). These results raise the possibility that Shh could be transported in axons in CNS neurons, perhaps to influence progenitor cell proliferation.

In the cerebellum, Shh is expressed by Purkinje cells and is required for granule cell progenitor proliferation (Figure 1.4C). Perturbation of Shh function from Purkinje neurons does not affect cerebellum patterning but results in decreased granule cell proliferation, leading to abnormal dendritic arborisation and a reduction of the size of the cerebellum (117, 121, 122, 197-199). Purkinje neurons have large dendritic arbours in the external granular layer (EGL) where these neurons are a source of mitogens and establish synapses with granule cell progenitors (206). Shh protein has been immunolocalised to the EGL layer and granule cell precursors express Shh signalling components indicating that these cells have the capacity to respond to a Hh signal (8, 194, 195). Thus, Purkinje neuron-derived Shh regulates the size of the cerebellum through regulation of granule cell precursor proliferation.

The Shh signalling pathway is also necessary for the proliferation of progenitors within the SGZ and SVZ, which generates dentate granule cells of the hippocampus and new neurons destined for the olfactory bulb, respectively (200-204). The proliferation rates of these progenitor cells are dependent on the degree of Hh pathway activation and require the presence of a Cardin-Weintraub (CW) motif within Shh-N that mediates Shh-N binding to various proteoglycans, such as HSPGs (200-204, 207). However, the cellular origin of Shh protein that mediates progenitor cell proliferation both in the SGZ and SVZ is unclear. These neurogenic regions are innervated by both afferent and efferent axons, and are surrounded by basal lamina, glial cells and blood vessels (208). Interestingly, Shh protein, but not the mRNA, is detected in the hippocampus and at several structures of the adult basal forebrain that project to the dentate gyrus through the fornix (194, 195). Severing this connection by transecting the fornix decreased progenitor proliferation, raising the possibility that Shh may be of axonal origin (202). Overall, neuron-derived Shh may signal from a distance by axoplasmic transport to drive progenitor cell proliferation in the SVZ and SGZ.

Although Shh is anterogradely transported in RGC axons to the superior colliculus where these axons synapse (173), the functional significance of Shh presentation at axon terminals is unknown. However, there is evidence for a proliferative effect of hh signalling on neural progenitors in the brain. In *Drosophila*, hh axonal transport by retinal neurons and its secretion at the neurite terminals regulates neurogenesis in the brain (47, 174-176). Due to the high degree of evolutionary conservation in the distribution of Hh in *Drosophila* and vertebrate visual systems, it raises the intriguing possibility that Shh may also influence cellular function of RGC axon targets. For instance, anterograde transport of growth factors from RGC axons is critical for the development and maintenance of neurons in the superior colliculus and lateral geniculate nucleus (209-213). Thus, it is possible that other RGC axon-derived factors, including Shh, may influence neurogenesis at RGC targets in the brain.

Hh proteins also have been immunolocalized in axons of different neuronal subtypes in the CNS (8, 205). Using a sensitive immunohistochemistry approach, Hh protein has been localized to commissural axons, axons in the ventral root of spinal nerves, nerve fibres emanating from inner ear ganglion, and in the dorsal telencephalon including Cajal-Retzius neurons, cortical plate neurons and GABAergic interneurons (8, 205). Overall, these examples indicate that axon transport of Shh might be a more general phenomenon in the CNS, possibly by maintaining niches such as neural stem cells.

1.6 Polarized protein trafficking in neurons

Neurons are polarized cells that contain extensive membrane protrusions (axons and dendrites) and have highly specialized secretory pathways. The majority of protein synthesis occurs in close proximity to the soma and newly synthesized proteins are actively transported down axons and dendrites. Production and trafficking of essential factors is critical for

intercellular communication and the stabilization of synapses of neurons with glia and other neurons. These factors are trafficked through either of two available secretory pathways in neurons, constitutive and regulated (Figure 1.6). All cell types are capable of targeting secretory proteins through the constitutive secretory pathway (CSP), whereby proteins in constitutive secretory vesicles (CSV) are either rapidly trafficked to the plasma membrane and are released immediately in an unstimulated manner or pass through intermediate endosomal compartments (e.g. early/late endosome and recycling compartments) before reaching the plasma membrane (214). On the other hand, specialized cells, including neurons and neuroendocrine cells, have a regulated secretory pathway (RSP), which includes synaptic vesicles (SVs) and secretory granules (SGs) whereby these vesicles store and release cargo upon stimulation by a secretagogue. The two classes of regulated secretory vesicles (RSVs) are distinguished by the cargo content, mechanism of organelle translocation in the endomembrane system and characteristics of cargo secretion. Sorting cargo to the correct compartment is regulated early in the secretory pathway. The TGN functions as a sorting platform to distribute secretory proteins to the CSP or RSP, and is subjected to tight regulation by specific factors and mechanisms that allow sorting of the correct protein into the appropriate secretory vesicle (215).

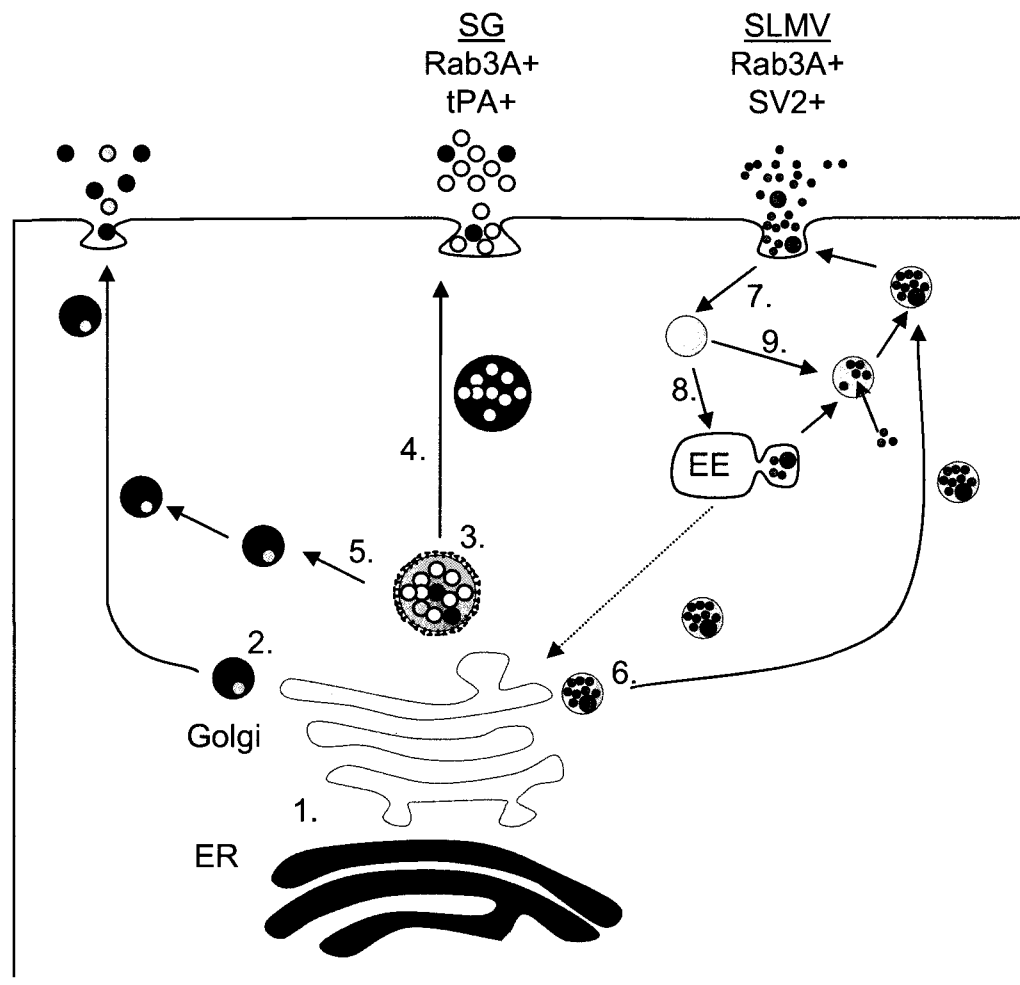
1.6.1. Synaptic vesicles

Synaptic vesicles are one of the distinguishing features of neurons, as no other cell type contains these small vesicles that are primed to secrete their cargo upon Ca^{2+} stimulation. SVs store and secrete small peptides and neurotransmitters that mediate the propagation of action potentials between synapses. The insight into intracellular trafficking of SVs has stemmed from not only neurons, but also neuroendocrine cells, such as the rat

Figure 1.6. Available secretory pathways in neurons. (1) Proteins and peptide neurotransmitters are synthesized within the rough endoplasmic reticulum (ER), inserted into the ER cisternae and transported to the Golgi apparatus. Trafficked proteins and peptides are packaged into constitutive (2) or regulated secretory vesicles comprised of secretory granules (SGs; 3) or synaptic vesicles (SV; synaptic-like microvesicles [SLMV] in PC6 cells; 6). The constitutive secretory pathway is available in all cell types, and does not require external signals for vesicle exocytosis. Immature SGs bud from the trans-Golgi (3) followed by a number of maturation steps to yield a mature SG (4), including acidification to activate prohormone convertases and carboxypeptidases necessary for processing for secretory pathway proteins, removal of constitutive secretory proteins (5) and lysosomal enzymes inadvertently packed into immature SGs, loss of the clathrin coat and condensation of granule contents. Mature SGs then traffic to the cell periphery and secrete their cargo upon stimulation with a secretagogue. (6) Synaptic vesicles are thought to exit the trans-Golgi network in a constitutive secretion manner and undergo an endocytotic step to generate synaptic vesicles (7). Generation of mature synaptic vesicles occurs through endocytosis through clathrin-dependent or independent mechanisms, which may be sorted to early endosomes (EE; 8) or re-filled with neurotransmitters (9). Rab3A marks both types of regulated secretory vesicles and tissue plasminogen activator (tPA) and SV2 can be used to distinguish SGs and SVs, respectively.

Constitutive secretory pathway

Regulated secretory pathways



pheochromocytoma-derived line PC12 which form SV-like organelles called synaptic-like microvesicles (SLMV; 216). Ultrastructurally, SLMVs resemble the small and uniform size of SVs, contain many of the integral membrane proteins found in SVs and are capable of storing and releasing neurotransmitters (216-218).

Studies into SV trafficking using both primary neurons and PC12 cells indicate that SV biogenesis begins with the rapid transport of constitutive-like secretory vesicles to the nerve terminal (219-221). The tubulovesicular vesicles from the TGN do not contain a complete set of SV membrane proteins, as different components of SVs are transported separately, requiring additional trafficking steps to form mature SVs (222). Following presentation of these organelles at the plasma membrane, membrane proteins destined for incorporation into SVs undergo consecutive cycles of endocytosis and exocytosis (221, 223). Within these cycles, mature SVs are generated from two donor membranes, the plasmalemma and endomembrane system (217, 223, 224).

Remodelling of SVs at synapses arises from two main endocytotic pathways; kiss-and-run and full-fusion. In the kiss-and-run model, SVs form transient pores with the plasma membrane in which SV cargo is secreted and the pore is quickly closed to disconnect the vesicle from the plasma membrane (225-227). As a result, the SV maintains its specialized identity and is rapidly returned to the readily releasable pool. The full-fusion model involves complete fusion of SVs with the plasma membrane and subsequent recycling is mediated by endocytosis of clathrin-coated vesicles which either uptakes new cargo from the surrounding cytoplasm or is delivered to an endosomal membrane from where new SVs bud off (228, 229).

1.6.2 Secretory granules

Secretory granules are a class of RSVs present in several different cell types that store diverse proteins that are released upon stimulation. The biogenesis of SGs have been examined in detail using primary neurons, neuroendocrine cells including the PC12 and AtT-20 cell lines, and other secretory cells, such as exocrine and haematopoietic cells. Secretory granules are often referred as dense core granules (DCGs), large DCGs (LDCGs) and large dense core vesicles (LDCVs). SGs are large organelles (~300 nm) and are typically distinguished from other large vesicles by the presence of a dense core containing pigmented granules that is easily resolved by electron microscopy. Unlike SVs that typically contain a highly restricted set of neuronal membrane proteins and cargo, SGs contain a subset of membrane proteins typically found in SVs as well as neuropeptides, neurophins, hormones, proteases and signalling molecules which facilitate diverse cellular function such as neuronal survival, plasticity, synaptic transmission and learning. Similar to SVs, the contents of SGs are released by exocytosis in response to a secretagogue.

Secretory granule biogenesis follows a different route than SVs and does not require endocytosis for SG maturation. Secretory granule cargo and membrane proteins are sorted at the TGN where the first step is the generation of a short-lived immature SG (ISG) within the TGN (230). While the ISG is located in the periphery of the TGN, selective removal of non-SG cargo, and processing and packaging of SG cargo results in increased condensation of the cores. As ISGs bud from the TGN, the vesicle contains a clathrin coat (231). Maturation of ISGs includes acidification within the vesicle that is critical for activation of prohormone convertases for bulk processing of proneuropeptides within these organelles (232, 233). Concurrently, the clathrin coat is removed from the short-lived ISG and the mature SG is

trafficked to its final destination where SGs release their cargo upon stimulation. Following secretion, the SG membrane is recycled to the Golgi complex to be refilled with SG cargo (232-234).

Sorting of SG cargo and membrane proteins are regulated by several different mechanisms. Since the CSP is the default pathway (232, 233), cargo must be sorted within the TGN to be packaged into ISGs. Currently, there are two main models for SG protein sorting; sorting by entry and sorting by retention. Sorting by entry involves high Ca^{2+} concentrations and a low pH in the TGN to promote aggregation of specific proteins destined to be sent to SGs (232-236). These aggregates may be enwrapped by ISG-specific membranes within the TGN and interact with other cargo to drive SG formation and maturation (237, 238). Concurrently, membrane-cargo interaction through specific sorting signals or interaction with sorting receptors that latch onto ISG-destined membrane directs cargo proteins to ISGs. As well, cargo proteins may associate with membrane rafts at the TGN through direct interactions with lipids or by sorting receptors that are concentrated at these raft microdomains (239-242). The second model, sorting by retention, states that proteins not destined for regulated secretion are initially encapsulated into nascent ISGs and these proteins are progressively removed towards the CSP (243). Thus, those proteins destined to be constitutively secreted are progressively extruded in low-density vesicles as the SG matures. As it is unlikely that these two models are mutually exclusive, the second model may act as an advanced sorting station. Overall, SG-destined proteins are segregated from proteins destined for the constitutive secretory pathway at the TGN by aggregation, followed by membrane association that is mediated by direct interaction with lipids or protein-sorting receptors. A secondary sorting step includes removal of constitutive secretory proteins that have inadvertently entered into SG vesicles.

The regulation of SV and DCG exocytosis share some common mechanisms. Both SVs and SGs undergo exocytosis in response to secretagogues that elevate intracellular calcium levels and the mechanism of docking, priming and release is mediated by many of the same molecules (244). However, SG cargo release occurs at a slower rate than SV secretion due to neutralization of the acidic SGs (234). As well, while SVs undergo local recycling to refill with peptides, SG membranes are retrogradely transported, and cells are also stimulated to upregulate transcription of SG proteins (e.g., granins) to replenish SGs (232-234).

1.6.3 Intracellular transport of secretory proteins – the case of BDNF

Like Hh in *Drosophila* and mammals, secretory factors such as neurotrophins can undergo anterograde axonal transport (245). Neurotrophins are a family of secreted proteins that are important for survival, development and synaptic plasticity, as well as maintenance of neural progenitor cells (246). The neurotrophin brain-derived neurotrophic factor (BDNF) is a well-characterized example of a secreted protein that is sorted to the CSP or RSP in a context-specific manner. BDNF is translated as a pre-propeptide with a signal peptide that is removed in the ER to yield a propeptide that is glycosylated and is processed intracellularly by furin or prohormone convertases to yield mature BDNF (247-250). BDNF is anterogradely transported in axons to synaptic terminals where release of BDNF has functional consequences on target cells (209, 211, 251, 252). In contrast to other neurotrophic factors (e.g., NGF and NT-4), which are sorted to the constitutive secretory pathway, BDNF is primarily associated with SGs in hippocampal neurons and neuroendocrine cells (249, 253-256). As a result of sorting of BDNF to SGs, BDNF release is regulated by neuronal activity (249, 253, 255, 257, 258). Interestingly, BDNF is also

packaged into CSVs (249) which results in differences in neuronal site of BDNF release; constitutive secretion occurs in the vicinity of the soma while regulated secretion is occurs in the distal neuronal processes (253).

Sorting of BDNF into SGs is dependent on the presence of two separate motifs in the pro and mature domains of BDNF. Interaction of the pro domain of BDNF with the sorting receptor, Sortilin, targets BDNF to SGs. Mutations within the Sortilin binding motif in the pro domain results in sorting of BDNF to the CSP and leads to failure in the trafficking of BDNF to synapses, ultimately resulting in aberration in neuronal function and memory (247, 255, 259). Additionally, interaction of the mature domain with the sorting receptor carboxipeptidase E (CPE) also guides BDNF to SGs and failure to remove the pro domain or perturbation of this interaction results in BDNF sorting to the CSP (260). Thus, the regulation of BDNF biosynthesis and presence of two motifs within BDNF that bind to sorting receptors influences sorting of BDNF to the appropriate secretory pathway.

1.6.4 Anterograde transport of secretory vesicles in neurons

The trajectories and intracellular distribution of different secretory vesicles are regulated by specific transport motor proteins. Vesicular transport in neurons is primarily dependent on microtubule-based molecular motors and, in axons and dendrites, microtubules are orientated with the microtubule plus end facing towards neurite terminals. The specificity of microtubule-based anterograde transport is mediated by kinesin motors that preferentially transport cargo towards the microtubule plus ends (261). Secretory vesicles are transported in an anterograde direction through interaction of kinesin motor proteins with vesicle-specific accessory molecules, such as coated proteins, scaffolds, adaptors, GTPases or transmembrane proteins (262). For example, the cytoplasmic region of the transmembrane

protein CPE interacts with the adaptor dynactin, which subsequently recruits and binds kinesins such as Kif1A for SG anterograde transport (263-267). Interestingly, unused SGs are returned to the cell body for recycling or degradation (265) and this retrograde transport is mediated by the transport motor dynein that preferentially travels towards the minus ends of microtubules, and dynactin also serves as the adaptor that bridges SGs to dynein (265, 266). Overall, the specificity of polarity transport is dependent on the interaction of secretory vesicle-specific accessory proteins that couples the appropriate transport motors for intracellular transport.

1.7 Statement of Hypothesis and Objectives

Trafficking of secreted proteins is controlled by regulatory steps that include PTMs and transient protein–protein interactions. Hh proteins undergo complex PTMs, including cleavage and lipidation that impacts Hh biological activity. Although the molecular aspects of nonneuronal-derived Shh has been well-researched for the past 15 years, little is known about Shh production, trafficking and release in neurons. Moreover, we do not adequately understand how the highly specialized morphology and physiology of neurons contributes to the secretion and range of Shh activity. Therefore, my overall objective is to address how the specialization of these cells contributes to the trafficking, secretion and reception of Shh. To address this objective, I hypothesize that Shh intraneuronal trafficking is dependent on PTMs and requires specific protein interactions for intra- and interneuronal transport. To test this hypothesis, I have set the following aims:

1. Determine whether PTMs are necessary for transport, localization and secretion in neuronal cells

2. Identify the vesicular pathway that is involved in Shh intracellular trafficking in neurons
3. Identify novel Shh-interacting proteins involved in CNS development

Understanding how Shh is regulated at the cellular level is critical in advancing our knowledge of how Shh influences CNS development. Overall, these objectives will identify the molecular regulation of Shh intracellular trafficking.

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2. TRAFFICKING OF THE SONIC HEDGEHOG MORPHOGEN TO THE REGULATED SECRETORY PATHWAY IN MAMMALIAN NEURONS

2.1 Abstract

In the developing nervous system, neuronal secretion of Sonic hedgehog (Shh) is important for progenitor proliferation, cell fate diversification and axon guidance. In some instances, these cellular processes are mediated by Shh secretion from different subcellular compartments in neurons. However, little is known about the regulation of Shh transport and secretion in neurons. To study this process, I compared the subcellular distribution of wild-type and mutant Shh isoforms in differentiated rat pheochromocytoma cells and primary hippocampal neurons by fluorescence microscopy and biochemical fractionation. In both neuron types, wild-type Shh exhibited an intra- and extracellular punctate distribution pattern along the length of the neurite and at the neurite terminal. Shh⁺ puncta exhibited bidirectional movement with fast and slow kinetics and colocalized with markers of synaptic vesicles (SVs) and dense core granules. Shh posttranslational modifications were required for sorting to SVs and cell surface association. Finally, consistent with its association with regulated secretory vesicles, Shh secretion could be induced under depolarizing conditions. Taken together, these observations suggest that long-range Shh transport and signalling in neurons involves trafficking into the regulated secretory pathway and cell surface accumulation of Shh on axons and suggests a link between neuronal activity and Shh release.

2.2 Introduction

Sonic hedgehog (Shh) is an essential morphogen that regulates patterning and growth in diverse developmental processes including formation of the central nervous system (CNS)

(1, 2). In the CNS, neurons are a major source of Shh and there is emerging evidence that Shh is transported from the cell body along axons and is released during transport and at the nerve terminals to modulate cellular functions. While several factors that influence Shh secretion and long-range transport in non-neural cells have been described, our current understanding of the mechanism of long-range transport and secretion of Shh in neurons is limited.

Shh is translated as a full-length precursor protein that undergoes autoproteolysis that is catalyzed by residues in the carboxy terminus to generate an amino terminal fragment (Shh-N) and a carboxy terminal fragment (Shh-C). To date, all of the biological activity ascribed to Shh is mediated by the Shh-N fragment. Shh-N is further processed by cleavage of its endoplasmic reticulum (ER) signal sequence and two lipid modifications: cholesteroylation at the carboxy terminus and *N*-palmitoylation on an N-terminal cysteine to generate the fully processed protein, ShhNp (3, 4). The lipid modifications are necessary for Shh association with lipid rafts, membrane tethering, multimerization and long-range signalling (3-9).

Hh functions as a morphogen in many systems by forming an extracellular concentration gradient that directly regulates cellular functions at sites distant from Hh-expressing cells (10, 11). Intracellular transport of Hh from the cell body to axons represents another potential mechanism of long-range transport, which does not appear to result in the formation of a Shh gradient (12, 13). In flies and mammals Hh proteins are transported in an anterograde direction from their site of production in the neuron cell body to axons and nerve terminal and release of Hh from these subcellular compartment is critical for normal development in the brain and eye (12-18). The mechanism of long-range Shh transport in mammalian neurons has not been elucidated; however, it may involve sorting of Shh to

specialized secretory vesicles, as secretory cargo that is trafficked by the constitutive secretory pathway is usually retained within the cell body (19).

As Shh expression is widespread throughout the nervous system and it is likely that axonal transport of Shh is prevalent in the CNS (14, 20-22), understanding the mechanisms of Shh trafficking will provide insights into Shh function in the developing and adult CNS. Here I investigated the role of Shh posttranslational modification (PTM) on Shh distribution and trafficking in mammalian neurons.

2.3 Materials and Methods

All materials and methods are listed in more detail in Appendix II.

2.3.1 DNA constructs

The ShhN, ^{C25S}Shh, ^{C25S}ShhN, ^{C199A}Shh and ^{C25SC199A}Shh isoforms were generated by site-directed mutagenesis of mouse Shh cDNA (Genbank Accession NM_009170; Table 2.1).

Incorporation of yellow fluorescent protein (YFP) into Shh constructs was as follows: a NheI restriction enzyme site was introduced between the codons for amino acids 196 and 197 of mouse Shh to generate Shh(NheI). YFP was amplified by PCR from pEYFP-C1 (Clontech) with primers containing flanking SpeI restriction sites and was inserted into the NheI site of Shh(NheI), resulting in placement of the tag three amino acids upstream from the cleavage site (Table 2.1). ShhC::CFP encodes a carboxy-terminal mouse Shh protein tagged with cyan fluorescent protein (CFP). ShhC::CFP was created by PCR amplification of the cDNA sequence of mouse Shh corresponding to amino acids 199-437, which was then cloned in frame with ECFP (Clontech) to generate a fusion protein with a carboxy-terminal tag (Table 2.1). Nucleotide sequence corresponding to the mouse Shh signal peptide (1-24 aa) was

Table 2.1. Schematic of Shh constructs used in Chapter 2.

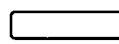
Shh isoform	Predicted processed form	
ShhNp	Wild-type	
Shh::YFP	Wild type and YFP tag	
ShhN	Cholesterol deficient	
ShhN::YFP	Cholesterol deficient and YFP tag	
C25S Shh	Palmitate deficient	
C25S Shh::YFP	Palmitate deficient and YFP tag	
C25S ShhN	No lipids	
C25S ShhN::YFP	No lipid and YFP tag	
C199A Shh	Uncleaved	
C199A Shh::YFP	Uncleaved and YFP tag	
C25SC199A Shh	Unprocessed	
C25SC199A Shh::YFP	Unprocessed and YFP tag	
ShhC::CFP	ShhC and CFP tag	

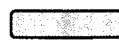
 Amino domain of Shh (Shh-N)

 Carboxy domain of Shh (Shh-C)

 Palmitate

 Cholesterol

 Yellow fluorescent protein (YFP)

 Cyan fluorescent protein (CFP)

cloned upstream of the ShhC::CFP construct to create SPShhC::CFP (Table 2.1). All Shh constructs were subcloned into the pcNDA3 expression vector and verified by sequencing.

Rab3a-EGFP was a kind gift from Miguel Seabra (Imperial College London, London, UK.). tPA-ECFP was a kind gift from Bethe Scalettar (Lewis and Clark College, Portland, OR). Syp-YFP and TfR-YFP were a kind gift from Flavia Valtorta (San Raffaele Scientific Institute, Milan, Italy).

2.3.2 Cell culture

PC6 cells were obtained from Randall Pittman (University of Pennsylvania, Philadelphia, PA) and were cultured in RPMI-1640 (Invitrogen) supplemented with 5% foetal bovine serum (FBS), 10% horse serum (HS) and 2 mM L-glutamine. Stable transfectants expressing Shh morphants were selected over 2 weeks in medium containing 400 µg/mL geneticin (Invitrogen). Clones were isolated and screened for low expression levels by immunoblotting. Neuronal differentiation was induced by plating cells on collagen-coated plates in RPMI supplemented with 0.75% FBS, 1.5% HS and 50 µg/mL NGF-2S (Millipore). Differentiated PC6 cells were transfected with Lipofectamine 2000 (Invitrogen), as per the manufacturer's instructions. In most experiments, cells were treated with 30 µg/mL cycloheximide for 20-30 minutes prior to imaging to halt *de novo* protein synthesis. To mark endosomes, PC6 cells were incubated with 3 µg/mL transferrin conjugated to AlexaFluor 555 (Invitrogen) for 20 min at 37°C.

2.3.3 Hippocampal neuron cultures

Primary cultures of rat hippocampal neurons were prepared by trypsinization (0.25% trypsin for 15 min) of dissected postnatal age 1 hippocampi, followed by plating in

Neurobasal/B27 (Invitrogen) on Poly-D-Lysine (PDL)-coated coverslips. After 24 hours, 3 μ M β -D-arabinofuranosylcytosine was added to inhibit glial cell growth. Fourteen day *in vitro* (DIV) primary hippocampal neurons were transfected using the calcium phosphate method as described previously (23).

2.3.4 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, followed by blocking with 25% goat serum in TBLS (50 mM Tris-HCl pH 7.4, 10 mM lysine, 145 mM NaCl and 1% BSA), and incubated in primary and AlexaFluor conjugated secondary antibodies. α -GFP (1:500 polyclonal), α -Shh-N (5E1 monoclonal, 1:20, DSHB) or α -SV2 (SV2 monoclonal, 1:100, DSHB) antibodies or FITC-conjugated cholera toxin B subunit (CTX-FITC, 3 μ g/mL, Sigma) were incubated with cells for 1 hour at RT followed by washing and incubation with AlexaFluor-coupled α -rabbit or α -mouse secondary antibodies (1:500; Invitrogen) at RT for 30 min.

2.3.5 Fluorescence imaging

Immunofluorescence images were acquired on a Zeiss Axiovert 200 equipped with 100X 1.4 NA PlanApochromat and 40X 0.95 NA PlanApochromat objectives using the Apotome module or a Zeiss LSM510 META confocal microscope using a 63X PlanApochromat objective. Exposure times and gain values were chosen such that saturation was avoided. Processing of images was performed by ImageJ (24). Quantification of fluorescence intensity was calculated with ImageJ software by determining the ratio of fluorescence density occupied at neurite terminals over fluorescence density along the entire length of the neurite using maximum intensity projection images from serial Z-stacks.

Colocalization of Shh and organelle markers was calculated using ImageJ plugins Colocalization Threshold to obtain the percentage of colocalizing pixels and Colocalization Highlighter (threshold settings = 40) was used to highlight overlapping pixels.

2.3.6 Lipid raft fractionation

Detergent-resistant membranes were prepared from differentiated PC6 cells stably expressing Shh. Cells were washed with TNE (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) and lysed in TNE containing 1% Triton X-100 and protease inhibitors (Roche). An equal volume of 80% sucrose in TNE was added to the lysate, layered on the bottom of a SW40 tube, overlayed with 30% sucrose and 5% sucrose in TNE, and centrifuged at 240,000 x g for 20 hours. Fractions were collected from the top and analysed by immunoblotting.

Detergent-free lipid raft association was performed as described (25). Differentiated PC6 cells stably expressing Shh were washed with PBS and dounce homogenized in base buffer (20 mM Tris-HCl (pH 7.8), 250 mM sucrose) supplemented with 1 mM CaCl_2 , 1 mM MgCl_2 and protease inhibitors (Roche). A postnuclear supernatant was mixed with an equal volume of base buffer containing 50% OptiPrep and placed in a SW40 tube, overlayed with 20%, 15%, 10%, 5% and 0% OptiPrep in base buffer, and centrifuged at 21,000 rpm for 90 min. Equal fractions were collected from the top and protein distribution analysed by immunoblotting.

2.3.7 Subcellular fractionation

PC6 cells stably expressing Shh isoforms were differentiated for 7-10 days and processed for sucrose or glycerol gradients. For sucrose gradients, cells were washed with PBS, scraped and dounce homogenized in 0.32 M Sucrose, 10 mM HEPES-KOH, pH 7.4, 5

mM EDTA and protease inhibitors (Roche). The homogenate was centrifuged at 1,000 x g for 10 min and the supernatant was layered on a continuous 0.6-1.8 M sucrose gradient prepared with 10 mM HEPES-KOH, pH 7.4 and 5 mM EDTA, and centrifuged at 40,000 rpm for 3 hours in a SW40 rotor. Equal fractions were collected from the bottom and analysed by immunoblotting. For glycerol gradients, cells were washed twice with PBS, scraped and dounce homogenized in 10 mM HEPES-KOH, pH 7.4, 0.15 M NaCl, 5 mM EDTA and protease inhibitors (Roche). The homogenate was centrifuged at 1,000 x g for 10 min and the supernatant was layered onto a continuous 5-25% glycerol gradient prepared in 10 mM HEPES-KOH, pH 7.4, 0.15 M NaCl and 5 mM EDTA, and centrifuged at 55,000 rpm for 45 min in a SW55 rotor. Equal fractions were collected from the top and analysed by immunoblotting

2.3.8 Cell surface labelling

Cell surface proteins on differentiated PC6 cells stably expressing Shh isoforms were labelled with the membrane-impermeant biotinylation reagent, Sulfo-NHS-SS-Biotin. PC6 cells washed with PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-Ca+Mg) and incubated with 1 mg/mL Sulfo-NHS-SS-biotin (Pierce) in PBS-Ca+Mg for 30 min at 4°C. Biotinylation was quenched by washing with PBS containing 100 mM glycine and cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (Roche). Biotinylated proteins were precipitated from clarified cell lysates with Ultralink NeutrAvidin beads (Pierce) overnight at 4°C and the beads were washed with 50 mM Tris-HCl (pH 7.5), 0.1% NP-40, 0.05% SDS and 500 mM NaCl. Bound proteins were eluted by addition of 2X SDS gel sample buffer (20% glycerol, 125 mM Tris-HCl, pH 6.8, 4% SDS, 0.01 mg/mL

bromophenol blue, 10% β -mercaptoethanol). Following western blotting, Shh immunoreactive bands were scanned and the band intensity was quantified using NIH ImageJ. Data is represented as the relative proportion of Shh protein present in the biotinylated fraction compared to the total lysate.

2.3.9 Western blotting

Aliquots of cell lysates and supernatants were subjected to 10% SDS-PAGE and transferred to HybondC Extra membranes (GE Healthcare) using standard protocols (26). Membranes were incubated overnight at 4°C with rabbit α -Shh (1:1000; Santa Cruz), mouse α - β -actin (1:10,000; Sigma), mouse α -TfR (1:1000, Zymed), mouse α -Flotillin1 (1:1000, Santa Cruz), α -tubulin (E7 monoclonal, 1:100, DSHB), mouse α -p38 (1:1000, Santa Cruz) or mouse α -CgB (1:1000, Santa Cruz) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20. After incubation with secondary antibodies (HRP-conjugated IgG; 1:10,000; Sigma), protein bands were detected using an ECL detection kit according to the manufacturer's instructions (GE Healthcare).

2.3.10 Analysis of induced secretion

Differentiated PC6 cells stably expressing Shh isoforms were washed with basal solution (5.6 mM KCl, 145 mM NaCl, 2.2 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose, 15 mM HEPES-KOH, pH 7.4 and 0.1 mg/mL BSA) and incubated for 20 min at 37°C with either the basal solution or stimulating solution (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose, 15 mM HEPES-KOH, pH 7.4 and 0.1 mg/mL BSA). Cell culture supernatants were collected, centrifuged for 5 min at 1,000 x g, and processed for ELISA for Shh-N according to the manufacturer's instructions (R&D Systems).

2.4 Results

2.4.1 Subcellular localization of Shh in neurons

The goal of this study was to characterize the subcellular distribution of wild-type and mutant forms of Shh in mammalian neurons. I was unable to detect endogenous Shh in cultured mammalian retinal ganglion cells (RGCs) using currently available anti-Hh reagents. To circumvent this problem, I used an exogenous expression approach by transfecting expression vectors coding for Shh isoforms (Table 2.1) into primary rat hippocampal neurons and PC6 cells, a subline of the rat PC12 pheochromocytoma cell line that exhibits reduced aggregation upon NGF-induced neuronal differentiation (27). PC6 cells do not express Shh; however, they do express *Hhat* and *Disp1*, which are required for posttranslational modification and secretion of Hh proteins (Figure 2.1). Because neurite outgrowth could potentially result in passive redistribution of Shh protein, initial characterization of Shh distribution was performed in transiently transfected differentiated neurons. Finally, to control for potential sorting artefacts caused by protein overexpression, only cells that demonstrated distinct intra- and extracellular puncta were scored in our analyses.

Wild-type Shh was distributed in a punctate pattern in the soma and neurite processes in hippocampal neurons (Figure 2.2A). To determine whether Shh distribution is polarized, neurons were cotransfected with Shh and either YFP-tagged transferrin receptor (TfR-YFP), which marks dendrites, or YFP-tagged synaptotagmin (Syp-YFP), which accumulates in axons (28). Surprisingly, Shh distribution is not polarized, as I detected Shh⁺ puncta in both Syp-YFP labelled axons and TfR-YFP positive dendrites in primary hippocampal neurons (Figure 2.2C,D). Titration of the Shh plasmid used for transfection resulted in sorting of Shh

Figure 2.1. PC6 cells express *Hhat* and *Displ*. cDNA isolated from undifferentiated and 10 DIV differentiated PC6 cells were subjected to RT-PCR with primers specific for *Hhat* and *Displ*. PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Controls included RT-PCR on non-reverse transcribed RNA (- MMLV).

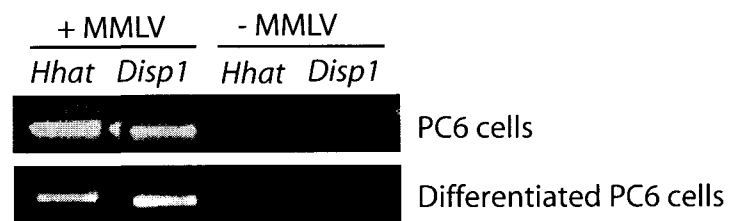
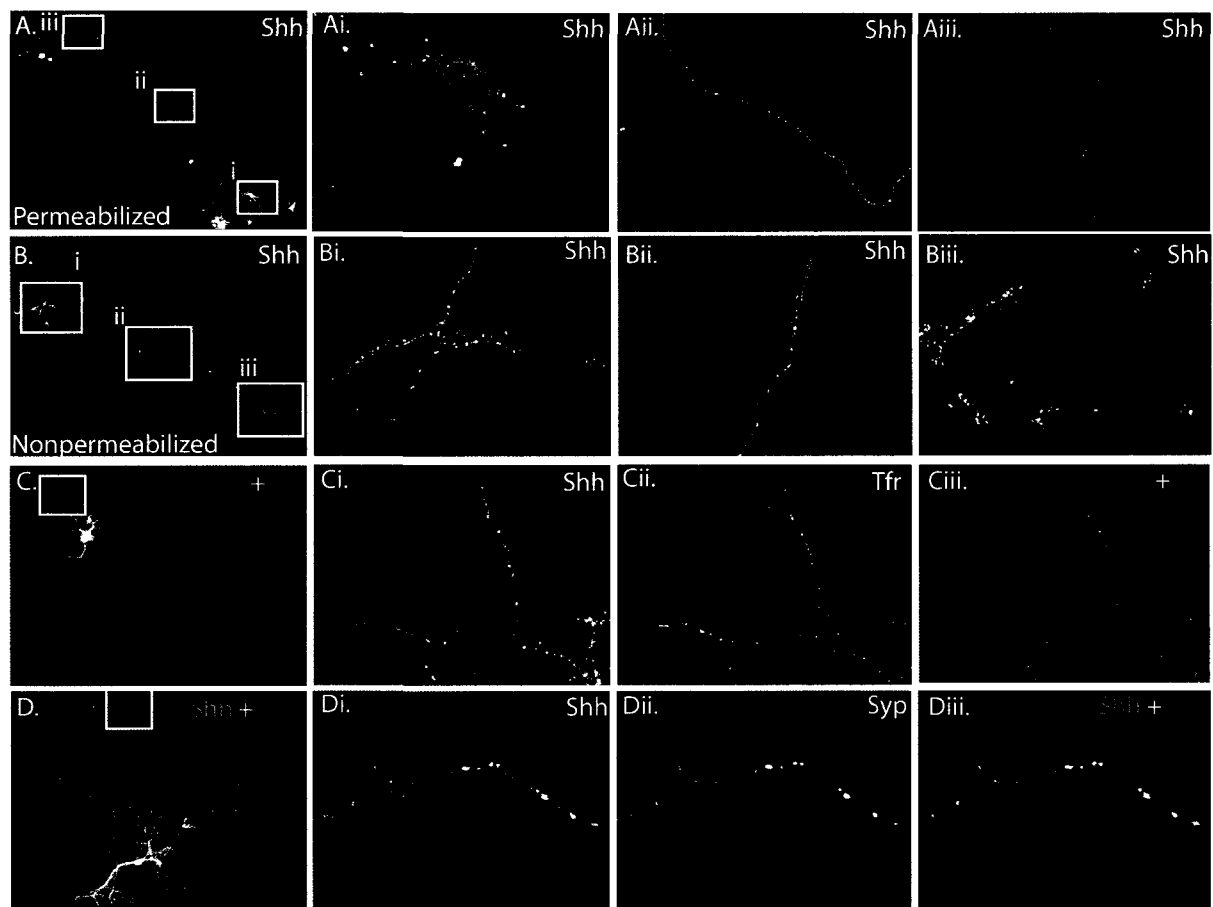


Figure 2.2. Shh is distributed in puncta and on the extracellular surface of neurites in primary hippocampal neurons. (A,B) 14DIV primary hippocampal neurons were transfected with Shh and stained with an anti-Shh-N antibody 24 hours posttransfection under permeabilized (A) and nonpermeabilized (B) fixation conditions. Shh⁺ puncta are visible within neurons and on the extracellular surface of the soma and neurites. Note: for (A) and (B), i, ii and iii are higher magnification images of the boxed areas in the leftmost panel in the same row. (C,D) Shh was cotransfected with YFP-tagged transferrin receptor (TfR-YFP) or YFP-tagged synaptophysin (Syp-YFP) into 14DIV primary neurons and analyzed for neurite polarity using TfR-YFP and Syp-YFP as markers for dendrites and axons, respectively. i, ii, iii are higher magnification images of the indicated transgene in the boxed area from the leftmost panel. White in merged images denotes colocalization between Shh and YFP-tagged proteins.



to both axons and dendrites (data not shown), indicating the lack of Shh polarized sorting is not due to protein overexpression. Moreover our transfection approach results in proper sorting of polarized proteins TfR and Syp, and is, therefore, likely to be revealing normal sorting of Shh protein in neurons. To determine whether Shh was present on the extracellular surface of neuronal processes, I performed immunocytochemistry for Shh in nonpermeabilized cells. In control experiments, I confirmed that cytosolic proteins were undetectable using these fixation conditions (Figure 2.3). Shh was distributed in puncta on the surface of axons and dendrites in hippocampal neurons (Figure 2.2B). Thus, in primary hippocampal neurons, Shh can be transported to both dendrites and axons and accumulates on the cell surface of neuronal processes.

Because of its tractability, I used PC6 cells for more in-depth analysis of Shh trafficking. Similar to hippocampal neurons, Shh was distributed in the soma and neurites of PC6 cells in a punctate pattern (Figure 2.4A). To determine whether Shh protein accumulates preferentially in the soma or neurites, cells were treated with cycloheximide to halt *de novo* protein synthesis and I determined the ratio of fluorescence density in the neuron versus neurites at a distance away from the soma equivalent to one cell length. I observed that $39 \pm 4\%$ (mean $\pm$ SEM) of total Shh is present in neurites one cell diameter from the cell body, demonstrating that Shh can be transported distally to neurites. Similar to primary neurons, Shh was present as punctate structures on the extracellular surface of the cell body and neurites (Figure 2.4B). Overall, the distribution profile of Shh in differentiated PC6 cells is similar to primary hippocampal neurons.

To quantify the relative amount of extracellular Shh in PC6 cells, I performed cell surface biotinylation experiments. Stably transfected differentiated PC6 cells expressing Shh

Figure 2.3. Staining controls for fixation conditions. (A,B) PC6 cells were transfected with cytosolic YFP and stained with anti-GFP antibodies under nonpermeabilized (A) or permeabilized (0.1% Triton X-100) (C) conditions. Note that cytosolic YFP is not detected in nonpermeabilized cells.

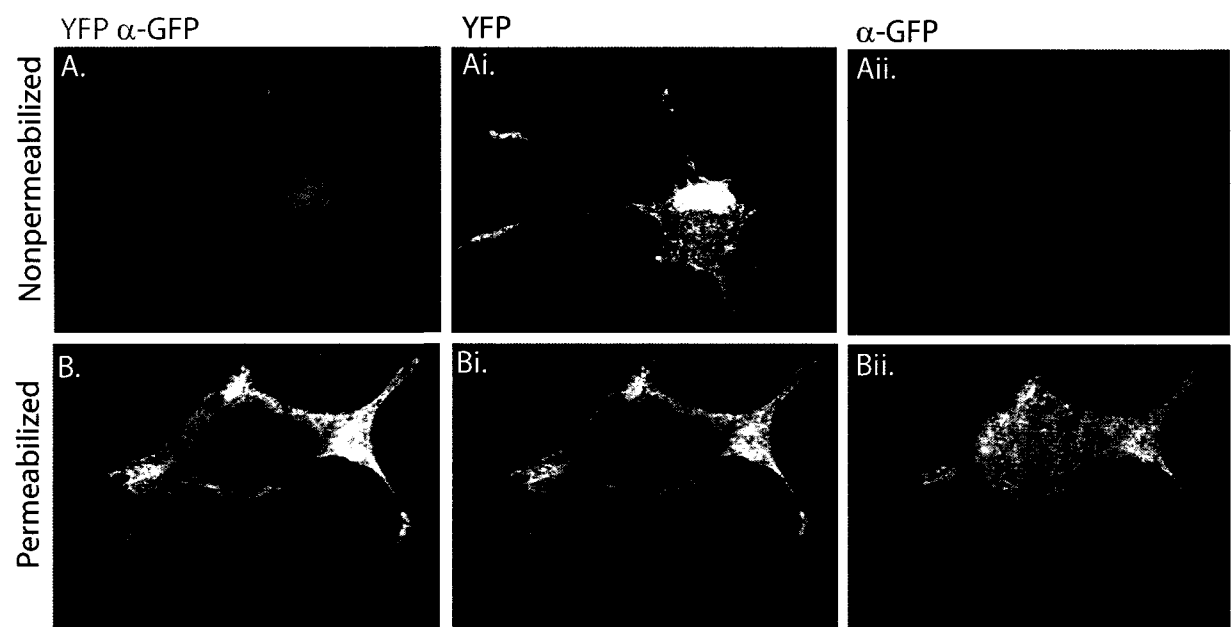
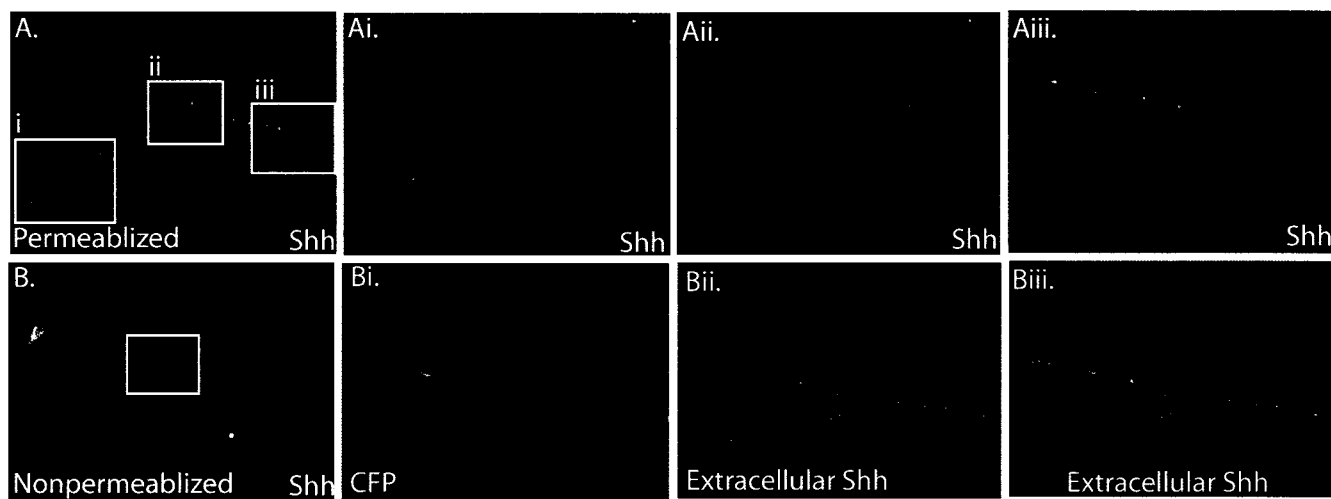


Figure 2.4. Shh distribution in differentiated PC6 cells. (A) 10 DIV differentiated PC6 cells were transfected with wild-type Shh and processed for immunocytochemistry with anti-Shh-N antibodies under permeabilized fixation conditions. Shh immunoreactivity is concentrated in puncta in the soma and neurites. i, ii and iii are higher magnification images of the boxed areas in the leftmost panel in the same row. (B) Cotransfection of wild-type Shh and cytosolic CFP into differentiated PC6 cells and subsequent immunocytochemistry for extracellular Shh under nonpermeabilized conditions reveals the presence of intra- and extracellular Shh⁺ puncta on neurites.



(Shh-PC6) were labelled with membrane-impermeant Sulfo-NHS-SS-Biotin and the biotinylated fractions of the total lysates purified by NeutrAvidin beads analysed by western blotting. Dually lipidated Shh-N (ShhNp) was distributed equally between the intracellular and extracellular pools, indicating that approximately half of the Shh in transfected cells is present at the cell surface (Figure 2.5). Overall, these results demonstrate that wild-type Shh in transfected neurons is trafficked to both axons and dendrites in neurons and approximately half of the total Shh protein is present in a punctate pattern on the cell surface.

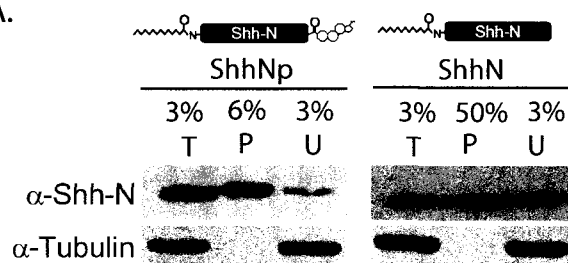
2.4.2 Shh is associated with lipid rafts in neurons

Lipid rafts are low-density membrane domains that are involved in a number of cellular processes such as trafficking and cell signalling (29-31) and Shh fractionates with lipid raft markers in the adult rodent brain and non-neuronal cell lines (4, 6, 8, 14). Moreover, the cell surface Shh accumulation pattern is reminiscent of lipid raft association. To examine if Shh associates with lipid rafts *in vivo*, I transfected primary hippocampal neurons with wild-type Shh and compared the distribution of Shh with cholera toxin B (CTX), which binds to the lipid raft marker ganglioside GM1, in permeabilized and non-permeabilized cells. Intracellular and extracellular Shh pools exhibited extensive colocalization with CTX⁺ lipid rafts in the soma and neurites (Figure 2.6A-D).

Because the anti-Hh antibodies can detect full length and processed ShhNp, the co-localization approach does not allow us to discriminate the form of Shh that is lipid raft associated. Therefore, I used a biochemical method to examine partitioning of ShhNp into detergent resistant membranes (DRM). Consistent with previous reports (4, 6, 8, 14), only a minor fraction of ShhNp was associated with DRMs in Shh-PC6 cells (Figure 2.6E). Due to the potential introduction of artefacts through the use of detergent and incomplete solubility

Figure 2.5. Cell surface presentation of Shh in differentiated PC6 neurons. (A) Western blot analysis of total and surface-biotinylated proteins from PC6 cells stably transfected with wild type Shh (ShhNp) or cholesterol-deficient Shh (ShhN). The indicated percentage of the total lysate (T), biotin pulled down fraction (P) and the residual nonbiotinylated fraction (U) were resolved by SDS-PAGE and immunoblotted with Shh-N and tubulin antibodies. Tubulin serves as a negative control to demonstrate that cell surface biotinylation did not label intracellular proteins. (B) The percentage of extracellular Shh protein of ShhNp, ShhN and uncleaved Shh (^{C199A}Shh), was quantified by densitometry of immunoreactive bands. Data is represented as the percentage of biotinylated Shh compared to total Shh protein (N = 3). Error bars denote standard error of the mean.

A.



B.

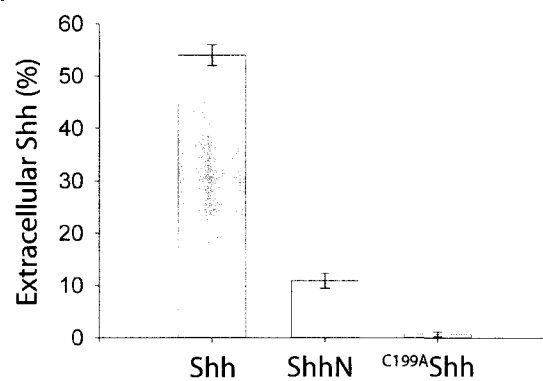
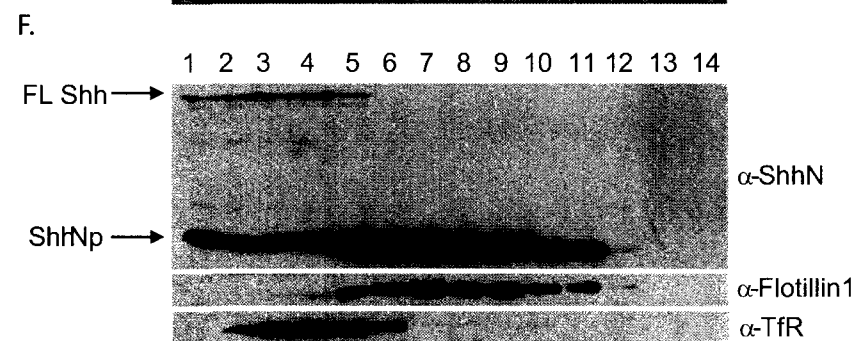
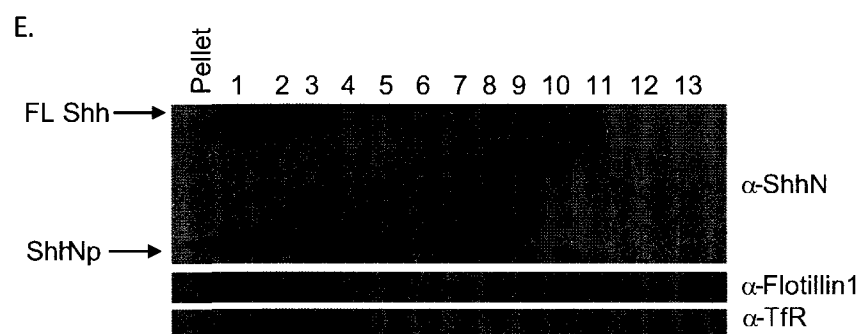
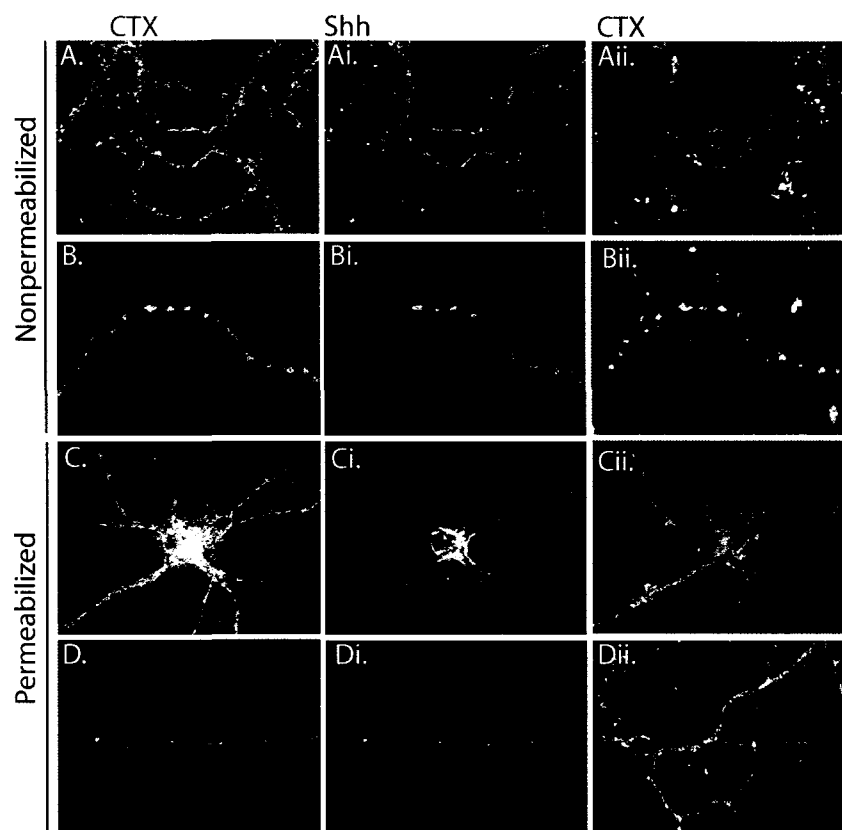


Figure 2.6. Shh associates with lipid rafts markers in hippocampal neurons and differentiated PC6 cells. (A-D) 14 DIV primary hippocampal neurons were transfected with Shh, fixed with 4% paraformaldehyde and double-labelled for Shh using an anti-Shh-N antibody and FITC-conjugated cholera toxin B subunit (CTX) under nonpermeabilized (A, B) and permeabilized (C, D) conditions. White in merged images denotes colocalized Shh and CTX pixels. (E) Triton X-100 solubilised cell lysates from stable Shh-PC6 cells were fractionated by a discontinuous sucrose gradient, individual fractions were resolved by SDS-PAGE. Membranes were blotted with antibodies specific for Shh-N, Flotillin1, which marks detergent resistant membrane (DRM) fractions, and TfR, a non-raft marker. A portion of fully processed Shh (ShhNp) cofractionates with Flotillin1. Unprocessed, full-length Shh (FL Shh) is not associated with the DRM fraction. (F) Lipid raft association of Shh was also analysed using a non-detergent method, as described in Materials and Methods. ShhNp predominantly associates with a Flotillin1 and FL Shh is restricted to the non-lipid raft fractions.



with Triton X-100, I also used a detergent-free method to fractionate lipid rafts (25). Using this technique, ShhNp was primarily associated with Flotillin1⁺ fractions (Figure 2.6F), indicating that ShhNp, but not incompletely processed Shh, is associated with lipid rafts. Overall, these results indicate that ShhNp is associated with lipid rafts on the cell surface and within the secretory pathway in PC6 and primary hippocampal neurons.

2.4.3 Shh is transported through both fast and slow mechanisms

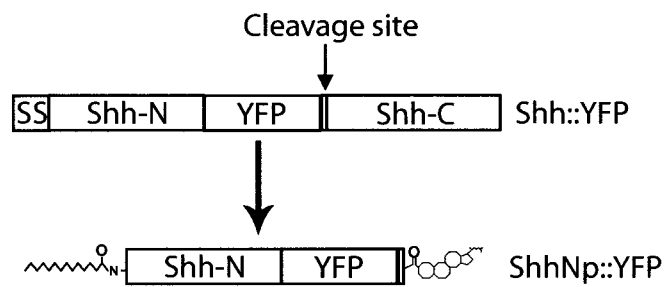
To gain insight into the mechanism of Shh movement in neurons, I used particle tracking software to measure the rate of Shh::YFP movement in differentiated PC6 cells. Consistent with previous reports (32, 33), I confirmed that placement of YFP proximal to the cleavage site does not significantly interfere with sorting, cleavage, activity and extracellular transport (Figure 2.7 and data not shown). YFP⁺ puncta in PC6 neurites moved bidirectionally with fast and slow kinetics in the range of the previously published rates for fast and slow axonal transport in PC12 cells (Table 2.2; 34). The majority of fast movement occurred in the anterograde direction, while there was no apparent directionality for slow transport. The fast axonal transport is consistent with the evidence that axonal Shh is actively transported *in vivo* (13, 14). While I cannot rule out the possibility that Shh::YFP movement also occurs on the cell surface, fast transport rates of Shh::YFP are consistent with the kinetics of microtubule-based transport (35).

2.4.4 Posttranslational modifications are necessary for plasma membrane association and subcellular distribution of Shh

To investigate the requirement for Shh PTMs for neuronal trafficking, I cotransfected cytosolic CFP with YFP-tagged Shh isoforms corresponding to uncleaved or lipid-deficient

Figure 2.7. Inclusion of YFP does not affect Shh processing or subcellular distribution. (A) Schematic of Shh::YFP showing the location of the YFP epitope tag proximal to the autocleavage site in Shh. Fully processed Shh (ShhNp) contains palmitic acid at the N-terminal and cholesterol at the C-terminal of Shh-N. ShhN is cleaved Shh without lipid modifications. (B) Untagged and YFP-tagged Shh were transiently expressed in COS7 cells and cell lysates were analysed 48 hours after transfection by western blotting with α -Shh-N and α - β -actin antibodies. (C) YFP-tagged wild-type Shh has a similar distribution pattern as untagged Shh in differentiated PC6 cells. i, ii, iii are higher magnification images of the indicated transgene in the boxed area from the leftmost panel.

A.



B.

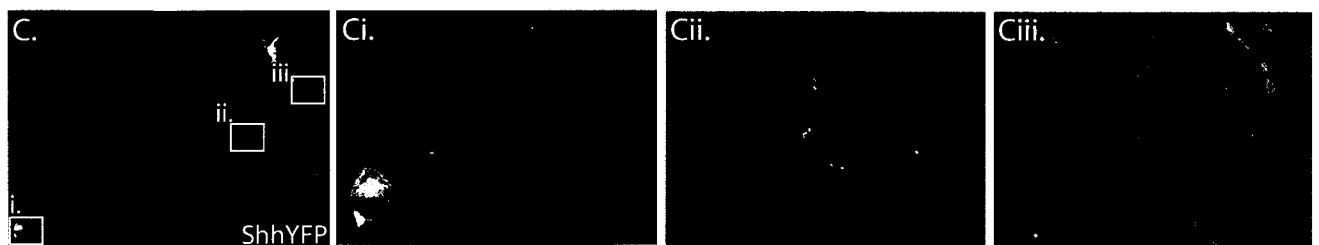
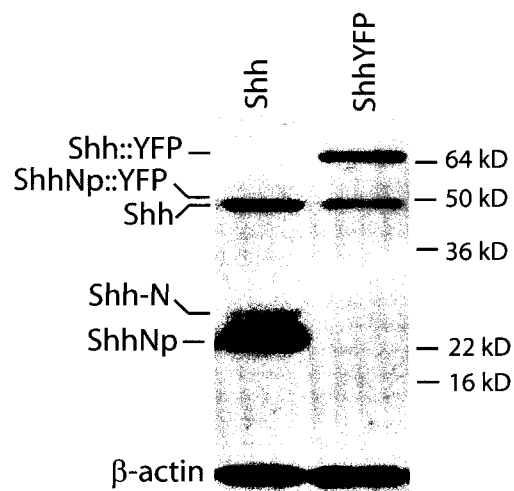


Table 2.2. Transport of ShhYFP⁺ puncta in PC6 neurites.

	Velocity (μm/sec)*	
	Fast	Slow
Anterograde	1.28 ± 0.55 (0.74-1.37)	0.047 ± 0.037 (0.011-0.047)
Retrograde	0.85 ± 0.10 (0.68-1.01)	0.17 ± 0.056 (0.0039-0.22)

*Values represent mean ± SEM.

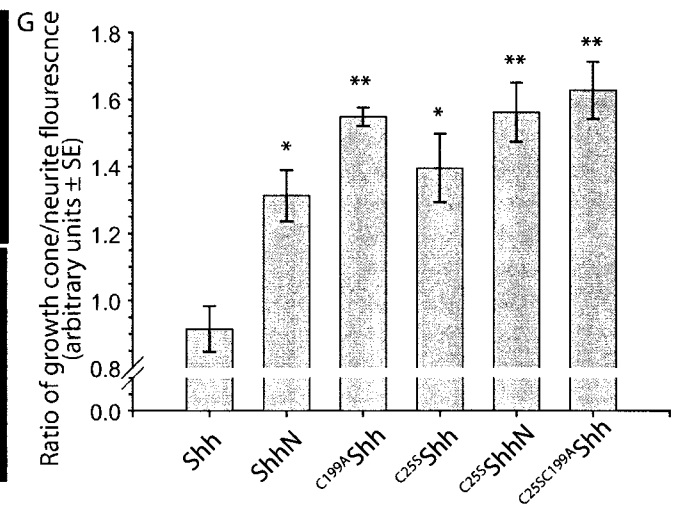
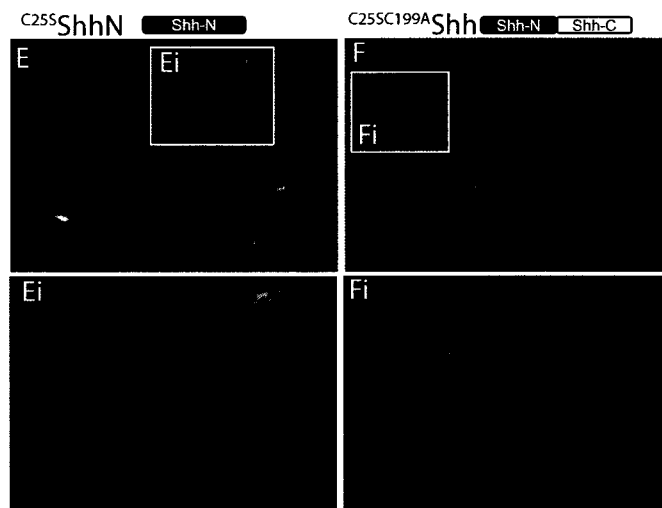
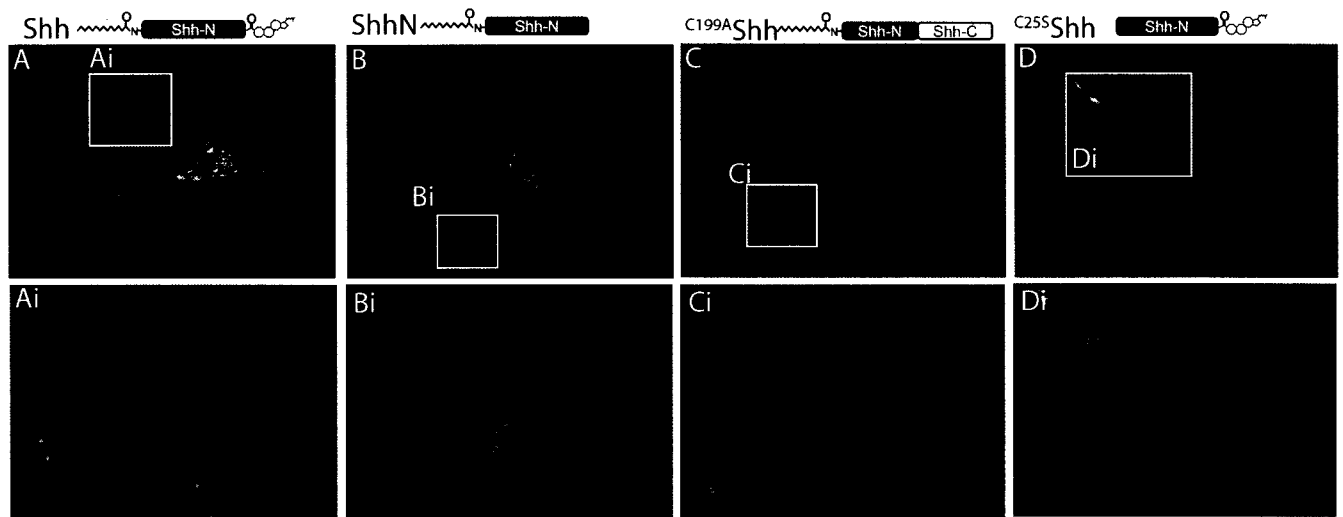
Shh into differentiated PC6 cells (Table 2.1). Compared with wild-type Shh, mutations that inhibited proteolysis or lipid modification of Shh resulted in accumulation of Shh protein at the neurite terminals (Figure 2.8A-F). This observation was quantified by calculating of the ratio of fluorescence density in the neurite terminals over the length of the neurite (Figure 2.8G). Untagged Shh isoforms also accumulated in the neurite terminal in differentiated PC6 cells (data not shown), indicating that the YFP tag is not the cause of altered distribution of Shh isoforms. Moreover, cholesterol-deficient and uncleaved Shh isoforms were localized to the somatodendritic region in transfected primary hippocampal neurons (data not shown).

In other cell types, lipid modifications to the amino domain of Shh are necessary for tethering of Shh protein to the plasma membrane (3, 6). Compared with wild-type Shh, cell surface staining of cholesterol deficient or uncleaved Shh was reduced in differentiated PC6 cells and primary hippocampal neurons (data not shown). These findings were also corroborated by quantification of surface biotinylated mutant Shh isoforms in stable PC6 cells. A minor portion of cholesterol-deficient ShhN was recovered in the biotinylated surface fraction, whereas uncleaved Shh was undetectable on the cell surface (Figure 2.5). These results show that PTMs are required for distribution and presentation of Shh on the extracellular surface in neurons.

2.4.5 Shh isoforms are associated with regulated secretory vesicles

I investigated the identity of Shh⁺ vesicles by performing colocalization experiments with known organelle markers in hippocampal neurons and PC6 cells. Remarkably, Shh overlapped with Syp-YFP, a marker of synaptic vesicles (SVs), in primary hippocampal neurons (Figure 2.2D), indicating that Shh may be sorted to the regulated secretory pathway. To further investigate this possibility, I compared the distribution of Shh with Rab3A, a

Figure 2.8. Perturbation of posttranslational modification results in accumulation of Shh isoforms in PC6 neurite terminals. (A-F) PC6 neurons were cotransfected with cytosolic CFP (red) and YFP-tagged Shh isoforms (green) and analyzed 24 hours posttransfection. Perturbation of Shh posttranslational modification, including cholesterol-deficient (B), uncleaved (C), palmitate-deficient (D), cholesterol- and palmitate-deficient (E) and delipidated, uncleaved (F) Shh results in an accumulation of Shh⁺ puncta at the nerve terminals. (G) Differentiated PC6 cells were transfected with YFP-tagged Shh isoforms and visualized using the Apotome function on a Zeiss epifluorescence microscope. YFP fluorescence along the length of the neurite and at the neurite terminals of maximum intensity projections of serial Z-stack images was obtained with ImageJ, and the proportion of fluorescence between the neurite terminals and neurite was calculated. Statistical analysis was performed with 1-way ANOVA and differences calculated with the Tukey post hoc test (* $p < 0.05$, ** $p < 0.01$). Error bars denote standard error of the mean.



GTPase required for regulated vesicle exocytosis (36). Cotransfection of Shh and GFP-tagged Rab3A (Rab3A-GFP) revealed a significant overlap of wild-type Shh⁺ puncta and Rab3A-GFP⁺ organelles (Figure 2.9A and Table 2.3). Surprisingly, Shh isoforms that were deficient in posttranslational modification were also preferentially associated with Rab3A-GFP⁺ puncta (Figures 2.9B and 10, Table 2.3). In PC12 cells, there are two classes of regulated secretory vesicles, dense core granules (DCGs) and synaptic-like microvesicles (SLMVs). DCGs are a specialized class of secretory vesicles that undergo exocytosis in response to a secretagogue (37). Only a fraction of wild-type Shh⁺ puncta overlapped with the DCG marker, YFP-tagged tissue plasminogen activator (tPA-YFP) in differentiated PC6 cells and primary hippocampal neurons (Figures 2.9C,D and 11, Table 2.3). In contrast, the majority of mutant Shh⁺ puncta exhibited extensive overlap with tPA in both differentiated PC6 cells and primary hippocampal neurons (Figures 2.11, Table 2.3, data not shown), which suggest that enrichment of these mutants at the neurite terminus in differentiated PC6 cells reflects their accumulation in DCGs. SLMVs are the equivalent of SVs in CNS neurons which mediate neurotransmitter release at synapses. I observed that wild-type, but not mutant, Shh⁺ puncta colocalized with the SLMV marker, SV2 (Figure 2.9E, Table 2.3).

To further investigate Shh trafficking to the regulated secretory pathway I used a biochemical fractionation approach to compare Shh and organelle marker distribution in stably transfected PC6 cells expressing wild-type Shh or ShhN. Sucrose density fractionation revealed that ShhNp cofractionated with the chromogranin B⁺ (CgB) DCG and synaptophysin⁺ (p38) SLMV fractions, whereas ShhN only cofractionated with DCGs (Figure 2.12A,B). While the sucrose gradient procedure separates heavier (DCG) from lighter (SLMV) fractions, other light membranes such as endosomes and plasmalemma also cofractionate with SLMVs (28). To determine if wild-type Shh associates with SLMVs, I

Figure 2.9. Shh is associated with regulated secretory vesicles in PC6 neurons. (A-E) Colocalization of Shh-N (green) and organelle markers (red) in PC6 cells. Differentiated PC6 cells were cotransfected with Shh (A, C, E) or cholesterol-deficient Shh (ShhN) (B, D) and Rab3A-GFP (A, B), tPA-YFP (C, D) and processed for immunohistochemistry with an anti-Shh-N antibody. (E) Shh-expressing cells were immunostained with anti-SV2 antibodies. Large panels are maximum intensity projected images of serial Z-stacks of merged images, (i) Shh, (ii) organelle marker and (iii) scatter plot of Shh isoforms and organelle marker. Lower panels are higher magnifications images in a single focal layer of the boxed regions in the upper panel. White in merged images denotes colocalization of Shh and organelle marker pixels using the ImageJ plug-in Colocalization Highlighter with threshold values set at 40.

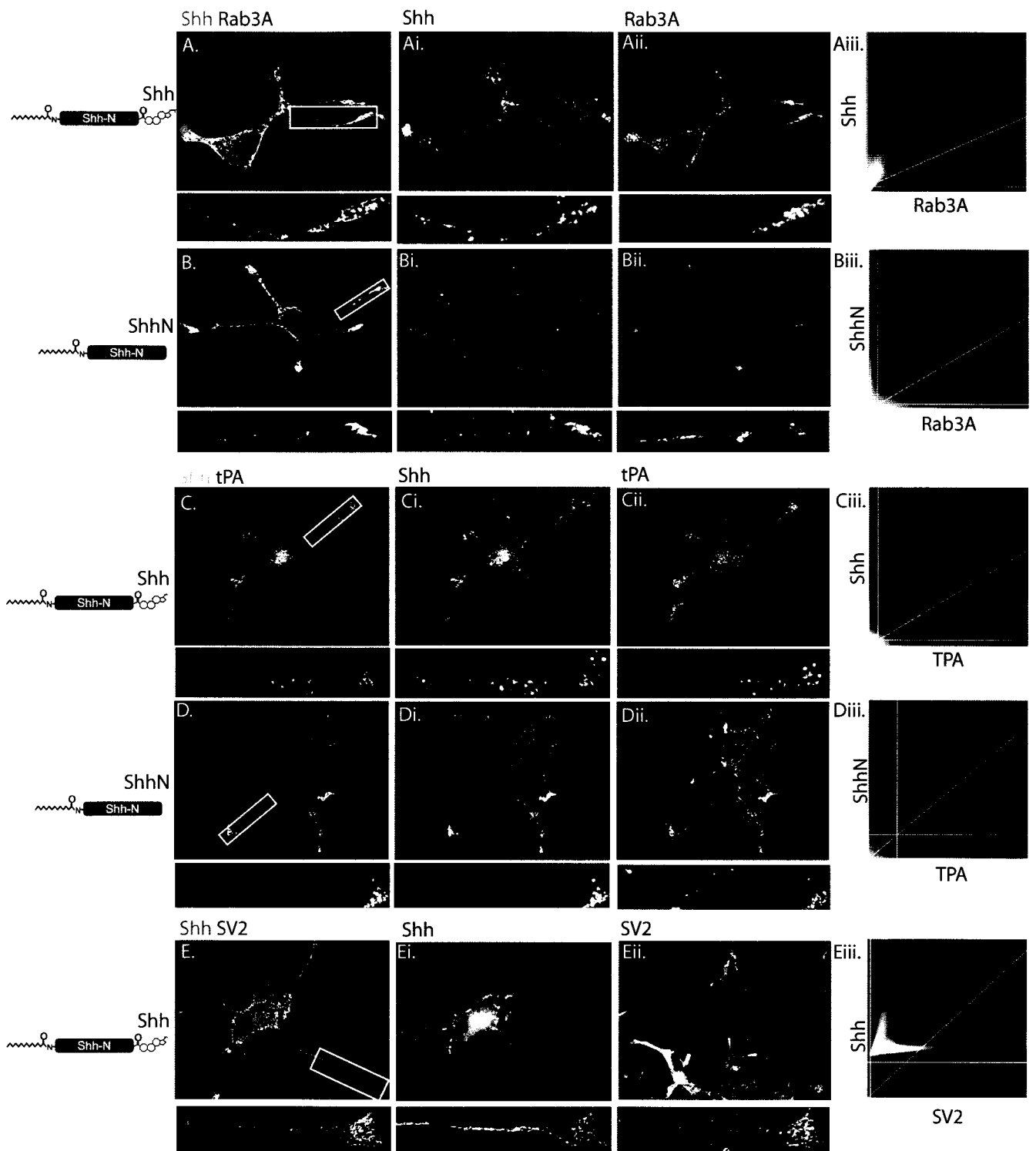


Table 2.3. Summary of Shh and organelle colocalization in differentiated PC6 neurons.

Shh isoform*	Predicted processed form	Rab3A colocalization (%)	tPA colocalization (%)	SV2 colocalization (%)	Tf555 colocalization (%)
Shh	Wild-type	37 ± 6 (~74)	11 ± 5 (~22)	18 ± 6 (~36)	3 ± 1 (~6) 1 ± 1 [#] (~2)
ShhN	Cholesterol deficient	67 ± 10 (~79)	63 ± 3 (~74)	4 ± 2 (~5)	3 ± 2 (~4)
C25S Shh	Palmitate deficient	76 ± 3	82 ± 6	N/A	8 ± 2
C25S ShhN	No lipids	80 ± 5	81 ± 4	N/A	8 ± 1
C199A Shh	Uncleaved	83 ± 4	85 ± 6	N/A	11 ± 4
C25SC199A Shh	Uncleaved and palmitate deficient	85 ± 3	89 ± 5	N/A	9 ± 4

* Shh and organelle marker constructs were cotransfected in differentiated PC6 cells. Cells were stained with anti-Hh-N antibodies and the colocalization of Shh with Rab3a-GFP, tPA-YFP, SV2 or Alexa555 conjugated-transferrin (Tf555) was quantified using Image J analysis as described in Methods. Values are reported as mean ± standard error. Numbers in parentheses are colocalization values that have been adjusted for the total intracellular Shh pool, as determined by quantitative analysis of total and surface biotinylated Shh protein in Shh-transfected PC6 cells. Approximately ~50% of wild-type Shh, 90% of ShhN and 100% of C199A Shh is intracellular)

[#] denotes values identified from primary hippocampal neurons. N/A: not available.

Figure 2.10. Mutated Shh isoforms colocalize with regulated secretory vesicles. (A-D) Differentiated PC6 cells were cotransfected with the indicated Shh isoforms and Rab3A-GFP, and 24 hours later the cells were immunostained with anti-Shh-N antibodies. The upper panels are maximum intensity projected images of serial Z-stacks of merged images, (i) Shh, (ii) Rab3A and (iii) scatter plot of Shh isoforms and Rab3A. Lower panels are higher magnifications images of the boxed regions in the upper panel. Note that palmitate-deficient (A), delipidated (B), uncleaved (C) and uncleaved and palmitate-deficient (D) Shh exhibit considerable overlap with Rab3A positive secretory vesicles. White in merged images denotes colocalized Shh and Rab3A pixels.

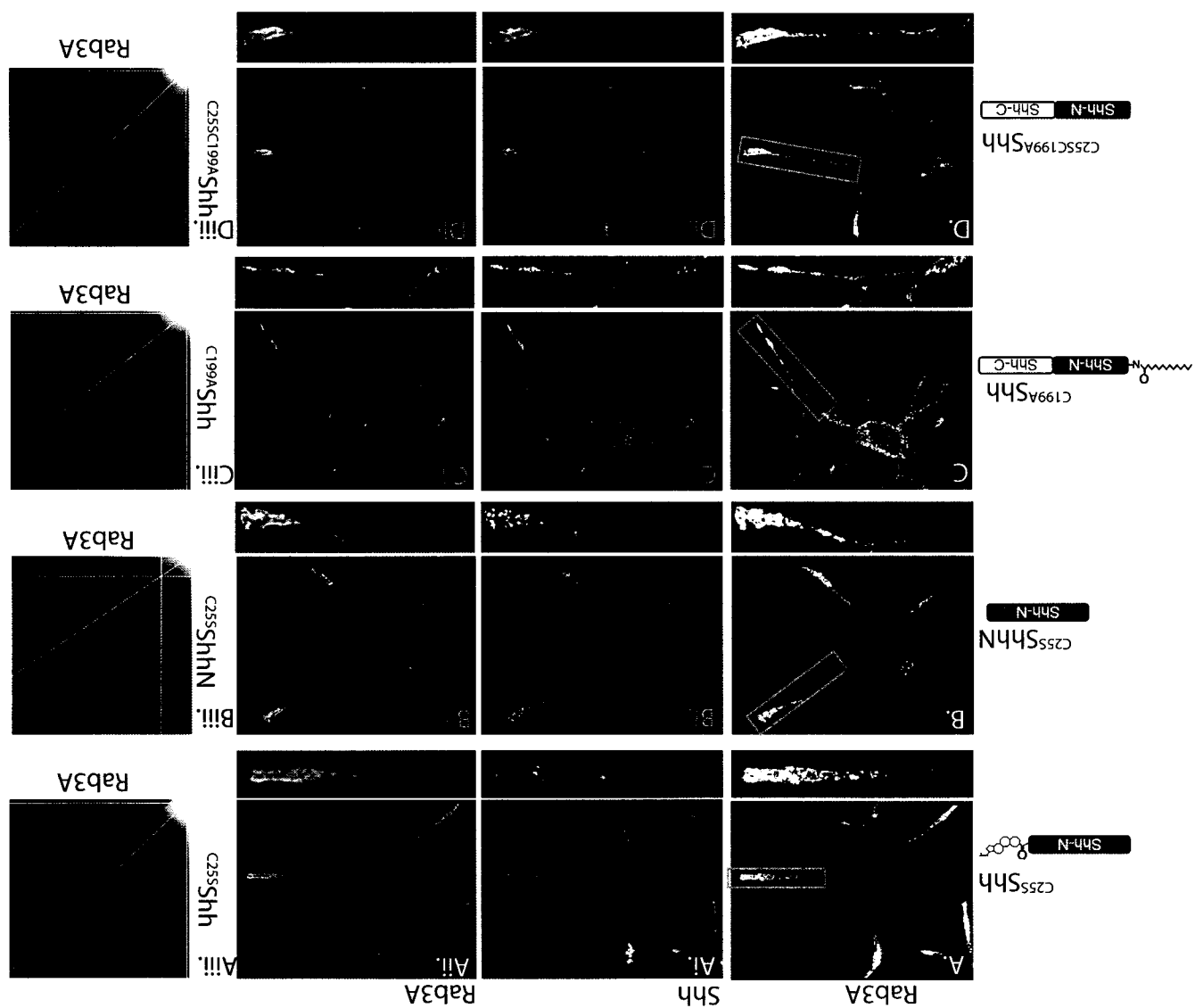


Figure 2.11. Mutated Shh isoforms are associated with the dense core granule marker tissue plasminogen activator (tPA) in differentiated PC6 cells. PC6 neurons were cotransfected with Shh isoforms and tPA-YFP, and cells were processed for imaging 24 hours after transfection with an anti-Shh-N antibody. The upper panels are maximum intensity projected images of serial Z-stacks of merged images, (i) Shh, (ii) Rab3A and (iii) scatter plot of Shh isoforms and Rab3A. Lower panels are higher magnifications images of the boxed regions in the upper panel. Note that palmitate-deficient (A), delipidated (B), uncleaved (C) and uncleaved and palmitate-deficient (D) Shh demonstrate considerable overlap with tPA positive secretory vesicles. White in merged images denotes colocalized Shh and tPA pixels.

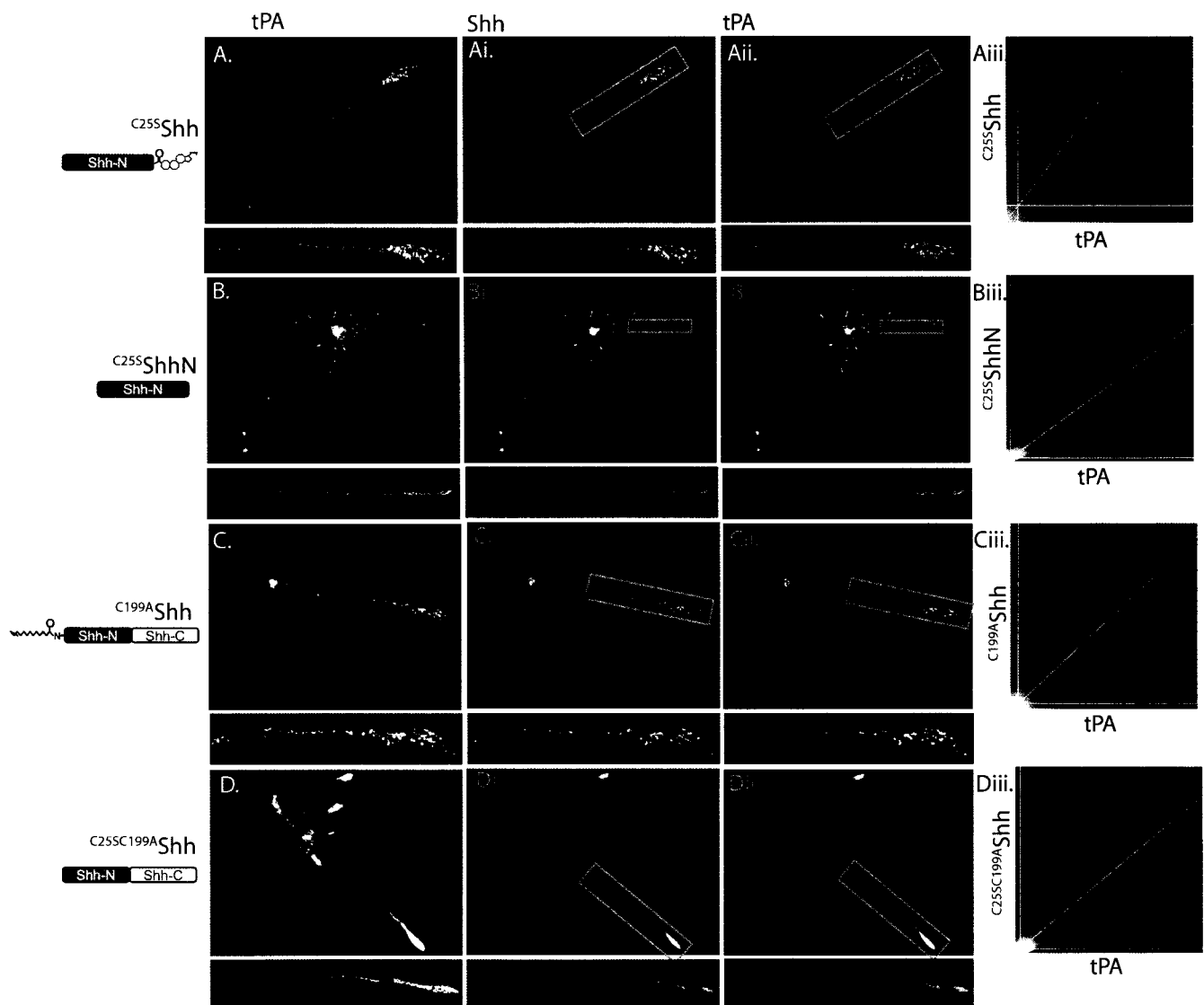
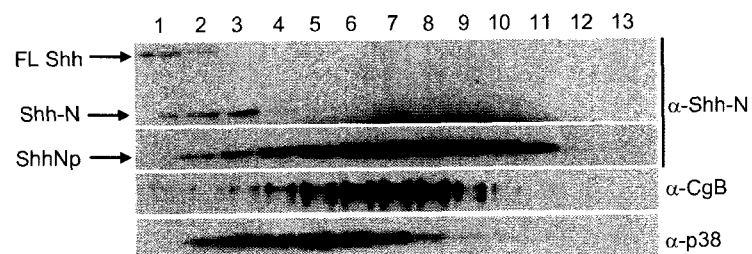
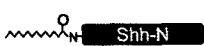
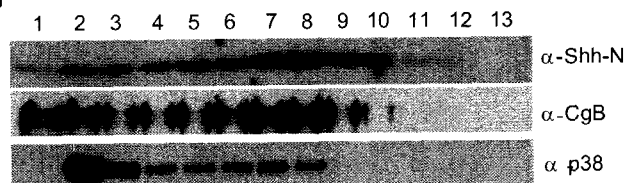


Figure 2.12. Shh cofractionates with dense core granules (DCGs) and synaptic-like microvesicles (SLMV). Postnuclear cell lysates of PC6 neurons stably expressing Shh (A) or ShhN (B) were fractionated on a continuous 0.6-1.8 M sucrose gradient followed by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies against Shh-N, p38 (SLMV marker) and CgB (DCG marker). (A) Fully processed Shh (ShhNp) cofractionates with both SLMV and DCG populations while full length Shh (FL Shh) or incompletely processed Shh (Shh-N) associates with the soluble fractions. Asterisk denotes non-specific band. (B) ShhN mainly copurifies with CgB. (C,D) Postnuclear cell lysates of PC6 cells stably expressing Shh or ShhN were fractionated in 5-25% glycerol gradients and assayed by immunoblotting. ShhNp (C), but not ShhN (D) partially cofractionates with SLMVs.

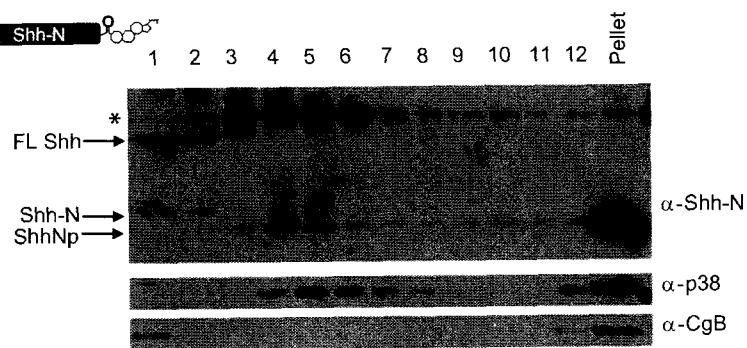
A. Shh 

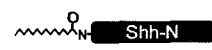


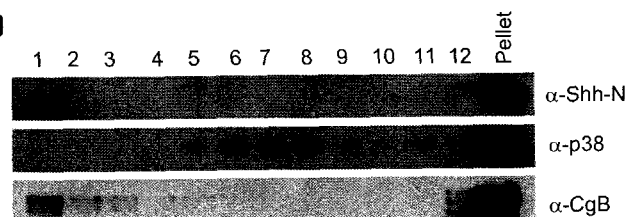
B. ShhN 



C. Shh 



D. ShhN 



applied postnuclear supernatants of Shh-expressing differentiated PC6 cells on glycerol gradients which separate SLMVs from other light organelles. ShhNp, but not ShhN cofractionates with SLMVs (Figure 2.12C,D), indicating that a subpopulation of intracellular ShhNp is present in SLMVs. The colocalization and biochemical fractionation results indicate that wild-type Shh protein may be targeted to both regulated secretory pathways while Shh mutants are targeted to DCGs.

To determine whether Shh isoforms may be also associated with endosomes, I incubated PC6 and primary hippocampal neurons expressing Shh with AlexaFluor555-conjugated transferrin (Tf555), which labels early endosomes (38, 39). There was minimal overlap between wild-type Shh and internalized Tf555 in both primary hippocampal and PC6 neurons (Figure 2.13A,B, Table 2.3), suggesting that Shh is not associated with endosomes. Similarly, Shh isoforms that are defective in posttranslational modification did not exhibit high colocalization levels with Tf555-positive endosomes (Figure 2.13C-G, Table 2.3). Lastly, there was minimal overlap between TfR-YFP and Shh in the dendrites of primary hippocampal neurons (Figure 2.2C). Overall, these results indicate that Shh is unlikely to be internalized and sorted to SLMVs through early endosomes.

2.4.6 Secretion of Shh isoforms

Association of Shh with the regulated secretory pathway suggests that Shh may be secreted by an activity-dependent process. To assess if cell depolarization would stimulate Shh secretion, differentiated PC6 cells stably expressing wild-type and mutant Shh were incubated with basal or high K^+ solutions and the concentration of Shh in the culture supernatant was quantified by ELISA. Exposure to high K^+ levels significantly increased the amount of mutant Shh isoforms detected in the cell culture medium (Figure 2.14), consistent

Figure 2.13. Shh puncta in neurites do not colocalize with early endosomes. (A-G) Distribution of Shh⁺ puncta (green) and transferrin (red) in 14 DIV primary hippocampal neurons (A) and differentiated PC6 cells (B-G). Neurons were transfected with Shh isoforms corresponding to wild-type (A,B), cholesterol-deficient (C), uncleaved (D), palmitate-deficient (E), delipidated (F) and uncleaved and palmitate-deficient (G), and 24 hours later cells were incubated with AlexaFluor555 conjugated Tf for 20 min and processed for immunocytochemistry with an anti-Shh-N antibody. (A-G) Merged images of Shh and Tf555, (i) Shh, (ii) internalized Tf555 and (iii) scatter plot of Shh and Tf555. Note that throughout the neurites, there is minimal colocalization of Shh isoforms and internalized Tf555. White in merged images denote colocalized Shh and Tf555 pixels.

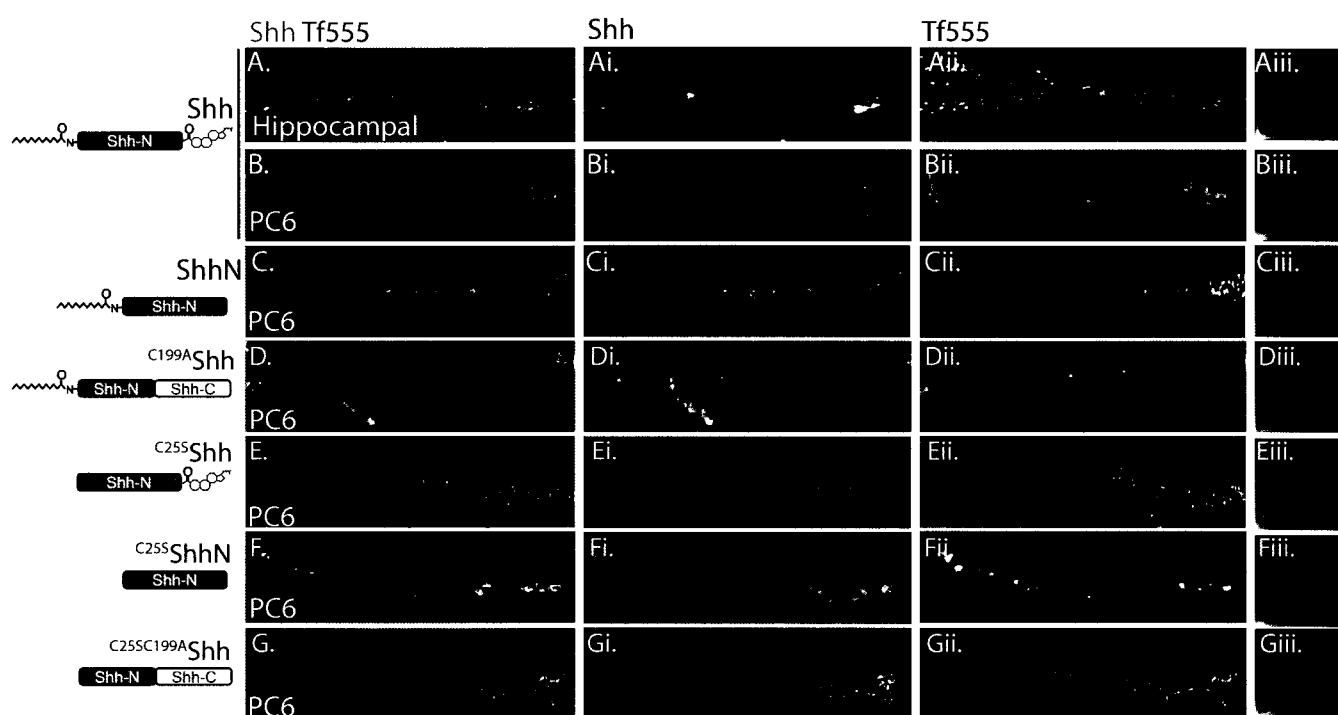
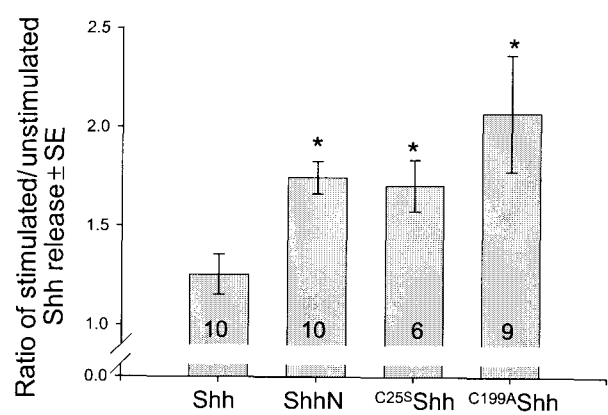


Figure 2.14. Regulated secretion of Shh from PC6 cells. Differentiated PC6 cells stably expressing the indicated Shh isoforms were washed and stimulated for 20 min at 37°C with physiological buffer or physiological buffer containing 56 mM KCl. After stimulation, cell culture supernatants were collected, cleared by centrifugation and Shh present in the supernatant was quantified by ELISA. Values represent the ratio of secreted Shh in the medium of stimulated versus unstimulated cells. Numbers shown in the bars indicate the number of replicates. Statistical analysis was performed using the Student's t-test to detect differences in Shh secretion between basal and stimulated conditions (* $p < 0.05$).



with secretion rates to those reported for neurotrophin secretion in transfected cells (40). However, I did not detect a significant increase in the amount of wild-type Shh in the medium under these conditions (Figure 2.14), possibly as a result of tethering of newly secreted Shh to the plasma membrane. Nevertheless, these results indicate that Shh has the potential to be released under stimulating conditions.

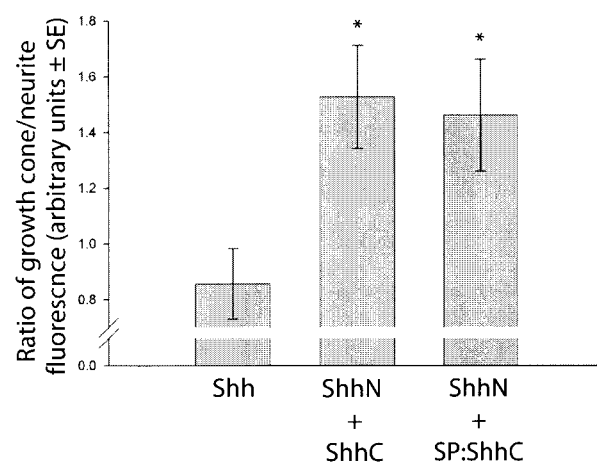
2.4.7 ShhC does not influence ShhN subcellular distribution

The carboxy terminal of Shh is essential for proteolysis of the Shh propeptide into Shh-N and Shh-C. A recent study reported that *Drosophila* hh also contains a sorting signal in the carboxy terminal of hh to direct hh-N towards neurite terminals, and both hh-N and hh-C appear to occupy the same transport organelles (12). Thus, I reasoned if mammalian Shh-C contains a sorting signal for the amino terminal of Shh, then the presence of Shh-C may rescue the subcellular distribution of ShhN in PC6 cells. To examine if the subcellular targeting role is conserved in mammals, I cotransfected ShhN::YFP and ShhC::CFP into PC6 neurons and monitored the subcellular distribution of ShhN::YFP⁺ puncta. Coexpression of ShhC with ShhN did not affect the accumulation of ShhN⁺ puncta in neurite terminals (Figure 2.15). Moreover, transfection of a Shh isoform lacking the critical residues identified to be essential for hhC sorting resulted in an unprocessed Shh protein with an identical distribution profile as delipidated Shh isoforms (data not shown).

2.5 Discussion

In this study I investigated the trafficking pattern of Shh in mammalian neurons. I report that Shh is distributed in a punctate pattern on the extracellular surface of the soma, dendrites and axons, and that Shh is trafficked to two major classes of regulated secretory

Figure 2.15. ShhC does not alter the subcellular distribution of ShhN in neurons. Differentiated PC6 neurons were transfected with wild-type Shh or cotransfected with ShhN::YFP and CFP-tagged ShhC isoforms and analyzed 24 hours after transfection. ShhC consists of the carboxy domain of Shh and SPShhC contains the Shh signal sequence upstream of the ShhC domain. YFP fluorescence along the length of the neurite and at the neurite terminals of maximum intensity projections of serial Z-stack images was obtained with ImageJ, and the proportion of fluorescence density between the neurite terminals and the neurite was calculated. Statistical analysis was performed with 1-way ANOVA and differences calculated with the Tukey post hoc test (* $p < 0.05$).



vesicles, DCGs and SVs. Lipid modification and proteolysis are not required for Shh trafficking to DCGs, but are required for the cell surface accumulation and trafficking of the protein to SVs. Consistent with a vesicular pattern of Shh in neurons, movement of Shh⁺ puncta is bidirectional, occurs with fast and slow kinetics and Shh secretion can be induced by depolarization. These observations provide a potential mechanism for axon-based Shh secretion and signalling to glia in nerves and also suggest a link between neuronal activity and Shh secretion.

2.5.1 Potential issues arising from overexpression studies

Ideally one would want to monitor the distribution pattern of endogenous proteins; however, if analysis of endogenous proteins is not feasible, gain-of-function approaches are standard tools in the protein trafficking field. To address any concerns arising from the gain-of-function approach, I examined several potential pitfalls that could yield nonphysiological results. First, titration of the Shh plasmid used for transfection did not reveal any differences in Shh distribution and polarity in primary hippocampal neurons, suggesting that the high levels of Shh expression did not cause Shh missorting into inappropriate compartments. Second, I confirmed that the distribution patterns of two well-characterized polarized proteins, TfR-YFP and Syp-YFP, were consistent with what has been reported in the literature. Third, the distinct subcellular distribution patterns of wild-type versus mutant Shh suggest that my observations in transfected neurons are not an artefact. Fourth, biochemical techniques were used to address the possibility that the overlap of Shh⁺ puncta with vesicle markers observed by confocal microscopy was random. Thus, the gain-of-function approach employed in this study likely did not result in an artifactual distribution profile as a result of overexpression.

2.5.2 Posttranslational modifications are required for proper Shh sorting

Posttranslational modifications of Hh proteins, including cleavage and lipidation are required for proper neuronal trafficking in both *Drosophila* (12) and mammals (this study). To investigate the requirement for PTMs for Shh vesicle trafficking in neurons, I compared the distribution of wild-type and mutant Shh. Inhibition of any PTM resulted in lack of Shh accumulation on the cell surface and exclusive trafficking to DCGs in both differentiated PC6 cells and hippocampal neurons. In the fly visual system, uncleaved hh, but not cholesterol-deficient hhN, is trafficked to presynaptic targets (12). As well, efficient trafficking of hh-N to these neurite terminals also requires several residues in the carboxy terminus (12). In contrast, I observed that uncleaved Shh, like cholesterol-deficient Shh, was largely absent from axons in primary hippocampal neurons. Moreover, providing ShhC *in trans* did not rescue the distribution pattern of ShhN in PC6 cells, suggesting that the carboxy domain is not sufficient to overcome neuron trafficking defects associated with loss of the cholesterol modification to Shh. The differences in Hh trafficking in flies and mammals could be attributed to DCG trafficking patterns in mammals. For instance, secretory cargo that is transported in DCGs preferentially traffics to the somatodendritic compartment (41-43). Overall, these findings indicate that the axon trafficking requirements for fly and mammalian Hh are different.

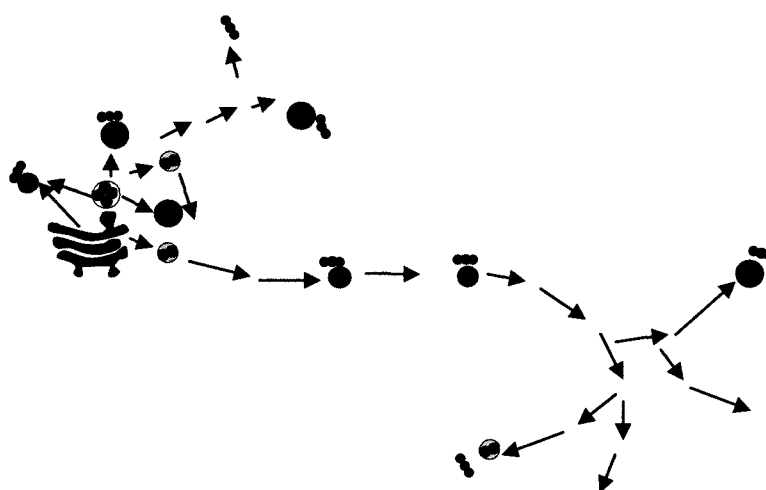
2.5.3 Sorting of Shh into the regulated secretory pathway

The regulated secretory pathways have distinct trafficking patterns and our data suggest that Shh may be sorted to these pathways by different mechanisms. For example, Shh targeting to SVs, as opposed to DCGs, is dependent on all of the PTMs, as perturbation of any PTM blocked trafficking to SVs. Synaptic vesicles traffic to the plasma membrane in

constitutive-like secretory vesicles, and mature through fusion with the plasma membrane and endosomes (44, 45). In agreement with Chu et al (12) who found that hh-N overlapped with presynaptic vesicle markers in fly retinal axons and synapses, I observed that ShhNp is sorted to SLMVs in differentiated PC6 cells. There was minimal colocalization of Shh with Tf, which colocalizes with SVs in newly endocytosed vesicles, raising the possibility that Shh association with SLMVs is mediated by copackaging of Shh into SVs that are transported to presynaptic sites. However, I cannot exclude the possibility that Shh is endocytosed or trafficked to Tf-deficient endosomes. Interestingly, ShhNp is also sorted to DCGs. As DCGs are enriched in the somatodendritic compartment (41-43), this result suggests that Shh may be differentially released at axons or dendrites as a result of sorting into different secretory vesicles (Figure 2.16). Overall, I conclude that neurons sort Shh into the regulated secretory pathway which is the same system that is used for neurotrophins, neurohormones and neuropeptides.

There are several mechanisms of protein sorting arising at the level of the Golgi. In epithelial cells, several apical proteins that complex with membrane rafts are believed to function in the trans-Golgi network (TGN) as sorting platforms for apical delivery (29-31, 44). Not surprisingly, intracellular ShhNp is strongly associated with lipid raft makers in both PC6 cells and primary hippocampal neurons. As delipidated Shh is not associated with lipid rafts (8) and perturbation of Shh PTMs including cleavage or lipidation resulted in near-complete accumulation of Shh isoforms with DCGs, these results indicate that lipid raft association early in the secretory pathway is not a requirement for proper Shh sorting to DCGs. Alternative mechanisms that may regulate Shh sorting to DCGs include aggregation within the TGN and interaction with sorting receptors (46). Candidate receptors include Carboxypeptidase E and Sortilin, which are known to interact with motifs in brain-derived

Figure 2.16. Schematic of Shh trafficking in neurons. Shh is processed in the ER-Golgi network, and a portion of Shh protein is routed through the regulated secretory pathway which includes synaptic vesicles (SVs) and dense core granules (DCGs). The association of Shh with SVs is likely carried out by the constitutive-like precursors of SVs since Shh does not colocalize with transferrin-positive organelles. The role of the constitutive secretory pathway in long-range Shh transport is unknown.



- Constitutive
- Synaptic vesicle
- Immature DCG
- Mature DCG

neurotrophic factor (BDNF) to sort it to DCGs (47, 48). Therefore, the efficiency of Shh PTM may be necessary for sorting of processed Shh to a non-DCG compartment.

Furthermore, it is possible that mammalian Shh possesses a sorting signal in the N-terminal which may interact with other sorting receptors.

2.5.4 Biological significance of Shh trafficking to the regulated secretory pathway

As Shh is localized within regulated secretory vesicles, this raises the possibility that Shh secretion could be regulated by neuronal activity. Indeed, mutated forms of Shh can be secreted by stimulation with high $[K^+]$ in differentiated PC6 cells. As mutant Shh is not packaged into SLMVs, this stimulated release under these conditions likely reflects Shh secretion from DCGs. However, this same treatment did not increase Shh levels in the supernatant of wild-type Shh-PC6 cells, despite the biochemical and light microscopy evidence of association of wild-type Shh with DCG and SLMVs. One possible explanation for this discrepancy is that secreted ShhNp would largely be cell associated (6, 49), and therefore not detected using our approach. These results also highlight the possibility that Shh release could be regulated by neuronal activity. Synaptic release of Shh from the hippocampus has been proposed to modulate progenitor cell proliferation within the subgranular zone (50, 51). It is possible that DCG-associated Shh in axons can be secreted by neuronal activity whereas Shh that is trafficked in SV precursors is not released in an activity-dependent manner. For instance, silencing electrical activity in RGCs did not alter astrocyte proliferation in the optic nerve (52). Overall, these results indicate that neuron-derived Shh vesicular targeting may be critical during development and for adults.

2.5.5 Functional relevance of cell surface Shh in neurons

The axonal transport of Shh that I observed is consistent with previous reports of anterograde Hh transport and with its *in vivo* biological effects in the visual system (12-18, 53-55). I found that mature Shh accumulates with lipid raft domains on the surface of neurons. These observations raise the possibility that surface-localized Shh mediates signalling to glia in the developing optic nerve. Indeed, reduction of RGC-derived Shh by application of antibodies that neutralize Shh function in optic nerves *in vivo* decreased Hh-target gene expression and proliferation of glial cells (13, 56). The cell surface Shh presentation on axons also raises the issue of how the timing of Shh-signalling is regulated in the optic nerve. Shh expression is maintained in adult RGCs, but Hh target gene expression and proliferation in astrocytes in the optic nerve are extinguished after P7 (13, 57). This indicates that there are potentially different mechanisms that could drive this phenomenon including temporally regulating Shh distribution or secretion, or Shh-responsiveness of target cells.

The apical versus basal release of hh elicits distinct cellular responses in the developing fly epithelia (12, 58, 59). Basal Shh secretion from the cell body/dendrites of RGCs stimulates proliferation and regulates cell fate in the retina. Apical localization of Shh from RGC axons also stimulates astrocyte proliferation in the optic nerve, but also affects guidance of axons in the retina, optic disc and optic nerve (53, 55). Interestingly, I did not observe any obvious polarity in the distribution pattern of Shh in primary neurons, which is consistent with the findings of Chu et al (2006) who monitored the distribution of wild-type hh in the fly visual system. If there are subcellular secretion site-dependent functional outcomes of Shh signalling, the level of regulation may not rest with the distribution of Shh;

rather, it may be based on sorting to the appropriate secretory vesicle and any cofactors that Shh is secreted with, as well as the composition of the extracellular matrix and receptors in both RGCs and target cells.

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3. IDENTIFICATION OF SHH INTERACTING PROTEINS IN THE CENTRAL NERVOUS SYSTEM

3.1 Abstract

An affinity purification screen was used to identify novel Sonic hedgehog (Shh) interacting proteins in the central nervous system. I generated glutathione S-transferase (GST)-tagged fusion proteins of amino-terminal Shh (GST::ShhN) and carboxy-terminal Shh (GST::ShhC) domains as bait and used solubilised membrane fractions from perinatal rat brain for GST pulldown experiments. Interacting proteins were identified with in-gel trypsin digestion and mass spectrometry analysis of excised bands. The list of candidate proteins for further analysis was prioritized based on the MASCOT score, peptide abundance, protein function, subcellular localization and potential for Hh interactions based on interactions with homologues in other species. Based on these criteria, 22 potential Shh interacting proteins with possible roles in posttranslational modification, intracellular trafficking, extracellular transport and reception were identified. With the exception of two candidates, these proteins have not been implicated in Shh signalling. I selected three proteins for further *in vitro* validation: Glypican 5 (Gpc5), Low density lipoprotein receptor-related protein 1 (Lrp1) and Sortilin (Sort1). Coimmunoprecipitation experiments in transfected COS cells expressing Shh and candidate proteins suggest that these candidates are bona fide Shh interacting partners.

3.2 Introduction

The morphogen Sonic hedgehog (Shh) interacts with diverse proteins that are involved in protein biogenesis, secretion, extracellular transport and reception. Multiple

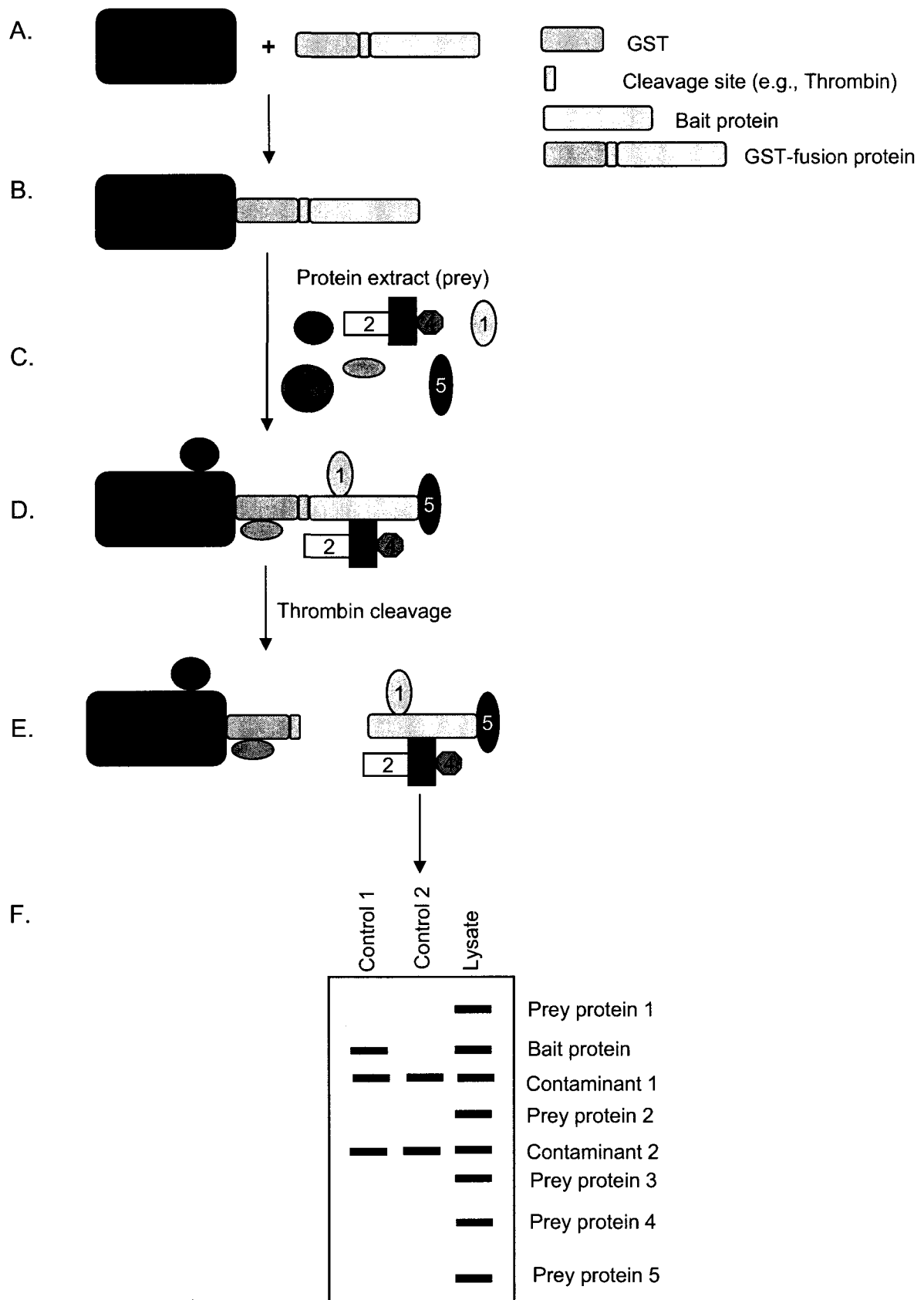
studies have revealed context-specific roles for these interacting proteins during development. For instance, Shh interacting proteins Lrp2, Cdo, Boc, Hhip and Gas1 have different expression profiles and regulate Shh signalling in a spatiotemporal manner (1-16). Probing for additional interacting partners in the central nervous (CNS) may yield more stage- and cell-specific interacting partners that regulate Shh activity. As outlined in Chapter 2, Shh sorting, intracellular trafficking and secretion in the CNS likely involve interaction with specialized factors that are present exclusively in neurons. Thus, identification of additional factors that are required for Shh signalling in the CNS would provide valuable insight into the mechanisms of Shh regulation.

While a number of Hh signalling components have been identified over the past two decades, it is unlikely that we have obtained a comprehensive profile of the factors involved in Hh signalling. The majority of Hh binding proteins and signalling components to date have been identified genetically. Genetic screens are a powerful approach to identify components of signalling pathways because the screens have the potential to discover factors that are involved in the Hh pathway and are also not subject to artefacts typical from extract preparations or *in vitro* techniques; however, there are limitations to this approach. The genetic approaches do not reveal if these proteins directly interact with Hh. Moreover, the loss-of-function phenotypes may not be clearly attributed to the Hh pathway and it is also difficult to identify mutated genes when employing a nonspecific mutagenesis screen (17). As well, it is possible that bona fide Hh interacting partners do not display Hh^{-/-} phenotypes when inactivated in a model organism (e.g., due to temporospatial-specific regulation, involvement in other pathways or functional redundancy from related genes). Although several genetic and cell-based large-scale studies have been conducted to discover additional molecules implicated with the Hh pathway (7, 10, 17), no other study to date has used an *in*

vitro protein:protein interaction screen to identify Hh interacting proteins. Moreover, there are no reports that have identified any potential interacting partners for the carboxy-terminus of Hh (Hh-C). Furthermore, there is emerging evidence that Shh may activate a noncanonical pathway that likely acts in parallel with the classical Ptc-Smo-Gli pathway (18, 19). Thus, cell-based screens that employ Ptc- and Gli-dependent activation of Hh-target genes or Hh reporter constructs would fail to identify interacting partners that does not activate the canonical pathway (e.g., 17). The glutathione S-transferase (GST)-pulldown method is an example of an affinity purification technique whereby proteins harvested from tissues or cells are incubated with GST fusion proteins, and after extensive washing, bound proteins are eluted, resolved by SDS-PAGE and identified by mass spectrometry (Figure 3.1). The affinity purification approach has resulted in identification of novel protein interactions involved in the sorting, trafficking or reception of secretory proteins (20-23). Thus, the GST-pulldown technique has the potential to identify novel Shh interacting partners.

Given the crucial role that the Hh signalling pathway plays during embryonic morphogenesis, it is possible that time- and tissue-specific interaction of Hh proteins with a diverse number of molecules is critical for normal development. The GST-pulldown approach was used to identify potential novel Shh-interacting factors from brain membrane preparations. I identified several potential novel interacting proteins that interacted with the amino and carboxy domains of Shh and these proteins are potentially involved in Shh posttranslational modification, intracellular trafficking, extracellular transport and reception. I selected three proteins to validate the GST-pulldown screen by coimmunoprecipitation (coIP).

Figure 3.1. Overview of the GST-pulldown technique. (A) Bacterially expressed soluble GST-fusion proteins are immobilized on a glutathione matrix and after stringent washes, unbound proteins are removed (B). (C) Protein extract from tissues or cells are added to the immobilized fusion protein. (D) Unbound proteins are removed by washing and with varying stringencies. (E) Proteins are eluted from the immobilized fractions by thrombin cleavage. (F) Protein:protein interaction complexes are resolved by SDS-PAGE, gels stained by silver staining and unique bands are identified by mass spectrometry.



3.3 Materials and Methods

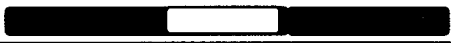
All materials and methods are listed in more detail in Appendix II.

3.3.1 DNA constructs

Full-length Shh cDNA (Genbank Accession NM_009170) was used to generate GST fusion constructs. Shh-N (amino acids 25-198) and Shh-C (amino acids 199-437) were amplified by PCR and subcloned into the GST recombinant vector pGEX-4T-1 (GE Healthcare) to generate GST::ShhN and GST::ShhC, respectively.

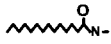
ShhN, ^{C25S}ShhN and ^{C199A}Shh isoforms were generated by site-directed mutagenesis of mouse Shh cDNA (Table 3.1). Incorporation of yellow fluorescent protein (YFP) into Shh-N was as follows: a NheI restriction enzyme site was introduced between amino acids 196 and 197 of mouse Shh to generate Shh(NheI). YFP was PCR amplified from pEYFP-C1 (Clontech) with primers containing flanking SpeI restriction sites and was inserted into the NheI site of Shh(NheI) resulting in placement of the tag three amino acids upstream from the cleavage site (Table 3.1). SPShhC::CFP encodes a carboxy-terminal mouse Shh protein containing the Shh signal peptide and tagged with cyan fluorescent protein (CFP). Shh::CFP was created by PCR amplification of the cDNA sequence of mouse Shh corresponding to amino acids 199-437, which was then cloned in frame with ECFP (Clontech) to generate a fusion protein with a carboxy-terminal tag (Table 3.1). Nucleotide sequence corresponding to the mouse Shh signal peptide (1-24 aa) was cloned upstream of the ShhC::CFP construct to create SPShhC::CFP (Table 3.1). All Shh constructs were subcloned into the pcDNA3 expression vector.

Table 3.1. Schematic of the constructs used in Chapter 3.

Shh isoform	Predicted processed form	
ShhN::Fc	Cholesterol deficient and Fc tag	
ShhN::YFP	Cholesterol deficient and YFP tag	
^{C25S} ShhN::YFP	No lipid and YFP tag	
^{C199A} Shh::YFP	Uncleaved and YFP tag	
ShhC::CFP	ShhC and CFP tag	
Sort1::myc-his	Sortilin 1 and myc-his tag	
Hhip::myc-his	Hhip and myc-his tag	
Gpc5::3XFLAG	Glypican 5 and 3XFLAG tag	
mLrp1	Lrp1 minireceptor 1 and HA tag	
mLrp2	Lrp1 minireceptor 2 and HA tag	
mLrp3	Lrp1 minireceptor 3 and HA tag	
mLrp4	Lrp1 minireceptor 4 and HA tag	

Shh-N 

Shh-C 

Palmitate 

Fc 

YFP 

CFP 

myc-his 

HA 

3XFLAG 

Hhip and Sort1 were obtained from the IMAGE Consortium and subcloned into pcDNA3.1myc-His(-)C by PCR amplification. Gpc5 was tagged with 3XFLAG upstream of the cleavage site, as described by Kurosawa et al (24) and subcloned into pcDNA3.

Lrp1 minireceptors (mLrp1-4) were kindly provided by Guojun Bu (Washington University School of Medicine, St. Louis, MO). ShhN::Fc was kindly provided by Chen-Ming Fan (Carnegie Institution of Washington, Washington, DC). CgA-HA was kindly provided by Laurent Taupenot (University of California, San Diego, CA).

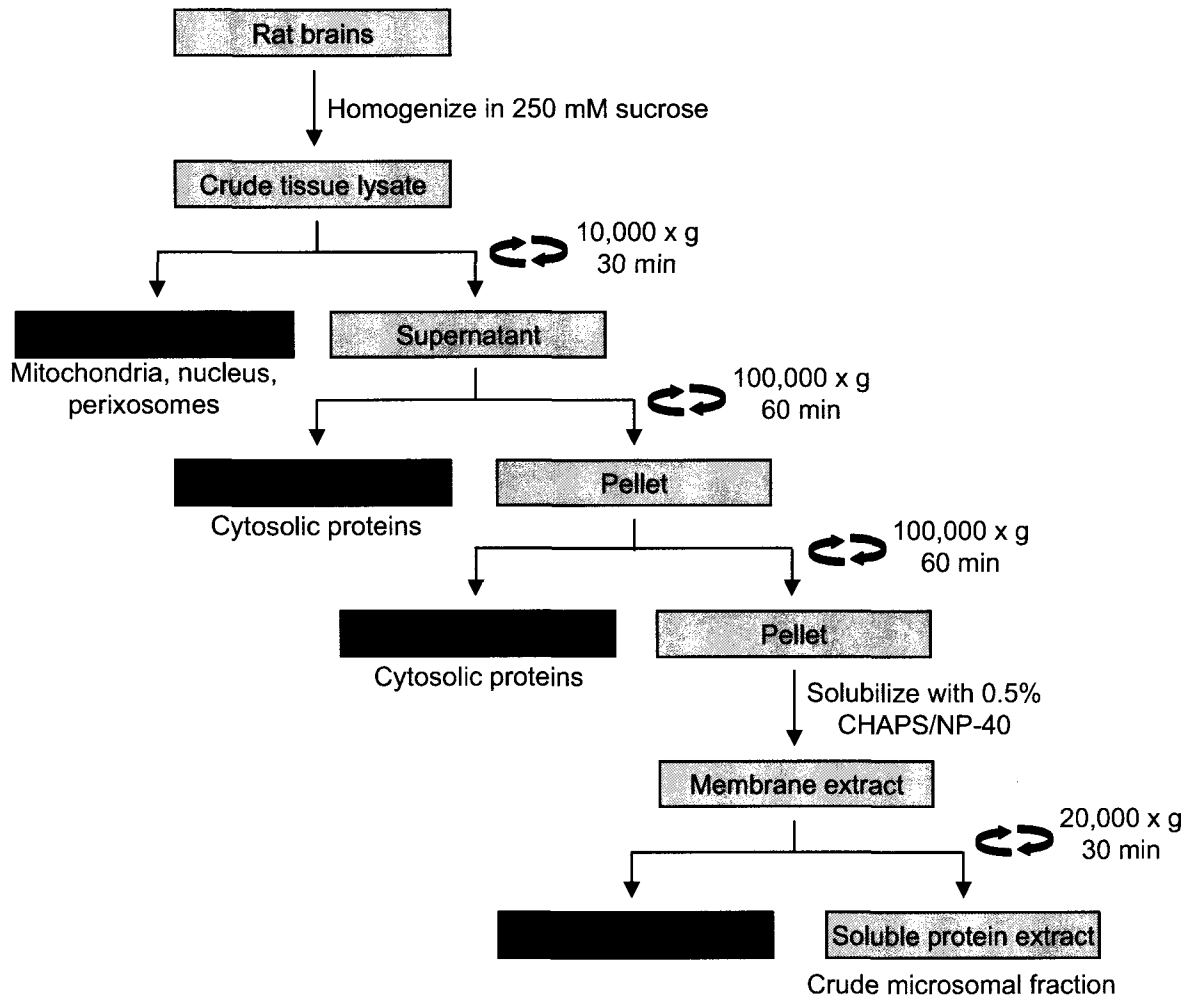
3.3.2 Expression of Glutathione S-Transferase Fusion proteins

GST recombinant vectors were transformed into BL21(DE3) cells. Fusion protein expression was induced with 0.1 mM IPTG and soluble bacterial extracts were incubated with glutathione-coupled Sepharose beads (GE Healthcare) according to the manufacturer's instructions. Beads were subsequently washed with high salt (20 mM HEPES-KOH pH 7.4, 500 mM NaCl, 5% Glycerol), Triton X-100 (20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 1% Triton X-100), and protein interacting buffer (20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 1 mM MgCl₂).

3.3.3 Preparation of rat brain microsomal fraction

Rat brains from postnatal day 2-3 were washed in DPBS and dounce homogenized in SIM buffer (250 mM sucrose, 5 mM imadazole, 1 mM MgCl₂, pH 7.4) containing protease inhibitors (Roche; Figure 3.2). Brain extracts were centrifuged at 10,000 x g for 30 min and the supernatant was sequentially centrifuged at 100,000 x g for 60 min. The pellet was dounce homogenized in SIM buffer and recentrifuged at 100,000 x g for 60 min. The microsomal pellet was solubilised with either 0.5% CHAPS or NP-40 in protein interacting

Figure 3.2. Schematic of the subcellular fractionation method. P3 rat brains were homogenized in 250 mM sucrose and centrifuged for 30 min to generate a supernatant containing microsomal membranes and cytosolic proteins. The supernatant was subsequently centrifuged at 100,000 x g for 1 hour. The pellet was homogenized in sucrose solution and recentrifuged to generate a pure microsomal pellet. The microsomal pellet was solubilised with 0.5% NP-40 or CHAPS and centrifuged for 30 min at 20,000 x g. The supernatant contained luminal and membrane proteins from the microsomal preparation.



buffer and clarified by centrifugation at 20,000 x g for 30 min.

3.3.4 Affinity purification

Solubilised rat brains were precleared by incubation with glutathione-coupled Sepharose beads. The precleared fraction membrane fraction was incubated with equimolar amounts of GST, GST::ShhN or GST::ShhC. Beads were washed sequentially with buffer (20 mM HEPES-KOH, pH 7.4), high salt buffer (20 mM HEPES-KOH pH 7.4, 300 mM NaCl), detergent buffer (20 mM HEPES-KOH pH 7.4, 300 mM NaCl and 0.1% CHAPS or NP-40) and PBS. Bound proteins were eluted by cleavage with thrombin and subjected to SDS-PAGE using linear 8-16% gradients. Gels were Silver stained, and bands were excised and identified by mass spectrometry (Ottawa Institute of Systems Biology, Ottawa, ON).

3.3.5 Coimmunoprecipitation

COS cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS. COS cells were cotransfected using Lipofectamine 2000 according to the manufacturer's instructions and incubated for 48 hours prior to harvesting. Cells were scraped in lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol and 1% NP-40) and centrifuged at 10,000 x g for 10 min. Clarified cell lysates were immunoprecipitated with either Protein A magnetic beads (Invitrogen) for ShhN::Fc coIP, α -FLAG M2 affinity beads (Sigma) for Gpc5 coIP or α -His coupled to α -mouse IgG beads (Roche) for Sort1 coIP. Proteins were washed with detergent buffer containing NP-40 and eluted using Laemmli's sample buffer.

3.3.6 Western blotting

Aliquots of cell lysates and immunoprecipitated material were subjected to 8 or 10% SDS-PAGE and transferred to HybondC Extra membranes (GE Healthcare) using standard

protocols (25). Membranes were incubated overnight at 4°C with rabbit α -GFP (1:2000, Invitrogen), mouse α -myc (1:1000, Santa Cruz), mouse α -HA (1:1000 Santa Cruz) and α -FLAG (1:5000, Sigma) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20. After incubation with secondary antibodies (HRP-conjugated IgG; 1:10,000; Sigma), protein bands were detected using an ECL detection kit according to the manufacturer's instructions (GE Healthcare).

3.4 Results

3.4.1 GST-pulldown using ShhN and ShhC isolated several candidates

A GST-pulldown approach was performed to identify novel Shh-interacting factors in brain tissue. Equimolar amounts of GST, GST::ShhN or GST::ShhC coupled to glutathione Sepharose beads were used to probe for interacting proteins from solubilised microsomal fractions extracted from perinatal rat brains (Figure 3.2). After washes with medium stringency, bound proteins were removed by thrombin cleavage, resolved by SDS-PAGE under denaturing conditions and the gels were Silver stained. Over 40 unique bands present in the GST:ShhN or GST:ShhC lanes compared to the GST control lanes were excised from gels and identified by mass spectrometry. A list of candidate proteins for further analysis was prioritized based on the MASCOT score, peptide abundance, protein function, subcellular localization and potential for Hh interactions based on interactions with homologues in other species. From this analysis, 22 proteins were identified (Table 3.2), and the majority of these proteins have no prior links with Shh signalling. The candidates that have previously published links in Shh signalling are Lrp1, a homologue of Megalin, and Gpc5, a homologue of Gpc3, which provides support that the GST pulldown was successful in isolating known

Table 3.2. Summary of GST::ShhN and GST::ShhC binding candidate proteins identified through GST pulldowns from NP-40 and CHAPS solubilised microsomal fractions of P2-3 rat brains.

<u>ShhN</u>	<u>ShhC</u>	<u>Protein</u>	<u>Full Name</u>	<u>Putative Role</u>
+	*	Agm	Aggrin	Receptor
	+	C1orf22	Putative alpha-mannosidase C1orf22	Processing
+		Cspg5	Chondroitin sulfate proteoglycan NG2	Transport
+		Fat3/4	FAT tumor suppressor homolog 3/4	Receptor
+		Gpc5	Glypican 5	Transport
+	***	IGF2R	Insulin-like growth factor 2 receptor	Receptor/Endocytosis
+	*	Kif1a	Kinensin family member 1a	Intracellular trafficking
+		Kif1b	Kinensin family member 1b	Intracellular trafficking
***	+	Lrp1	Low density lipoprotein receptor-related protein	Receptor
	+	Lrrn1	Leucine rich repeat protein 1	Receptor
	+	Lrrnc4B	Leucine rich repeat containing 4B	Receptor
***	+	NCAM1	Neural cell adhesion molecule	Transport
	+	Ngln3	Neurologin 3	Receptor
+		Nrp1	Neuropilin 1	Receptor
+	*	Nrxn1/2	Neurexin 1/2	Receptor
+		Podxl2	Podocalyxin-like 2	Transport
***	+	Robo1	Roundabout homolog 1	Receptor
	+	Rtn4	Reticulon 4	Intracellular trafficking
+	***	Sort1	Sortilin 1	Intracellular trafficking
	+	SV2	Synaptic 2 glycoprotein	Intracellular trafficking

+: peptides identified in the corresponding GST-fusion protein pulldowns

*: peptides identified from the NP-40 and CHAPS solubilised protein fractions.

***: identified peptides were more abundant in the indicated GST pulldown fraction. For example, Lrp1 had over 50 identified peptides from the GST::ShhN fraction while the GST::ShhC fraction had 4 identified Lrp1 peptides.

Shh interacting partners (11, 14, 16, 26-36).

From literature searches, the candidates were subclassed based on their putative role in the Shh pathway. In the intracellular trafficking class, five candidates (Kif1A/B, Rtn4, Sort1 and SV2) may be involved in Shh intraneuronal transport. Four candidates were grouped within the transport class (Cspg5, Gpc5, Ncam1 and Podxl2) whereby these proteins may mediate Shh extracellular transport in either producing or receiving cells. Nine of the putative interacting proteins were classified as receptors (Agn, Fat3/4, Igf2R, Lrp1, Lrrc4B, Lrrn1, Ng13, Nrp1, Nrnx1/2 and Robo1) that are potentially involved in the stabilization of extracellular Shh, Shh internalization or coreceptor function for bridging Shh to Ptc. The last class includes C1orf22, which may be involved with Shh-C glycosylation in the ER-Golgi (37, 38).

3.4.2 Validation of GST pulldown candidates

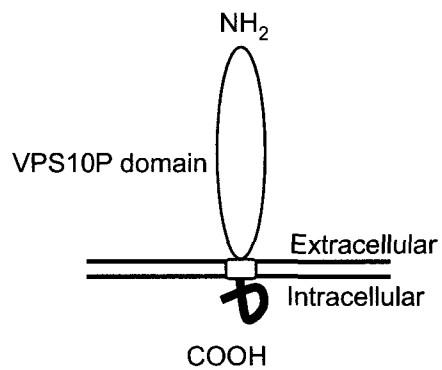
As the GST pulldown approach does not directly prove that these newly identified proteins are bona fide interacting proteins, coIPs from cotransfected COS cells were carried out to validate these potential interactions.

3.4.2.1 Sortilin (Sort1)

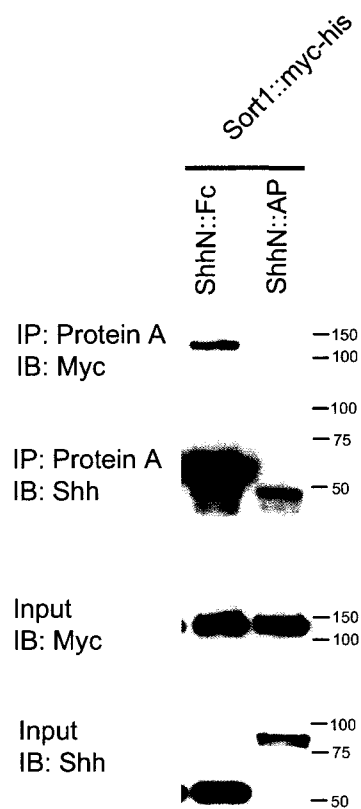
Sort1 is a multifunctional receptor that binds to a variety of ligands to regulate diverse cellular processes. Sort1 lacks the typical features of a signalling receptor, consisting of a large luminal domain and a short cytoplasmic tail that interacts with accessory molecules involved in intracellular transport (Figure 3.3A; 39). While Sort1 is found on the plasma membrane and functions as an endocytotic receptor, the majority of Sort1 resides in the Golgi apparatus and is involved with intracellular trafficking (20, 39-42). To confirm that

Figure 3.3. Sort1 interacts with ShhN and ShhC. (A) Structural organization of Sort1 showing the vacuolar protein sorting 10 (VSP10P) luminal/extracellular domain, transmembrane region and cytoplasmic domain. Adapted from (41). (B) Epitope tagged Sort1 (Sort1::myc-his) was cotransfected with either ShhN::Fc or ShhN::AP in COS cells and subjected to coimmunoprecipitation using Protein A beads. Sort1::myc-his coimmunoprecipitated with ShhN::Fc but not with the negative control ShhN::AP. (C) Sort1::myc-his was cotransfected with either ShhN::YFP or SPShhC::CFP in COS cells and clarified cell lysates were immunoprecipitated with an α -HIS antibody. Under these conditions, ShhN::YFP and SPShhC::CFP coimmunoprecipitated with Sort1::myc-his and the positive control, epitope-tagged Hhip (Hhip::myc-his). pcDNA3.1-myc-his served as a negative control. Asterisk denotes contaminating heavy IgG subunit. Numbers represent molecular weight (kDa)

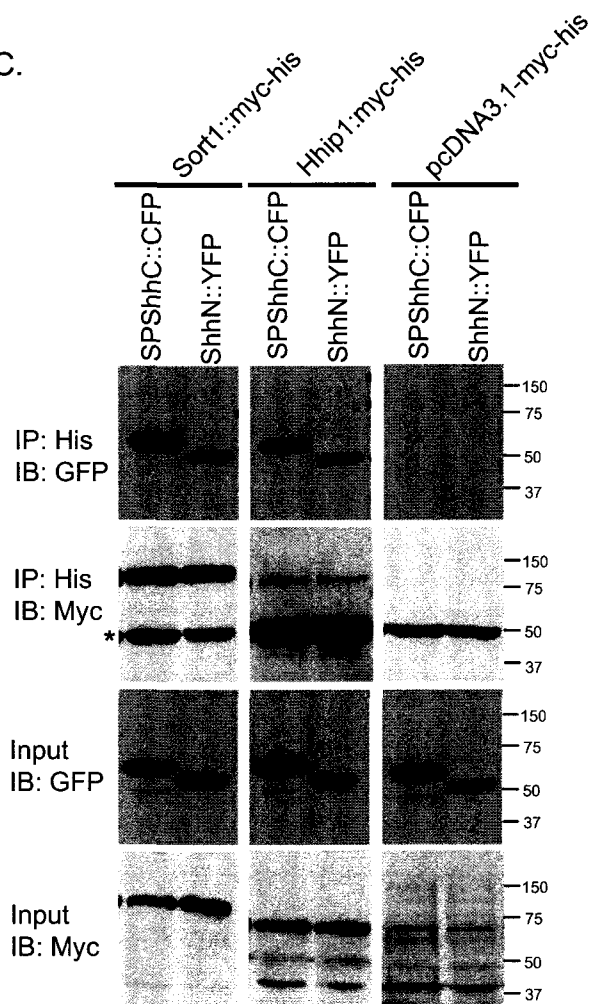
A.



B.



C.



Shh binds to Sort1, I inserted a myc-his epitope tag at the carboxyl terminal (Sort1::myc-his; 43), and cotransfected Sort1::myc-his with Fc-tagged ShhN (ShhN::Fc) or alkaline-phosphatase-conjugated ShhN (ShhN::AP). Subsequent coIP using protein A beads reveals that Sort1::myc-his was detected with ShhN::Fc, but not ShhN::AP immunoprecipitates (Figure 3.3B). As ShhN::AP served as a negative control for nonspecific binding of proteins to the Protein A beads, the ShhN::Fc and Sort1::myc-his immunoprecipitate suggests that ShhN interacts with Sort1.

As I was unsuccessful in creating an Fc-tagged Shh-C construct, interaction of Sort1 with Shh-C was investigated in a coIP using fluorescent-tagged Shh constructs. To verify that both Shh domains interact with Sort1, I cotransfected Sort::myc-his with fluorescent tagged ShhN (ShhN::YFP) or Shh-C fused to the signal peptide of Shh (SPShhC::CFP). Subsequent coIP with an α -His antibody reveals that both ShhN::YFP and SPShhC::CFP were detected in Sort1 immunoprecipitates (Figure 3.3C). As ShhN::YFP and SPShhC::CFP did not coimmunoprecipitate with the empty vector pcDNA3-myc-his, these results indicate that ShhN and ShhC interacts with Sort1. ShhN::Fc also coimmunoprecipitated with the positive control myc-his tagged Hhip (Hhip::myc-his), and surprisingly, SPShhC::CFP was also detected with Hhip::myc-his immunoprecipitates. This observation is the first indication that Hhip interacts with Shh-C.

3.4.2.2 Glypican 5 (Gpc5)

Gpc5 demonstrates exciting potential to be a Shh interacting protein. Firstly, Gpc5 is a heparan sulphate proteoglycan (HSPG) whose homologues have been demonstrated to bind to Hh proteins in both flies and mammals (14, 28-36). Secondly, the punctate pattern of Shh accumulation on the surface of neurons resembles the dlp-dependent accumulation of

Drosophila hh on S2 cells which depends on a Cardin-Weintraub (CW) sequence in the hh-N fragment (44). The CW-sequence in mammalian Shh is conserved and has been shown to bind to HSPGs (45, 46), and interaction of Shh with Gpc3 has recently been reported (27). Lastly, Gpc5 has a restricted expression pattern in the perinatal rodent brain. To verify that Gpc5 interacts with ShhN, I cotransfected 3XFLAG-tagged Gpc5 (Gpc5::3XFLAG) with either ShhN::Fc or ShhN::AP in COS cells and performed coIP using Protein A beads. In this assay, Gpc5::3XFLAG coimmunoprecipitated with ShhN::Fc, but not the negative control ShhN::AP (Figure 3.4B), indicating that Gpc5 interacts with ShhN.

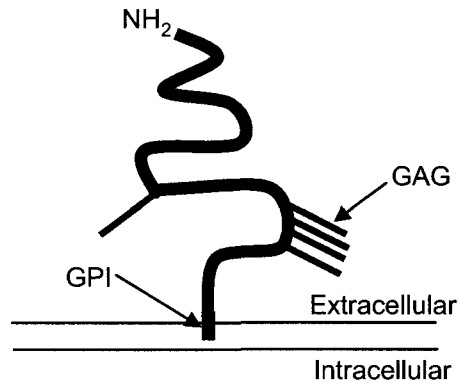
I subsequently provided further verification that Gpc5 interacts with Shh by performing a reciprocal coIP in COS cells expressing Gpc5::3XFLAG and various fluorescent protein tagged Shh isoforms. coIP of these cotransfected cells using M2 affinity beads reveals that fluorescent-tagged ShhN (ShhN::YFP), non-lipid modified Shh (^{C25S}Shh::YFP), uncleaved Shh (^{C199A}Shh::YFP) and unprocessed Shh (^{C25SC199A}Shh::YFP) coimmunoprecipitated with Gpc5::3XFLAG (Figure 3.4C). Surprisingly, SPShhC::CFP also coimmunoprecipitated with Gpc5::3XFLAG. These results are not due to nonspecific binding of Shh to the beads as the fluorescent protein-tagged Shh constructs did not immunoprecipitate with the M2 affinity beads. Since the ^{C199A}Shh isoform is retained within transfected cell lines (Chapter 2; unpublished observations in COS cells; 38), these results indicate that Gpc5 can interact with Shh-N within the secretory pathway. Furthermore, these results indicate that Gpc5 interaction is independent of Shh-N cleavage or lipidation.

3.4.2.3 Low density lipoprotein receptor-related protein 1 (Lrp1)

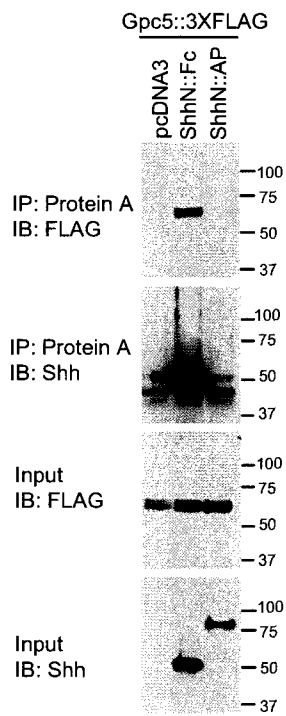
Lrp1 is a multifunctional receptor that participates in multiple signalling pathways and endocytosis (48-52). Due to the presence of multiple ligand binding motifs in the

Figure 3.4. Cspg5 interacts with Shh-N and Shh-C. (A) Structural organization of Gpc5. Gpc5 is a heparan sulphate proteoglycan (HSPG) that is tethered to the cell surface by a glycosylphosphatidylinositol (GPI) anchor and contains four to five HS glycosaminoglycan (GAG) attachment sites adjacent to the plasma membrane. Adapted from (47). (B) Epitope tagged Gpc5 (Gpc5::3XFLAG) was cotransfected with pcDNA3, ShhN::Fc or ShhN::AP in COS cells and subjected to coimmunoprecipitation using Protein A beads. Gpc5::3XFLAG coimmunoprecipitated with ShhN::Fc but not with the negative control ShhN::AP. Additionally, Gpc5::3XFLAG did not bind nonspecifically to the Protein A beads. (C) Gpc5::3XFLAG was cotransfected with fluorescent tagged amino terminal Shh (ShhN::YFP), delipidated Shh (^{C25S}ShhN::YFP), uncleaved Shh (^{C199A}Shh::YFP), unprocessed Shh (^{C25SC199A}Shh::YFP) or carboxyl-terminal Shh (SPShhC::CFP) in COS cells. Clarified cell lysates were immunoprecipitated using α -FLAG M2 affinity Sepharose beads and analysed by western blotting. Under these conditions, all Shh isoforms coimmunoprecipitated with Gpc5::3XFLAG. In contrast, none of the fluorescent Shh isoforms nonspecifically coimmunoprecipitated with the negative control pcDNA3.1-3XFLAG. Asterisks denote contaminating heavy and light IgG subunits. Numbers indicate molecular weight (kDa).

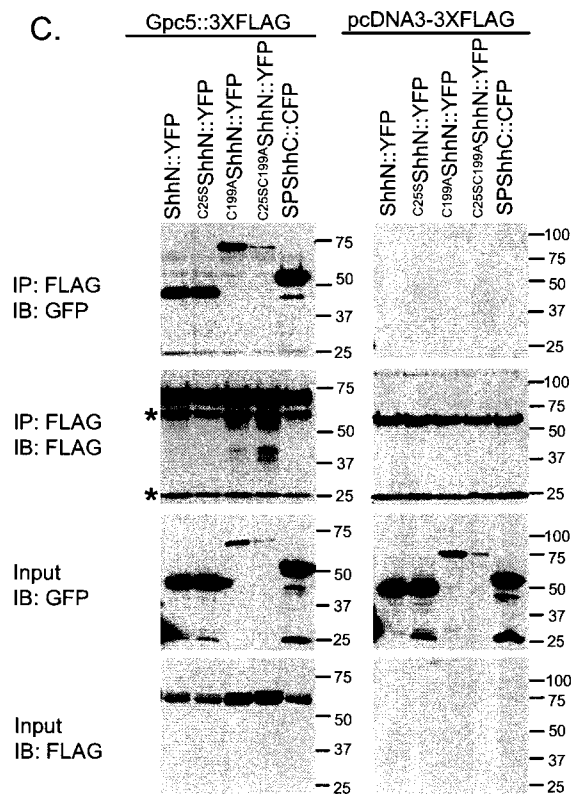
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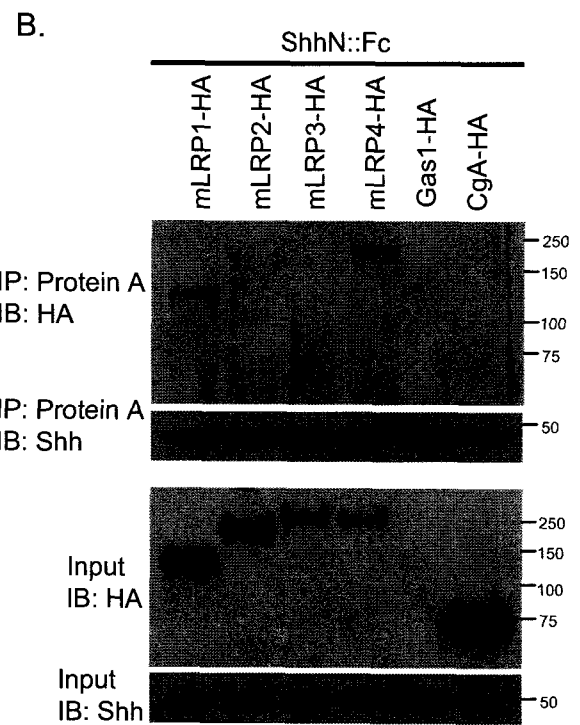
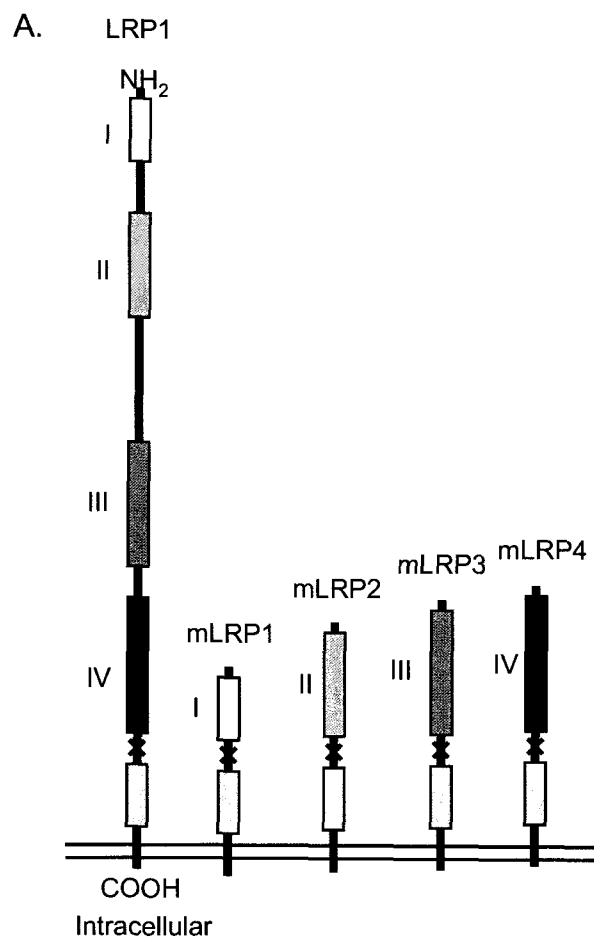
extracellular domain, Lrp1 binds a variety of ligands, including lipoproteins, hormones and growth factors (48-50), and a recent study has indirectly implicated Lrp1 in Shh signalling (26). In cultured granule neuron precursor cells, Hh-dependent activation of *CyclinD1* is blocked by application of the Lrp1 ligand binding inhibitor, Protease nexin 1 (Pn-1; 26). Moreover, Lrp1 is a relative of Lrp2 (also known as Megalin), which binds and internalizes Shh (11, 16). Thus, Lrp1 demonstrates the capability to be a Shh-N interacting protein.

To verify that ShhN interacts with Lrp1, I cotransfected ShhN::Fc with membrane-bound Lrp1 minireceptors (mLrp) that represent each of the four putative extracellular ligand-binding domains of Lrp1 (Figure 3.5A; 53). In addition to a ligand binding domain, each mLrp construct contains an amino terminal HA tag, the carboxyl-terminal region and the furin-cleavage site. Following furin cleavage, each mLrp is processed into two subunits, with the carboxyl terminus non-covalently linked to the ligand-binding domain. Cotransfection and subsequent coIP with Protein A beads reveals that mLrp1 and mLrp4, corresponding to domains I and IV of Lrp1, respectively, binds with ShhN::Fc. Importantly, HA-tagged chromogranin A (CgA-HA), a protein that is sorted to the secretory pathway and is localized on cell surface, did not coimmunoprecipitate with ShhN::Fc (54).

3.5 Discussion

In this study, a GST-pulldown method was performed to identify novel Shh-interacting proteins in brain tissue. A microsomal extract from rat brains was used as prey and bacterially-expressed Shh-N and Shh-C GST fusion proteins were used as bait. Perinatal rat brains were used as the source material because Shh signalling in the brain is still active at this developmental stage and I could obtain sufficient brain material for pulldowns. To obtain a more comprehensive protein interaction profile, I performed independent GST-

Figure 3.5. ShhN interacts with domains I and IV of Lrp1. (A) Schematic representation of the Lrp1 minireceptors (mLrp). Each of the four mLrp is depicted in comparison to the full-length Lrp1. The four putative extracellular ligand binding domains are labelled with Roman numerals I-IV. The site of furin cleavage is indicated with a red X. Adapted from Obermoeller-McCormick et al. (53). (B) mLrp1-4 tagged with HA, Gas1-HA or CgA-HA (serves as a negative control) were cotransfected with ShhN::Fc in COS cells and clarified cell lysates were immunoprecipitated with Protein A magnetic beads. Note that Gas1-HA did not express in this experiment. Numbers indicate molecular weight (kDa).



pulldown experiments with protein preparations that were solubilised with two different detergents, NP-40 and CHAPS. The rationale for screening for Shh-C interacting proteins is based on from work in *Drosophila* showing that a tyrosine residue in hh-C is required for hh axon trafficking and activity at the growth cone (55). As well, the carboxy-domain of Hh might have targeting motifs that act independently of Hh-N, as the hh-C fragment is trafficked within synaptic vesicles and is enriched at the growth cones, while a large portion of hh-N is retained at the cell body (55). Furthermore, the only known function of the carboxyl terminus of mammalian Hh is cleavage of the Shh propeptide into N- and C-terminal halves (37, 56, 57). Thus, it is reasonable to speculate that the carboxy-terminal has a yet-undefined role in mammalian cells. Although it is possible that there may be proteins that interact with uncleaved Shh, my initial attempts at purifying soluble full-length Shh resulted in insufficient amounts of material and, therefore, I was unable to perform the GST-pulldown screen with full-length Shh.

While the affinity purification approach is a useful tool to screen for novel interacting proteins, there are several caveats with this technique. First, this approach would not identify proteins that interact with lipid modified Shh as I used nonlipid modified Shh protein for the GST-pulldown; however, there are several examples of Shh-interacting proteins including Ptc, Hhip, Cdo, Boc, Gas1, Gpc3 or Lrp2 that do not require lipid modification of Shh for binding (2-4, 7, 10, 16, 27, 44, 58, 59). Second, this affinity screen involves mixing a heterogeneous protein extract with molar excess of bait proteins in a nonphysiological setting. As a result, some of the proteins identified in this approach could be false positives due to nonphysiologically relevant interactions. For instance, highly abundant proteins, such as metabolic enzymes and heat shock proteins are a common background artefact in affinity purification screens (60, 61). Several of the metabolic and heat shock proteins were

identified in the GST-pulldown and thus were excluded from further analysis. As well, the introduction of mixed protein extract can result in false positives if the identified interacting proteins are normally segregated *in vivo* due to different tissue or subcellular distributions. However, many of the identified candidates do not have a widespread distribution pattern in the brain, and the expression patterns of candidate proteins are consistent with Shh localization within the CNS.

Third, the *in vitro* milieu that is used to probe for interaction of Shh fusion proteins with the solubilised protein extract does not resemble *in vivo* conditions. Specifically, the protein interaction solution used in this study included NaCl, MgCl₂ and detergent, and as a result, the protein interaction solution might not be permissive for certain interactions. For instance, Ca²⁺ is required for the binding of bacterially-produced Shh/Ihh and Cdo in a cell-free interaction assay (5), and thus, I would fail to identify interacting proteins that require Ca²⁺ for interaction with Shh. As well, the detergents NP-40 and CHAPS were used to solubilise the microsomal fraction and maximize protein extraction of membrane-bound proteins. While NP-40 and CHAPS are commonly used to solubilise numerous proteins, these detergents do not solubilise the majority of membrane-bound proteins (62). Repeating the affinity screen using different protein interacting solutions may overcome this limitation.

Fourth, it is possible that I did not isolate all of the putative interacting partners from the GST-pulldown method. I attempted to specifically isolate the unique, dominant bands observed on a 1-D acrylamide gel and it is possible that the dominant bands masked any faint neighbouring bands. As well, it is possible that proteins with higher affinity for either Shh-N or Shh-C would outcompete any weaker affinity proteins, resulting in a reduction of the protein interacting profile. For this reason, I likely only identified a subset of potential Shh interactors.

Lastly, the technique used to prepare the microsomal extract was designed to maintain as much of the *in vivo* protein complexes as possible. As a result, there are cytoplasmic proteins or seemingly unrelated proteins that, at first glance, would be discarded as a false positive. For example, Shh is unlikely to directly interact with the cytoplasmic-localized transport motor Kif1A; however, it is possible that Shh indirectly interacts with this motor protein through a complex containing a Shh-bound transmembrane protein that tethers to Kif1A through an accessory protein (63).

Despite the potential caveats of the GST-pulldown technique, this screen identified Lrp1 and Gpc5, two proteins that have homologues that are important for normal Hh signalling, which provides support that the affinity purification procedure was able to isolate known Hh signalling components.

3.5.1 Identification of ShhN and ShhC interacting candidates

The *in vitro* screen for novel Shh interacting proteins identified a number of excellent candidate proteins. These candidates are postulated to function in Shh PTM, intracellular trafficking, extracellular transport and reception. While there are a number of excellent candidates to validate by preliminary coIPs in cotransfected COS cells, I chose three candidates because their putative functions are predicted to influence Shh intracellular transport, extracellular movement or reception.

3.5.1.1 Sort1

Sort1 (also known as neurotensin receptor-3 [NTS3] or GP95) is a lipid raft-associated single-pass transmembrane receptor that binds to a variety of unrelated ligands (41). The majority of Sort1 resides in the Golgi apparatus and is involved with intracellular

trafficking of neurotensin, sphingolipid activator protein, lipoprotein lipase and brain-derived neurotrophic factor (BDNF; 20, 40-42, 64). Sorting of proteins that lack cytoplasmic domains to the regulated secretory pathway is mediated by protein aggregation, sorting motifs and/or interactions with sorting receptors in the lumen (65-67). Both wild type and several mutant isoforms of hh can be targeted to axons in the *Drosophila* visual system (55) and to the regulated secretory pathway and neurites in mammalian neurons (Chapter 2). Moreover, several critical residues in the hh-C domain are important for transport of hh-N to the growth cones (55). These observations are consistent with the possibility of additional receptor-mediated mechanisms of Shh sorting in neurons.

Sort1 contains two main domains; a luminal/extracellular region and a cytoplasmic tail. The luminal domain exhibits high homology to the *Saccharomyces cerevisiae* protein VPS10P, a sorting receptor that directs the trafficking of lysosomal enzymes from the Golgi to the vacuole (68). Sort1 is initially produced as a propeptide that is enzymatically cleaved in the TGN by the endopeptidase furin (20). The propeptide has two functions; prevention of premature ligand binding and facilitating Sort1 transport in the biosynthetic pathway (69, 70). Sort1 also binds to the endoplasmic reticulum (ER)-resident receptor-associated protein (RAP) which prevents premature binding of ligands to Sort1 (20, 69). While Sort1 also has the potential to shed its extracellular domain (71); the functional significance of ectodomain shedding is unclear. Lastly, Sort1 has a short cytoplasmic tail that contains a YXX Φ and several dileucine motifs that mediate endocytosis and intracellular trafficking of bound ligands (39).

Sort1 expression is localized in several discrete regions within the CNS. Sort1 is expressed in pyramidal cells of the cerebral cortex, granule cells in dentate gyrus and pyramidal cells in hippocampus, Purkinje cells in cerebellum and RGCs (20, 72-74). In

neurons, Sort1 has three main roles, (1) facilitating intracellular sorting of proteins, (2) mediating endocytic uptake and bioavailability of ligands and (3) regulating p75^{NTF}-dependent apoptosis. Sort1 is a well-characterized sorting receptor that facilitates vesicular trafficking of proteins to endosomal, lysosomal or secretory organelles assisted by specific adaptor proteins that tether to the cytoplasmic tail of Sort1. On arrival of cargo proteins at the trans-Golgi network (TGN) for trafficking to the secretory pathway, proteins are sorted to the appropriate organelle compartment. For example, Sort1 is present in both the TGN and immature secretory granules (ISGs) and directly interacts with ligands such as BDNF (42). Interference with Sort1-BDNF interaction impairs sorting of BDNF to mature SGs without affecting constitutive secretion (42). The minority of cell surface Sort1 rapidly internalizes bound ligands, including neurotrophins, and is trafficked by retrograde sorting mechanisms (39, 75, 76). Sort1 also engages in intracellular signalling by triggering apoptosis upon formation of a trimeric complex with proneurotrophins and the p75 neurotrophic receptor (77).

As Shh coimmunoprecipitated with Sort1 in a reciprocal fashion in cotransfected COS cells, these results suggest that Shh could interact with Sort1 whereby Sort1 may regulate Shh endocytosis or mediate intracellular Shh trafficking as a sorting receptor. For instance, Sort1 mediates rapid lipoprotein internalization that is trafficked to lysosomes, late endosomes and TGN with no recycling of bound lipoproteins to the cell surface (78). Thus, Sort1 may act as an endocytic receptor that sequesters mature Shh-N (ShhNp) and targets ShhNp to lysosomes for degradation. Alternatively, Sort1 may function as a coreceptor with Ptc to activate the Hh signal pathway. As the Shh-C domain is secreted (37, 56), it is possible that Sort1 can bind and internalize secreted Shh-C. Second, as Shh is sorted to SGs, Sort1 could be involved in targeting Shh to SGs via interaction with motifs in the Shh protein. For

example, the *Drosophila* carboxyl domain of hh contains several residues that are required for proper presentation of hh-N to growth cones in photoreceptors (55), implicating this region as a sorting motif that could interact with sorting receptors. However, motifs within mammalian Shh-C are unlikely to serve a similar function for Shh-N targeting, as all Shh isoforms were sorted to SGs. Nevertheless, it is possible that Sort1 may interact with motifs within the Shh-N domain to facilitate Shh-N intracellular sorting. To test whether Sort1 is involved in cell surface binding, one can incubate Sort1-expressing cells with increasing concentrations of ShhN::AP and test if the binding pattern follows a dose-response curve (7). If this is found to be the case, then one can follow up with cell-based assays and subcellular colocalization approaches to determine whether Sort1 functions to augment Shh signalling or to sequester Shh by endocytosis. To determine whether Sort1 functions as a sorting receptor, one can analyse the subcellular distribution of Shh after RNA interference-mediated knockdown of Sort1 or introduction of a Sort1 dominant negative (79) in neurons transfected with Shh.

3.5.1.2 Gpc5

Glypicans are a family of HSPGs that are tethered to the cell surface through a glycosylphosphatidylinositol (GPI) anchor. In *Drosophila*, there are two glypicans (dally [dly] and dly-like protein [dlp]) and six family members in mammals (Gpc1 to Gpc6; 80). Glypicans share conserved domains and consensus sites for addition of HS glycosaminoglycan (HS-GAG) chains, which likely results in similar tertiary structures among the glypicans (80). Several studies have implicated mammalian and *Drosophila* glypicans in regulation of morphogen activity in a stage- and tissue-specific manner through

stabilization and/or promotion of these morphogens with their cognate receptors or by shaping morphogen gradients (80).

Glypicans undergo several maturation steps early in the biosynthetic pathway. Glypicans have a secretory signal peptide and a hydrophobic domain that is required for addition of the GPI anchor. During glypican biosynthesis, HS-GAG chains are added to core proteins in the Golgi apparatus and these chains are further modified by sulphation and epimerization before transportation of the mature protein to the cell surface (80). HS modifications confer the ability of glypicans to bind to a wide variety of ligands (80). Glypicans also contain a C-terminal signal that is necessary for GPI anchor attachment that results in association of glypicans to lipid rafts (81). Alternatively, cleavage of the GPI anchor may be a mechanism of regulated release of proteins bound to glypicans located in the extracellular matrix (82-85). As well, the GPI anchor also allows glypicans to internalize ligands and these ligands can potentially be recycled to the cell surface (27, 86).

Several studies have highlighted the importance of glypicans in Hh signalling. Patients that have mutations within the human *Gpc3* gene and *Gpc3*^{-/-} mice exhibit a number of developmental abnormalities, including tissue overgrowth, polydactyly and fused vertebrae (87, 88). Specifically, *Gpc3* serves as a negative regulator of body size by sequestering Shh through competition with *Ptc1*, and induces Shh endocytosis that targets Shh for lysosomal degradation (27). Interestingly, the HS-GAG chains of *Gpc3* are not required for interaction with Shh (27), which is unlike the interaction between heparan sulfate (HS) and Shh (89). In contrast to *Gpc3*, *dlp* (which is the *Drosophila* homologue of mammalian *Gpc4/6*) is a positive regulator of hh signalling (17, 30, 36, 90). *dlp* colocalizes with hh and *ptc* in endocytic vesicles in hh-receiving cells, and in *dlp* mutant tissues, hh target gene expression is reduced in intensity and width (36, 90). Removal of the GPI anchor

of dlp alters the subcellular localization of hh and inhibits Hh signalling (36, 90). Conversely, in the absence of ptc, there was no dlp-dependent hh endocytosis in receiving cells (36). Thus, dlp appears to function by increasing ptc-dependent internalization of hh, resulting in a high level of hh pathway activation. The differences between dlp and Gpc3 could reside on the type of Hh binding to these glypicans; stable interactions between hh and dlp are only possible with a tripartite complex with ptc and Shh directly binds to Gpc3 with high affinity.

All of the Shh isoforms, including delipidated and uncleaved Shh, coimmunoprecipitated with Gpc5, which is consistent with a direct, lipid-independent interaction of Gpc5 with Shh. As Gpc5 belongs to the same subfamily as Gpc3 (80, 91), it is likely that Gpc5 also negatively regulates the Shh pathway during development. *Gpc5* is expressed in neural precursors of the striatum primordium and ventral diencephalic wall at E10, at the anterior telencephalon at E14.5, and in post-mitotic neurons in the brain (92, 93). Moreover, *Gpc5* is expressed in the developing limb bud, coinciding with the onset of mesenchymal proliferation and condensation (93). As Shh is expressed in these tissues at these stages, it is possible that Gpc5 interacts with Shh to negatively regulate Shh signal transduction in a manner similar to Gpc3. Interestingly, Gpc5 interacted with uncleaved Shh and with the Shh-C domain. As uncleaved Shh is not secreted in a number of nonneuronal cell lines (Data not shown, 38), this observation suggests that Gpc5 interacts with Shh early in the secretory pathway, likely before transport of Shh to the cis-Golgi. As clustering of hh into large punctate structures on the cell surface is dependent on dlp (44), it is possible that Shh interacts and binds to Gpc5 in the ER-Golgi and these proteins are cotransported to the cell surface in which Gpc5 facilitates nanoscale clustering of Shh and subsequent promotion of multimeric complexes. It would be of considerable interest to determine whether Gpc5

mainly functions in producing or receiving cells, if the CW motif of Shh-N is required for Gpc5 binding, and if there is a physiological impact between interactions of Gpc5 and Shh-C.

In vitro and *in vivo* approaches can be used to address if Gpc5 regulates Shh transport or activity. For instance, one can examine whether co-expression of Gpc5 and Shh in producing cells is required for Gpc5-dependent clustering of Shh-N or Shh-C on the cell surface, formation of Shh multimers or for efficient secretion. As well, one can examine if Gpc5 is responsible for Shh-N internalization in receiving cells to sequester Shh from Ptc or whether Gpc5 functions to augment the Shh pathway. Alternatively, an investigator can produce a Gpc5 knockout mouse model and examine if Gpc5^{-/-} mice leads to dysregulation of the Shh signalling pathway.

3.5.1.3 Lrp1

Lrp1 is a large single-pass transmembrane receptor that is expressed in numerous tissues. Lrp1 has diverse roles in development and tissue homeostasis, including lipoprotein metabolism, degradation of proteases, activation of lysosomal enzymes and viral infection (50). Although deletion of Lrp1 leads to lethality by E12, the cause of this phenotype is not clear (94). However, the analysis of tissue-specific knockout mice indicates that Lrp1 is important for the developing vascular system and function in the adult CNS, macrophages and adipocytes (95). The wide variety of important roles of Lrp1 in these systems likely results from the interaction of its extracellular domain with numerous ligands, the ability of the cytoplasmic domain to associate with a number of adaptor proteins and its association with other transmembrane receptors (48-52, 95).

In the adult brain, Lrp1 is localized in the proximal processes of neurons in the external granule layer and Purkinje cells in the cerebellum and the granule and pyramidal layers of the dentate gyrus (96). Interestingly, Lrp1 has been demonstrated to interact with postsynaptic proteins (97). Moreover, Lrp1 has been immunolocalized to astrocytic foot processes (96). Several *in vitro* studies have indicated that Lrp1 has an important role in neuronal metabolism by modulating the extracellular environment through the endocytic uptake of extracellular proteins (95); however, Lrp1 function in CNS development is unknown. A conditional mouse knockout of Lrp1 in differentiated neurons results in mice with behavioural and motor abnormalities including hyperactivity, tremor and dystonia, and finally cachexia and premature death (97). Interestingly, no histological abnormalities were noted, indicating that gross developmental processes were not impaired and that the observed abnormalities are a result of a functional defect in Lrp1^{-/-} neurons (97). However, this model used tissue-specific gene disruption under the control of synapsinI promoter which is only active in differentiated neurons (97).

Lrp2 (aka Megalin) is another multifunctional member of the LDL family that demonstrates high homology to Lrp1. Like Lrp1, Lrp2 is classified as a scavenger receptor due to its multiligand binding properties including endocytosis and transport of ligands including lipoproteins, drugs, hormones, enzymes and Shh (98, 99). Lrp2 knockout in mice results in defects in ventral forebrain development, leading to HPE (100, 101). Furthermore, Lrp2 was demonstrated to bind and mediate endocytotic uptake of Shh (11, 16). Although Lrp2 is important for development, whether Lrp2 affects Shh extracellular transport has not yet been investigated.

Cotransfection of ShhN and Lrp1 minireceptors and subsequent coIP in COS cells revealed that ShhN interacts with domains I and IV of Lrp1. Thus, it is possible that Lrp1

may mediate Shh function in the developing and adult CNS. In cultured granule neuron precursor cells, Hh-dependent activation of *CyclinD1* is blocked by application of the Lrp1 ligand binding inhibitor, RAP (26). Similarly, addition of α -Lrp2 antibodies or RAP blocks ShhN uptake in both *in vitro* and *in vivo* models that express Lrp2 (11, 16). Moreover, a Lrp1 ligand, the serine-protease inhibitor protease nexin 1 (Pn-1), also interferes with Hh signalling in granule precursor cells, and Pn-1^{-/-} mice display Shh gain-of-function phenotypes when mutated in mice (26). Thus, it is possible that Lrp1 has a positive role in Shh signalling and that Pn-1 competes with Shh binding to Lrp1. For instance, Lrp1 may function as a coreceptor with Ptc and lead to heteromeric receptor complexes, similar to the receptor complex formation between Frizzled and Lrp5/6 (102). Additionally, since Lrp1 has been demonstrated to bind to a number of lipoproteins (48-52, 96) and *Drosophila* hh has been shown to copurify with lipoproteins (103), it is possible that *in vivo* Shh binds to Lrp1 in lipoprotein complexes. Overall, multiple interactions between Shh, Ptc1, Lrp1 and lipoproteins may regulate Shh signalling in CNS development.

3.5.2 Interaction of Shh-C with Hhip

The serendipitous finding that Shh-C coimmunoprecipitated with Hhip suggests that the carboxy terminal is involved in other functions. Importantly, the Hhip:Shh-C interaction does not appear to be an artefact, as Shh-C did not nonspecifically bind to the affinity beads. Hhip was initially identified from a cell-based cDNA screen to identify novel factors that binds to extracellular ShhN (10) and subsequent genetic and biochemical studies have implicated Hhip as a negative regulator of the Hh pathway by sequestering Hh-N on the cell surface (10, 12, 104-107). However, these studies did not consider the possibility that Hh-C may also interact with Hhip. Furthermore, with the exception of Chu et al. (55), there are no

existing publications that have implicated additional functions for Hh-C beyond Hh proteolysis and cholesteroylation. Interestingly, several residues in hh-C appears to be essential for intracellular hh-N transport in *Drosophila* photoreceptor axons (55). As well, hh-N and hh-C appear to occupy the same secretory vesicle in the photoreceptor axons (55). As cleavage of the Hh protein into the amino and carboxy domains likely occurs before the *cis*-Golgi (38, 55) and protein sorting occurs within the *trans*-Golgi network (108), it is possible that hh-N and hh-C are actively sorted to the same transport vesicle. Furthermore, Hh-C can be secreted *in vitro* when Hh is translated as a full-length construct (37, 56). Lastly, as the molecular composition of Hh-N large multimeric complexes is relatively uncharacterized, it would be of considerable interest to determine whether Hh-C is also part of these multimeric complexes. While Hh-C is indispensable for Hh cleavage and Hh-N cholesteroylation in *Drosophila* and mammals, it is unknown if the phenotype of cholesterol-deficient HhN mutants is due to the lack of cholesterol modification, is a consequence of the absence of Hh-C, or is a result from the combination of no cholesterol and the absence of Hh-C. Thus, it is possible that Hh-C may bind to existing and possibly novel proteins involved in the Hh pathway.

Several studies can be performed to validate this novel finding that Hhip and Shh-C do interact. To determine whether Shh-C interacts with proteins such as the candidates identified in the GST-pulldown or Hhip in a nonspecific manner, coIPs with unrelated receptors such as Transferrin receptor (TfR) can be performed. For instance, as Shh does not colocalize with TfR and internalized Tf in neurons (Chapter 2), it is unlikely that Shh-C interacts with TfR. If Shh-C does not coIP with TfR, then this assay would indicate that the Hhip:Shh-C interaction is not an artefact arising from the coIP method. Further validation studies, such as application of AP-tagged Shh-C in a dose-response fashion to Hhip-

expressing cells would delineate whether Shh-C binds to cell surface Hhip in a concentration-dependent manner. Furthermore, it would also be of considerable interest to determine whether the Hh large multimeric complexes include Hh-C.

3.5.2 Summary

The affinity purification screen identified several putative Shh interacting proteins and these identified candidates demonstrate potential to be involved in Shh trafficking, extracellular transport and reception. Although I only chose three candidates to perform preliminary coIP validation experiments, several other candidates have potential to be genuine interacting partners and able to shape the Shh signalling pathway.

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4. DISCUSSION

The objective of my thesis was to characterize the cellular regulation of neuron-derived Shh trafficking. To address how the highly specialized morphology and physiology of neurons contribute to the trafficking, secretion and reception of Shh, I investigated the intracellular trafficking pattern of Shh mammalian neurons and performed an affinity screen to identify novel Shh-interacting proteins. I found that mature Shh is distributed in a punctate pattern on the cell surface in neurons and is associated with lipid rafts. Additionally, Shh is sorted to two major classes of the regulated secretory pathway (RSP), secretory granules (SGs) and synaptic vesicles (SVs). Posttranslational modifications (PTMs), including proteolysis and lipid modification, are not required for Shh trafficking to SGs, but PTMs are required for cell surface accumulation and sorting of Shh to SVs. Consistent with a vesicular pattern of Shh in neurons, Shh⁺ puncta are bidirectional, occur with fast and slow kinetics, and Shh secretion can be induced by depolarization. The *in vitro* screen for novel Shh interacting factors identified a number of candidates that are predicted to function in Shh PTMs, intracellular trafficking, extracellular transport and reception. Because of the biological relevance of Shh signalling in CNS development, these findings will further our current knowledge of how the molecular mechanisms of Shh signalling patterns the CNS.

4.1 Shh is transported in neurons

Neurons are polarized cells with processes that can span long distances (e.g., 20-40 mm in human optic nerves and > 1 m in human motor neurons) and have a highly specialized RSP. How these features contribute to the range of growth factor and morphogen signalling in neurons is just beginning to be appreciated. There is an evolutionary conserved role for

axoplasmic transport of Hh proteins in neurons to pattern the brain. In the developing *Drosophila* visual system, blocking the ability of lamina precursor cells to respond to a Hh signal derived from photoreceptor axons interrupts lamina neuron differentiation and subsequent retinotopic map formation (1-4). In mammals, Shh is transported in an anterograde manner in retinal ganglion cell (RGC) axons to the superior colliculus where the RGC axons synapse and regulates gliogenesis in the optic nerve and possibly the brain (5-8). My *in vitro* findings demonstrate that Shh is trafficked to the RSP and accumulates the surface of neurites. These findings are consistent with this model for axon-mediated long range Shh signalling in neurons. While Shh trafficking and secretion has been studied primarily in nonneuronal cells where it is thought that long-range signalling is primarily mediated by transport of the protein in the extracellular space, my results provides additional support that neuron-derived Shh long range signalling is mediated by anterograde transport and regulated section.

My results indicate that neurons contain at least 3 pools of Shh, (1) on the cell surface, (2) SG and (3) SV precursors. The cell surface localization of Shh is consistent with its known function in signalling to progenitor cells in the retina and the optic nerve, and suggests that signalling in these tissues is mediated by direct contact of progenitors or astrocytes with RGC membranes. The novel observation that Shh is present in regulated secretory vesicles (RSVs) in vertebrate neurons raises additional question related to the function of this molecule in neurons. For instance, it implicates neuronal activity as a novel means of regulating Shh distribution in a tissue. As well, there may be additional sites of action of the molecule, for example at the targets of RGC in the lateral geniculate nucleus and the superior colliculus.

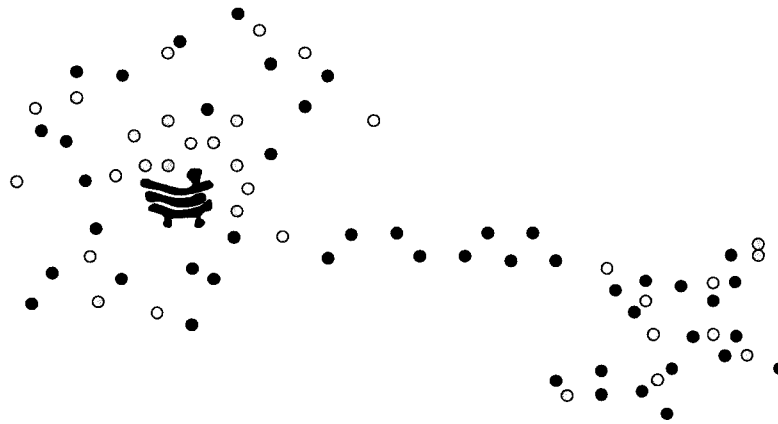
4.1.1 Shh protein accumulates on the cell surface in neurons

While Shh has been demonstrated to undergo axoplasmic transport, little is known about how Shh is targeted to axons or how secretion is regulated. In differentiated PC6 cells and primary hippocampal neurons, wild-type Shh was distributed in a punctate pattern along the cell body and neuronal processes (Figure 4.1). However, none of the delipidated or uncleaved Shh isoforms was retained on the cell surface (Figure 4.1). Thus, cleavage and dual lipidation may have a critical role for amino domain of Shh (Shh-N) subcellular localization in neurons.

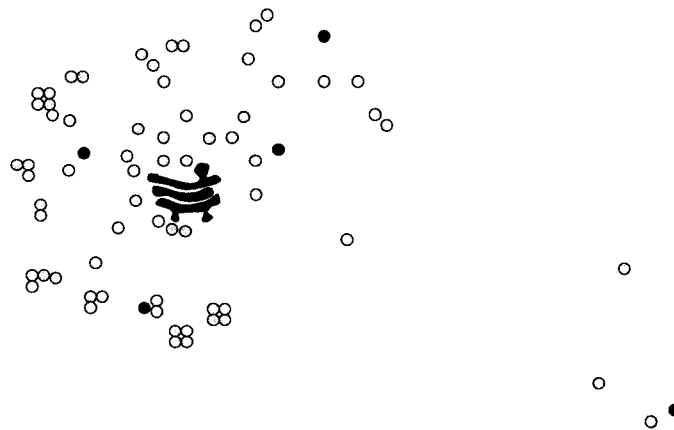
Although Shh is present in a punctate pattern on the extracellular surface, little is known about the composition of cell surface Shh puncta. A similar punctate accumulation of Hh proteins on the cell membrane has been observed in a number of cells and tissues, including wing imaginal discs, mammalian and *Drosophila* nerves, and in the neural tube (2, 9-13). The formation and distribution of cell surface puncta in Hh producing cells may be regulated through interactions of Hh with heparan sulphate proteoglycans (HSPGs), including the glypican family members. The punctate pattern of Shh accumulation that I observed on the surface of neurons resembles the dlp-dependent punctate accumulation of hh on *Drosophila* S2 cells (11). Indeed, an interaction of mammalian Shh with Gpc3 has recently been reported (14); however this study focused on the interaction of Shh and Gpc3 in receiving cells and did not characterize Shh-Gpc3 interactions in Shh-expressing cells. Interestingly, I identified Gpc5 as a putative Shh-N interacting protein. Surprisingly, Gpc5 interacted with an uncleaved form of Shh, which is believed to be retained within the *cis*-Golgi (15), suggesting that interactions between glypicans and Hh in producing cells may originate in the biosynthetic pathway. Interaction of Shh and these HSPGs within the ER-

Figure 4.1. Schematic of Shh trafficking in neurons. (A) Wild-type Shh is distributed intracellularly (green) and on the extracellular surface (red) of the soma and neurites in differentiated PC6 cells and primary hippocampal neurons. Shh is trafficked down axons in synaptic vesicles (SVs), dendrites in secretory granules (SGs) and likely undergoes local secretion through the constitutive secretory pathway. (B) Perturbation of Shh posttranslational modification results in a lack of Shh extracellular association and results in accumulation of Shh protein in SGs at the neurite terminus. As a result, the majority of Shh mutants are trafficked to the somatodendritic compartment.

A. Wild-type Shh - Transported in SGs and SVs



B. Mutant Shh - Transported in SGs



- Extracellular Shh protein
- Intracellular Shh protein

Golgi and subsequent transport to the cell surface may thus promote the formation of multimeric complexes, enable Shh to be released by Disp or facilitate interactions with other factors.

One of the limitations of conventional confocal microscopic analysis to study protein distribution is that confocal microscopy cannot distinguish whether cell-surface Shh is present as diffuse monomers that are in close proximity or oligomerizes into multimeric complexes (11). It remains to be seen if these punctated structures on the cell surface are capable of long-range signalling and whether Shh is packaged into multimeric complexes. However, there does not appear to be a graded Hh target gene expression or a detectable concentration gradient of Shh that emanates from neurons, suggesting that neuron-derived Shh is unlikely to undergo long-range extracellular transport (7, 9, 16). Currently, the developing neural tube and limb bud are the only mammalian developmental processes whereby a Shh protein gradient has been directly proven by immunohistochemical, in situ hybridization and genetic techniques (9, 12, 17-21). However, attempts from the Wallace laboratory to detect endogenous Shh distribution in retinal and brain tissues using conventional anti-Shh reagents have been inconclusive due to poor signal-to-noise ratio, likely related to the low level of expression of Shh in neurons compared with tissues that express high levels of Shh, such as the floor plate or notochord. Additionally, the in situ hybridization method only provides a crude readout of Hh gene target activity. Thus, any subtle gradients that might be present in the retina or from RGC axons would not be detected.

The finding that Shh forms punctate structures on the cell surface suggests that surface-localized Shh mediates short-range signalling to neighbouring cells through cell-cell contact. Abrogation of Shh expression from RGCs leads to RGC axon pathfinding defects,

reduced proliferation and total number of glial cells in the optic nerve, and Wallerian degeneration of RGC axons (6, 22). Supporting a role for Shh signalling in optic nerve development, application of antibody-secreting hybridoma cells that neutralize Shh function downregulated Shh target gene expression and attenuated proliferation of optic nerve astrocytes (7, 8), supporting the hypothesis that Shh is in the extracellular space in the nerve. As well, application of cyclopamine, a potent Shh inhibitor, also decreased the number of oligodendrocytes in the optic nerve (8). These studies indicate that Shh directly signals as a short-range molecule to influence development of the optic nerve. Moreover, Shh is also present on the somatodendritic surface and its presence on the cell body/dendrites is consistent with the *in vivo* biological effects of Shh in the visual system. Basal secretion from the somatodendritic region locally regulates retinal development by promoting cellular proliferation of retinal progenitor cells and modulating cell fate induction (23-30). For instance, in a mouse model where Shh expression is ablated in the peripheral retina, but Shh is still expressed in the central retina, Hh target gene expression is not rescued from the neighbouring Shh-expressing RGCs and the retinal progenitor cell population is depleted (24), indicating that Shh functions as a direct short-range signal in the retina. Overall, these reports suggest that neuron-derived Shh has the potential to signal to cells at the cell body, dendrites, along the length of axons, growth cones and at synapses.

The surface presentation of Shh on axons raises the issue of the timing of Shh signalling in the optic nerve. Shh expression is maintained in adult RGCs and the protein is detectable in the optic nerve and superior colliculus in adult rodents; however Shh target gene expression and glial proliferation in the optic nerve is extinguished by P7 (5, 7, 31-33). Thus, Shh is still trafficked in an anterograde direction within RGC axons at a stage where the classical Hh pathway in the optic nerve appears to be abolished. This discrepancy suggests

that there could be additional factors or mechanisms that regulate Shh signalling including temporally regulating Shh distribution and/or secretion, or even the Shh responsiveness of target cells. For instance, Hhip, an antagonist of the Hh pathway, is frequently observed in CNS regions where Shh immunoreactivity has been observed (34-36) and its presence likely results in differences of Shh responsiveness in receiving tissues.

In developing fly epithelia, the apical versus basal release of hh in producing tissues elicits distinct cellular responses (10, 13, 37). Interestingly, there was no obvious polarity in the distribution pattern of Shh in primary neurons, which is consistent with the distribution of epitope-tagged hh transgenes in the fly visual system (10). There are several possible pathways for dendritic and axonal protein sorting in neurons, including protein sorting at the Golgi that preferentially delivers cargo to the appropriate neurite or dendrite-to-axon transcytosis whereby axon-destined proteins are initially trafficked to the dendritic plasma membrane and are subsequently endocytosed and trafficked to axons (38). It is unlikely that membrane-tethered Shh is endocytosized and intracellularly trafficked via transcytosis as there was negligible overlap of wild-type Shh with Tf, an endosome marker. Moreover, the lack of Shh colocalization within endosomes in Shh-transfected neurons indicates that Shh is unlikely to be sorted to lysosomes or multivesicular bodies (39, 40). The absence of polarized Shh distribution in cultured neurons does not reflect a failure to polarize neurons in the cell culture model because I observed appropriate trafficking of transfected epitope-tagged dendrite and axon markers. Interestingly, the distribution of hh in the wing imaginal disc also does not appear to be dependent on transcytosis (41). A GFP-tagged hh transgene can be distributed to the anterior compartment in wing imaginal discs when endocytosis is blocked, indicating that hh does not undergo transcytosis (41). Therefore, if there are subcellular secretion site-dependent functional outcomes of Shh signalling from producing

cells, the level of regulation may not rest with the distribution of Shh; rather, it may be based on sorting to the appropriate secretory vesicle and any cofactors that Shh is secreted, as well as the composition of the extracellular matrix and receptors in producing and target cells.

4.1.2 Shh is transported in both fast and slow transported mechanisms in neurons

Shh was transported with fast and slow kinetics in anterograde and retrograde directions in neurons. This observation suggests that there are different molecular motors and multiple adaptors involved with Shh transport. Fast axoplasmic transport is consistent with the current paradigm that axonal Shh is actively transported *in vivo* (5, 42). Anterograde movement of Shh is likely mediated by kinesins which are the main plus-ended directed motors that transport organelles in neurons (43). Retrograde transport requires the activity of the cytoplasmic dynein motor protein (44, 45). Although the slow transport rate of Shh is likely intracellular transport, I cannot discount the possibility that the slow movement of Shh reflects Shh diffusion on the cell surface, as cell surface diffusion has similar kinetic rates (46, 47). Interestingly, two of the candidates identified in the GST::ShhN pulldown was Kif1A and Kif1B which have been shown to transport membrane organelles that contain synaptic precursor proteins such as synaptophysin and synaptogamin (48-50). As kinesins are connected to organelles via accessory proteins, such as coated proteins, scaffolds, GTPases or transmembrane proteins, there are multiple potential mechanisms of linking Shh-containing organelles to these motor proteins (51). Identification of intracellular motors is a key step of understanding how Shh movement is regulated.

4.1.3 Shh is associated with lipid rafts in neurons

As neurons are polarized cells with extensive protrusions, precise sorting and selective trafficking of proteins is essential for development and maintenance of specific structures and functions in neurons. Not surprisingly, mature Shh is associated with markers of lipid rafts, as several studies have implicated Shh to be a membrane raft resident protein (5, 52-54). Membrane rafts are believed to promote protein:protein and protein:lipid interactions in sterol and sphingolipid-enriched regions by facilitating membrane microdomain clustering of selected proteins and lipids (55, 56). Formation of raft microdomains originates in the Golgi, where raft proteins acquire detergent resistance and apicobasolateral sorting leads to differential segregation of proteins based on association with lipid raft microdomains (57, 58). Biochemically, detergent resistant membranes (DRMs) are defined by their resistance to detergents such as Triton X-100, but its use is highly contentious as this method is prone to artefacts and does not provide direct evidence that proteins which cofractionate with DRMs is associated with lipid rafts *in vivo* (59, 60). Therefore, I also examined the *in vivo* colocalization of Shh with a commonly used lipid raft marker, cholera toxin B subunit. As wild-type Shh is primarily associated with the lipid raft markers both intracellularly and on the cell surface, Shh is likely present with these raft membranes throughout the secretory pathway, including at the Golgi, in secretory vesicles and on the cell surface.

Association of Shh with lipid rafts may result in different Shh secretory mechanisms. Recently, hh has been demonstrated to be associated with a lipid raft-associated membrane-scaffolding protein Reggie-1 (61). Overexpression of Reggie-1 stimulates secretion of hh and augments hh extracellular diffusion. Conversely, loss-of-function of Reggie-1 attenuates hh

extracellular distribution, but maintains short range signalling (61). These authors speculated that hh may be secreted in two mechanisms that is dependent on association with Reggie-1; lack of association with this scaffolding protein leads to short-range hh signalling while the presence of functional Reggie-1 enables hh long-range transport. As the raft microdomains are hypothesized to compartmentalize cellular processes (56), association of HhNp with the raft compartments on the cell surface likely facilitates clustering of Hh puncta to promote oligomerization of Hh into multimeric complexes (11, 62). Interestingly, a recent study has implicated lipid raft-associated matrix metalloproteases for the shedding of HSPG-associated ShhNp complexes from the cell surface (63). The authors propose that the HSPG-dependent nanoscale clustering of Hh facilitates HhNp oligomerization into multimeric complexes at lipid rafts microdomains. Subsequently, HSPG-associated HhNp complexes that are capable of long-range signalling are released from the cell surface by metalloprotease-mediated ectodomain shedding. Thus, HhNp can be shed with associated HS chains, resulting in secretion of soluble and biologically active HhNp, whereas delipidated Hh-N is unable to form lipid raft-dependent preclustering, resulting in direct secretion of Hh-N (63). Overall, association of Shh within lipid raft domains likely facilitates clustering of Shh with interacting partners that leads to effective Shh secretion.

4.1.4. Shh is associated with the secretory pathway

A major finding from this thesis is the observations that processed ShhNp is associated with regulated secretory vesicles (RSVs). The first indication that Shh was trafficked by the RSP occurred when Shh was found to colocalize with Rab3A, a GTPase that binds selectively to RSVs (64, 65). Subsequent quantification of Shh and Rab3A colocalization revealed that ~75% of intracellular Shh⁺ pixels overlapped with Rab3A.

Secretory proteins associated with the constitutive secretory pathway (CSP) are proximally retained within the cell body and are not trafficked into axons or dendrites whereas proteins that are sorted to the RSP are trafficked to the neurite terminals (66). Thus, Shh protein is capable of being sorted to the distal ends of neurites. Surprisingly, ShhNp associated with both the SV and SG pathways. SVs traffic to the plasma membrane and mature through fusion with the plasma membrane and endosomes (67, 68). Since Shh minimally associated with Tf, a general marker for endosomes, this finding suggests that Shh is packaged into SVs at the trans-Golgi network (TGN). In fact, numerous SV precursor proteins originate from the TGN and undergo anterograde axoplasmic transport where these vesicles fuse with the plasma membrane. The association of Shh with SGs, which are typically enriched in the somatodendritic compartment (69, 70), raises the possibility that Shh may be differentially released at axons or dendrites. As the CSP is the default pathway, sorting into RSVs suggests that Shh is actively sorted to these pathways. Surprisingly, perturbation of Shh PTMs resulted in the accumulation of Shh isoforms with DCGs, indicating that PTMs are not required for Shh sorting to SGs. In contrast, mutations in the primary sequence and PTMs of BDNF results in missorting to constitutive secretory vesicles (CSVs) in neurons (71-74). Overall, these results indicate that neurons can sort Shh into the regulated secretory pathway which is the same system that is used for neurotrophins, neurohormones and neuropeptides.

Targeting of Shh to the RSP has several important implications in neuron-derived Shh signalling. Trafficking of Shh to the RSP may regulate axon guidance and enable Shh secretion at synapses while sorting of Shh to the CSP would allow Shh to signal locally to neighbouring cells. First, Shh has a cell-autonomous role in shaping axon guidance. A recent study demonstrated that Shh is expressed in a subset of RGCs, and inhibiting Shh signalling from RGC growth cones result in aberrant axon pathfinding defects (75). However, it is

currently unknown whether midline-derived Shh or axoplasmic transport of RGC-derived Shh guides RGC axon guidance in the optic nerve. Second, sorting to RSVs suggests a mechanism for Shh targeting and release at the synaptic targets of Shh-expressing neurons. Neurons that express Shh do synapse to progenitor regions, such as the SVZ and SGZ (76-86). Additionally, sorting of Shh into the RSP and subsequent trafficking to target sites may be critical steps for activity-dependent secretion of Shh. For instance, release of hh from photoreceptor axon terminals is essential for normal brain development in the fly (2, 4, 10). As well, Shh protein is detected in neurons in the adult forebrain that project to the SGZ through the fornix (87, 88). Severing this connection decreased progenitor dentate cell proliferation, which is consistent with the hypothesis that Shh undergoes axoplasmic transport (84). Interestingly, the mitogenic effect of Shh on progenitor cells in SGZ of the hippocampus is believed to be modulated by electrical activity (89). Third, it is likely that Shh is also sorted to the CSP, which implies that proximal secretion of Shh is mediated through CSVs and distal secretion is mediated through RSVs. Secretion from the CSP would allow Shh to be an autocrine signal; for example, as a local survival factor. For instance, packaging of BDNF into the constitutive or regulated secretory pathway influences the biological impact of BDNF (71-74). Overall, these findings indicate that Shh is capable of being sorted to distinct secretory vesicles that are targeted to different subcellular regions; SGs for somatodendritic secretion, CSVs for proximal secretion and SVs for release at presynaptic terminals.

4.1.5 Sorting mechanisms for Shh transport in neurons

The unexpected observation that Shh PTMs is not required for sorting to the RSP raises a few questions about the role of PTMs in Shh sorting. The finding that perturbation of

Shh PTMs in neurons results in accumulation of Shh isoforms within SGs and only a small fraction of these morphants are associated with Tf-positive endosomes is surprising as the CSV is believed to be the default pathway (90). As PTMs are required for sorting of Shh to SVs, this indicates that peptide motifs within the Shh protein, but not PTMs, might be important for directing Shh to the RSP. Interestingly, uncleaved hh can traffic to and be released from the synaptic terminals of photoreceptor neurons in *Drosophila* (10), whereas uncleaved Shh is retained in the pre-medial Golgi compartment in CHO cells (15). Thus, association of Shh with SGs is likely to be a cell-specific phenomenon. Supporting the finding that Hh proteins are sorted to different secretory pathways, *Drosophila* hh segregates away from a secreted form of GFP and, as a result, does not appear to enter the default secretory pathway in wing imaginal disc (91). Overall, it is likely that the presence of certain amino acid residues and possibly PTMs, including cleavage and dual lipid modification, of Shh leads to differences in cellular trafficking and movement within CNS tissues.

Sorting of proteins that lack cytoplasmic domains to the RSP is thought to be mediated by protein aggregation and/or interactions with sorting receptors, which results in the accumulation of the protein in lipid raft enriched microdomains in the TGN. In epithelial cells, apical proteins that complex with detergent-insoluble rafts are believed to function as sorting platforms in the TGN for apical delivery (67, 92-94). Even with one lipid modification, Shh still cofractionates with DRMs (52), indicating that partially modified Shh still associates with lipid rafts. However, delipidated and unprocessed Shh still traffics to SGs, which raises the possibility that lipid raft association is not an essential requirement for sorting to SGs. Supporting this, the nonlipidated form of hh is still targeted to axons in *Drosophila* visual system (10). Thus, it is probable that membrane rafts does not function as sorting platforms for targeting Shh to SGs. However, it is possible that dual lipidation is

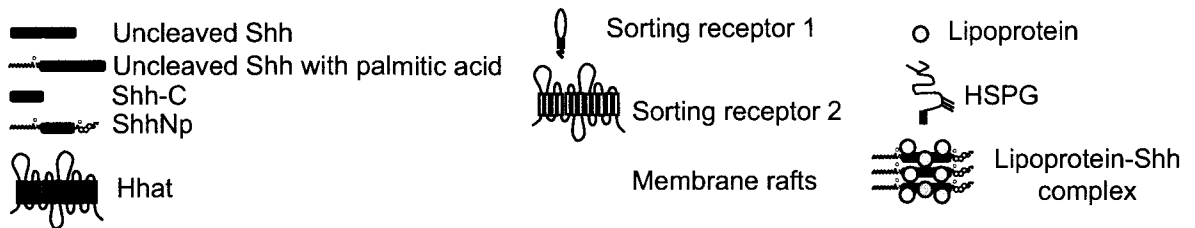
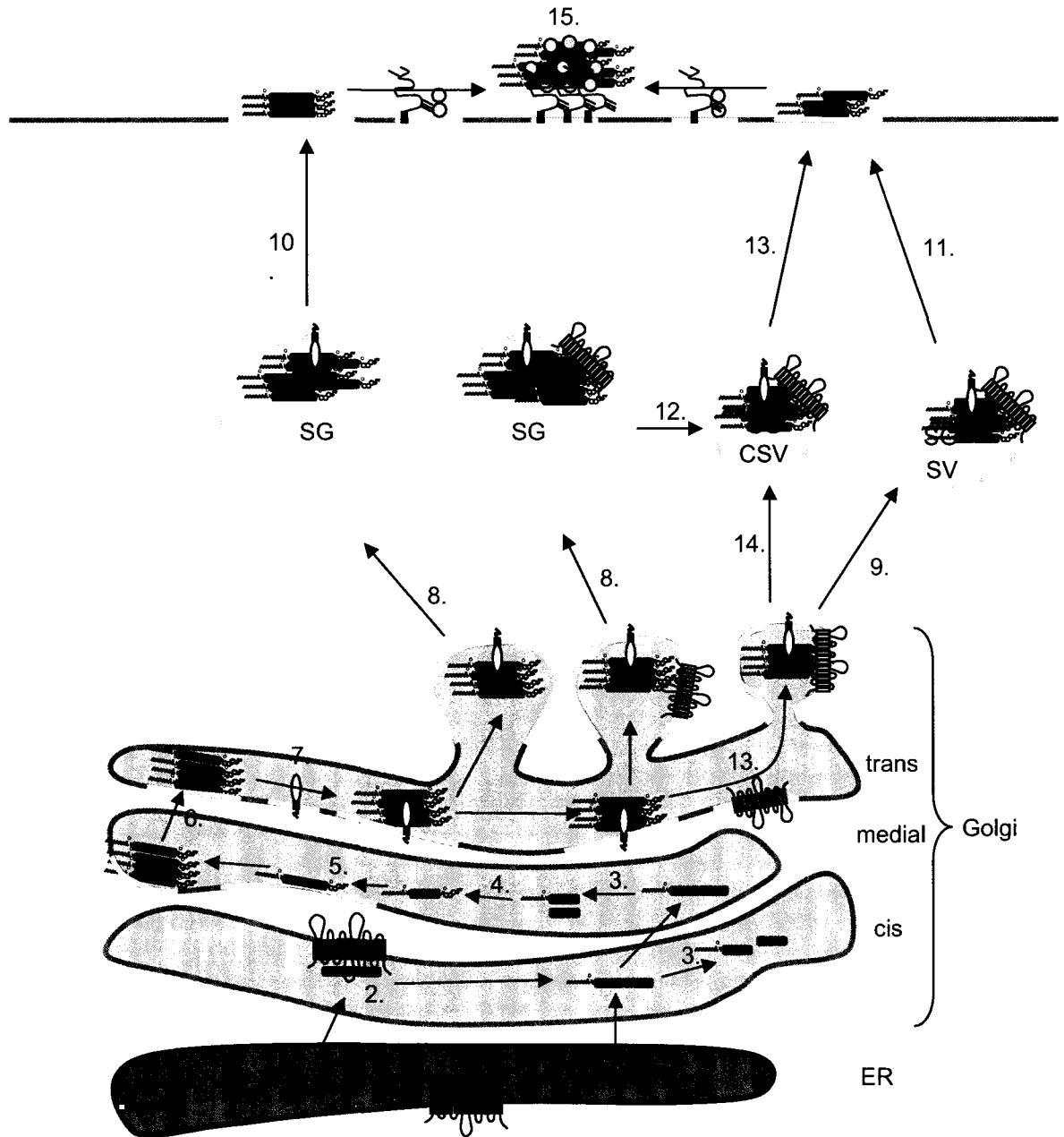
required for proper presentation of ShhNp to appropriate microdomains. For instance, oligomerization of secretory proteins originates in the medial Golgi, which is where membrane rafts originate (95), and dual lipidation may be a mechanism for Shh aggregation. Further characterization is required to address whether lipid raft microdomains function as a sorting platform for trafficking of Shh to the appropriate secretory pathway. Nevertheless, these results are consistent with the possibility of additional-receptor mediated mechanisms of sorting of Shh in neurons.

In addition, Shh may be sorted to the appropriate secretory organelle through interaction with different sorting receptors. The sterol-sensing domain of Disp may function as a sorting receptor for cholesterol-modified Hh (96-101). For instance, Disp may be a sorting receptor that sorts membrane-bound ShhNp to SVs or even from ISGs to CSVs. While Disp function is required in early spinal cord patterning (96-98, 101, 102), it is not required for paracrine Hh signalling in the bone (103). Thus, it is possible that Shh may interact with other sorting receptors, such as CPE and Sort1. For instance, sorting of BDNF requires interaction with these two sorting receptors in tandem; perturbation of either interaction results in resorting of BDNF to the constitutive secretory pathway (72, 74). Interestingly, I identified Sort1 as a potential interacting factor with the Shh-N and Shh-C domains. Thus, it is possible that Shh interacts with multiple factors that assist with Shh sorting to proper secretory vesicles.

4.1.6 Model of Shh intraneuronal transport

Based on my observations and data in the literature, I propose the following model of Shh trafficking in neurons (Figure 4.2). Although there has not been any studies that have unambiguously demonstrated the sequence of Shh PTMs, it is likely that palmitoylation

Figure 4.2. Proposed model of Shh synthesis, processing, sorting, transport and secretion in neurons. (1) Shh is translated as a 45 kDa preproprotein that contains a signal peptide for translocation of Shh into the endoplasmic reticulum (ER). (2) It is unknown whether Hhat-mediated palmitoylation of the Shh protein occurs in the ER or Golgi. (3) Shh then passes through the Golgi network and is cleaved into Shh-N (20 kDa) and Shh-C (25 kDa) domains either in the cis- or medial-Golgi. (4) Shh-N is further modified by cholesterol modification at the C-terminal to generate biologically active ShhNp. The combination of Shh localization with membrane raft microdomains (5) and perhaps aggregation (6) in the medial- or trans-Golgi and interaction with sorting receptors in the trans-Golgi (7) facilitates sorting of Shh to secretory vesicles of the regulated pathway (8 and 9). These vesicles are subsequently transported to appropriate sites for activity-dependent secretion. Most of the Shh in the regulated secretory pathway is transported to somatodendritic region by secretory granules (SG; 10) and undergoes anterograde axonal transport mainly through synaptic vesicles (SVs) while a small portion of SGs traffics to axon terminals (11). (12) ShhNp may also be trafficked to constitutive secretory vesicles (CSVs) by extrusion from nascent ISGs through interactions with another sorting receptor. Sorting of ShhNp into SVs may be dependent on additional sorting mechanisms, such as interactions with another sorting receptor or requires the presence of both lipids (13). As well, Shh is likely sorted to the CSVs for local secretion at the cell body (14). Upon secretion, Shh is tethered to the plasma membrane in membrane microdomains where Shh may be packaged into multimeric complexes containing lipoproteins (15).



occurs within the ER and both cleavage and cholesterol modification occurs in the cis-Golgi where the Golgi has a larger concentration of cholesterol and a more permissible environment for autocatalysis (15, 52, 104-108). At this point, Shh likely interacts with HSPGs such as glypicans and these complexes are cotrafficked throughout the biosynthetic and secretory pathways. Before presentation of Shh to the medial Golgi, Shh is cleaved and cholesteroylated. Shh then begins to associate with lipid raft microdomains by virtue of dual lipid modifications and may begin to form aggregates. It is possible that Shh interacts with sorting receptor such as Disp or Sort1, which traffics Shh into secretory vesicles of the CSP or RSP. Although I did not test the possibility that Shh is associated with the CSP, it is unlikely that Shh is trafficked exclusively to either the CSP or RSP. It is likely that ShhC is cotransported with ShhNp in these secretory vesicles (10). These Shh-containing vesicles are subsequently transported under the control of specific molecular motors to the appropriate sites for activity dependent secretion (SG and SV) or rapid secretion (CSV). Most of the Shh protein present in SGs is transported to postsynaptic dendrites but a small proportion of these vesicles undergoes anterograde axonal transport for activity-dependent release at both pre- and postsynaptic sites. After Shh is released through either pathway, Shh is tethered to the plasma membrane, likely in association with glypicans.

4.2 Identification of novel binding partners

Considering the multifaceted functions of the Shh pathway, it is likely that protein-protein interactions with Shh are context-dependent. In addition, it is possible that these interactions are not required for development; rather, these factors are important for post-developmental processes. As a result, this study has the potential to investigate context-specific protein interactions in the CNS.

I have identified several proteins that are promising candidates to play a role in regulating Shh signalling. Several of these proteins are likely involved in intracellular processing and transport. For instance, Kifs may be involved with transport of Shh-containing secretory vesicles down axons. Sort1 may regulate Shh sorting within the TGN or ISGs. Several of the candidates would be predicted to be involved with Shh extracellular transport, including several proteoglycans such as NCAM, Csgp5 and Gpc5. Thus, these proteins are likely to help shape Shh secretion or promote tethering to producing cells. Another class of identified potential candidates are transmembrane proteins that likely function as receptors. Of these receptors, several candidates are likely to be involved with endocytosis of secreted Shh, which may act in a negative fashion, or to function as coreceptors to augment Shh signalling.

4.3 Future directions

The results presented in this thesis are consistent with the possibility that Shh movement and secretion is dependent on context- and tissue-specific protein:protein interactions. While the transport and cell surface accumulation of Shh in neurites is consistent with the *in vivo* data implicating axons as a source of Shh signalling in the optic nerve, it also raises important questions regarding the regulation of trafficking and secretion of Shh in neurons. Several experiments can be carried out to characterize the factors that influence Shh signalling within the CNS. These suggested experimental directions, as outlined below, will provide more general insights into neuron-dependent regulation and secretion of other developmentally significant signalling molecules.

4.3.1 Characterization of endogenous Shh distribution

One of the unfortunate pitfalls of analyzing endogenous Shh distribution is that anti-Hh reagents do not have adequate sensitivity to detect Shh when expression levels are low. Although there have been reports demonstrating endogenous Shh protein distribution in tissues with high levels of Shh expression (e.g., floor plate and notochord) with different immunohistochemical techniques (9, 75, 109), repeated attempts by me and other laboratory members have not resulted in the reliable detection of endogenous Shh expression in neurons from retinal tissue sections and cultured primary CNS neurons using available α -Hh antibodies. An alternative approach is to use the recently established transgenic mouse line that expresses GFP-tagged Shh (Shh::GFP) to monitor the distribution of endogenous Shh in primary neurons. This approach has the advantage that the Shh::GFP signal can be amplified with commercially available α -GFP antibodies. This mouse was generated by targeted insertion of the GFP tag proximal to the cleavage site to produce a GFP-tagged Shh-N fragment that is lipid modified (12). Although cleavage of GFP-tagged Shh is less efficient compared to untagged Shh, Shh::GFP is biologically active and heterozygous mutants are patterned normally in early development (12) which will permit the analysis of Shh trafficking in neurons, including RGCs. This mouse model can be used for biochemical and developmental stage-dependent alterations in endogenous Shh distribution in the visual system and to study consequences of genetic and pharmacological perturbations on Shh distribution *in vivo*.

4.3.2 Specificity of Shh sorting

My results indicate that PTMs are required for cell surface accumulation of Shh in large puncta, as this was observed in cells transfected with wild-type Shh but not

nonlipidated or uncleaved Shh. This finding also indicates that PTMs are not required for sorting to SGs but are required for sorting to SVs. There are two possible interpretations of Shh sorting to the cell surface; the specificity for cell surface accumulation is at the level of secretion where PTMs are required at the point of secretion for the formation of cell surface aggregates or the specificity for cell surface accumulation is determined at the TGN where PTMs direct sorting of the protein to a secretory compartment that is separate from the regulated pathway. To distinguish between the two possibilities, one can address the requirement of regulated secretion for cell surface accumulation of Shh and compare the relative efficiency of Shh sorting to the two secretory pathways. Discriminating between these two possibilities can be accomplished by acute knockdown of factors that are involved in regulated secretory organelle trafficking and/or biogenesis and observing level of cell surface accumulation and secreted protein. Similarly, one can explore the role of the constitutive secretory pathway by performing pulse chase labelling experiments and differential density centrifugation to determine the proportion of Shh protein that is associated with constitutive secretory vesicles and ISGs. If cell surface accumulation occurs independently of SG release, then this result would implicate the nonregulated pathway in the deposition of Shh at the surface. Intracellular accumulation and loss of extracellular Shh immunoreactivity in siRNA/dominant negative treated cells would indicate that the regulated pathway is the source of the cell surface Shh and that PTMs are required for the accumulation of, but not transport to, Shh on the cell surface. This would be a novel mechanism of regulation of Shh release from neurons and would implicate Ca^{2+} -mediated events in the regulation of Shh signalling.

As the default pathway for secretory proteins is the CSP and inhibition of Shh PTM resulted in the association of Shh with SGs, these results indicates that Shh is recruited to

SGs. There are multiple mechanisms that are used to direct proteins to the appropriate secretory compartment, including aggregation, lipid-raft association, sorting motifs and interaction with sorting receptors. Notably, the Shh-C domain appears to not be required for targeting Shh to SGs, in contrast to the fly where certain residues in the carboxyl terminus of hh are required to target hh-N to the growth cones (10). One possibility to determine if Shh-N is actively sorted to SGs is to examine the association of Shh with known sorting receptors such as CPE or Sort1. To investigate the requirement for these sorting receptors in Shh trafficking in neurons, knockdown approaches by RNA interference or dominant negative techniques can be performed to monitor Shh subcellular distribution. Moreover, Disp has an essential role for secreting cholesterol-modified Shh-N, however, the exact mechanism is unknown. It would be of considerable interest to determine if Disp functions as a sorting receptor, allowing ShhNp to be sorted away from the SG compartment. Neurons from floxed Disp mice can be cultured and be induced to deactivate Disp expression by cre recombinase. If Disp function is important for Shh trafficking, one would expect to observe a reduction of Shh secretory vesicle in the cytoplasm, reduction of cell surface Shh and/or reduction of secretagogue-induced Shh secretion

4.3.4 Effects of Shh signalling in axons

To understand the biological relevance of neuronal trafficking of Shh and secretion, functional approaches to investigate the impact of Shh PTMs and sorting on neuron-derived Shh signalling can be performed. An approach is to use multicompartiment Campenot chambers where neurons are cultured such that the somatodendritic region, axons and terminals are physically separated, allowing investigations of anterograde transport of proteins between the soma and distal axons (110-112). This system can facilitate

investigation of the role of Shh PTMs on neuron-mediated Shh signalling in a coculture system. For instance, one can culture glial cells among the cell body, along the length of the axons or at the axon terminals, and examine the effect of neurons that express Shh isoforms on glial cells. This system can also be used for examining the requirement of Disp or other sorting receptors for Shh signalling.

The mechanisms of depolarized-induced Shh release can be assayed through the use of YFP-tagged Shh constructs. While bulk secretion under depolarizing conditions was assessed through ELISA of the cell culture supernatants, this approach lacks spatial information such as the site of secretion and temporal resolution (113). This caveat can be resolved by using the YFP-tagged Shh constructs to visualize decreases in intracellular fluorescent intensity upon stimulation using an inverted fluorescence microscope equipped with a perfusion chambers (66).

Previous studies using *in vivo* loss-of-function analyses with conditional mouse mutagenesis strains have shown that Shh is required for the induction of target genes and proliferation locally in the retina and at long range in the optic nerve (6, 22, 24, 30). However, because of the early embryonic lethality associated with complete gene inactivation (114), the role of lipid modifications in the context of the timing, range and biological consequences of neuron-mediated local and long range Shh signalling is unknown. One can address the requirement for PTMs by examining the retina and optic nerve phenotypes in mice with conditional expression of delipidated Shh or conditional inactivation of *Disp1* in the peripheral retina by crossing existing floxed mouse lines with mice that express Cre under the Pax6 promoter. This strategy would allow investigators to circumvent early lethality associated with ShhN animals. The retina and optic nerves in these mutants could be examined for Hh target gene induction, proliferation, RGC differentiation,

astrocyte proliferation, oligodendrocyte differentiation and myelination in the optic nerve (6, 22, 24, 30). As well, one can examine the role of retina-derived Shh on the axon fasciculation and guidance at the chiasm, as well as targeting to the lateral geniculate nucleus and superior colliculus by anterograde labelling in the peripheral retina with DiI (115). These approaches will allow investigators to interrogate the role of PTMs and other Hh pathway components on short- and long-range Shh signalling *in vivo*.

4.3.5 Validation of novel interaction partners

Novel receptors and protein-binding factors have been identified using a GST pulldown affinity purification approach (Chapter 3). Most of the candidates that are described in Table 3.1 have already been epitope tagged with myc-his and are expressed in transfected cell lines. Reciprocal coimmunoprecipitations (coIPs) of these myc-his tagged proteins and ShhN or SPShhC can also be performed, and if possible, coIP of the endogenous proteins and Shh can be carried out. As several of these candidates are transmembrane proteins that can be trafficked to the cell surface, these candidates can be tested for Shh-N binding using a cell-based assay. Specifically, candidate proteins are transfected into a cell line and are incubated with increasing concentrations of alkaline phosphatase-conjugated ShhN (ShhN::AP), washed and bound ShhN::AP is quantified through a colorimetric reaction (116-118). Candidates that exhibit interaction with Shh in the binding assays or coIPs can then be examined for colocalization with Shh and in primary neurons, and knockdown approaches can be used for investigating their requirement for Shh sorting in cultured primary neurons. Moreover, candidate proteins can also be tested in gain- and loss-of-function assays for their effects on Shh signalling in reporter cell lines (e.g., LIGHTII cells) and in primary tissues, such as retinal explants. Ultimately, it would be

important to examine genetic relationships using mouse mutant analysis and epistasis experiments to examine the *in vivo* interactions of these proteins with the Shh pathway.

4.4 Summary

Shh signalling in the developing and adult CNS has been studied intensively for almost two decades. As a result, we have accumulated an understanding of the various molecules that regulate Shh signalling. However, we have a limited understanding of how the highly specialized morphology and physiology of neurons contributes to the trafficking, secretion and temporal regulation of Shh. Since the initial studies that highlighted the role of axons in Shh signalling in the developing optic nerve, researchers have been intrigued by the possibility of long-range transport of Shh as a means to regulate cellular events at the targets of neurons. The results in this thesis brings us closer to understanding the mechanism of Shh transport, release and reception in neurons, which will lead to a deeper insight of how Shh functions in the developing and adult CNS.

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5. CONTRIBUTION OF COLLABORATORS

Heidi McBride, Scientist, University of Ottawa Heart Institute. Dr. McBride provided strategic advice for the GST pulldown and preliminary research plan for Chapter 2.

Robin Parks, Senior Scientist, Ottawa Hospital Research Institute. Dr. Parks constructed an adenovirus harbouring Shh::YFP and provided advice for DNA cloning.

Chantal Mazerolle, Research Technician, Ottawa Hospital Research Institute. Ms. Mazerolle assisted with construction of cDNAs and performed the coimmunoprecipitation listed in Figure 3.3B.

6. APPENDIX I – PRIMERS USED IN THIS THESIS

Table 6.1. Primers used in this thesis and their respective sequences.

ID	Sense	Sequence (5'→3')	RE
Shh	For	GCGGAATTCGCCACCATGCTGCTGCTGCTGGCCAGATGTT	EcoRI
	Rev	GCGCTCGAGTCAGCTGGACTTGACCGCCATTCCC	XhoI
Shh(NheI)	For	GCGGCTAGCTCCGGCGGGCGCATTCCC	NheI
	Rev	GCGGCTAGCTTTGGCCGCCACGGAGTTCT	NheI
ShhN	For	GCGGAATTCGCCACCATGCTGCTGCTGCTGGCCAGATGTT	EcoRI
	Rev	AGCGCTCGAGTCAGCCGCCGGATTTGGCCGGCCACGG	XhoI
^{C25S} Shh*	For	CGGGCTGGCCTCTGGGCCCCGGCAG	N/A
	Rev	CTGCCGGGGCCAGAGGCCAGCCCCG	N/A
^{C199A} Shh*	For	GCTAGCTCCGGCGGGCGCATTCGCCGGGATCCGC	N/A
	Rev	GCGGATCCCCGGGAATGCGCCGGCGGAGCTAGC	N/A
Shh ^{Δ404-437}	For	GCGGAATTCGCCACCATGCTGCTGCTGCTGGCCAGATGTT	EcoRI
	Rev	GCGCTCGAGTCAAGTCGGCTCCGCGCCCCCTCG	XhoI
EYFP(SpeI)	For	GCGACTAGTATGGTGAGCAAGGGCGAGGAGC	SpeI
	Rev	GCGACTAGTCTTGTACAGCTCGTCCATGCCGAGA	SpeI
Shh-N	For	AGCGGAATTCTGTGGGCCCCGGCAGGGGGTTTGG	EcoRI
	Rev	AGCGCTCGAGTCAGCCGCCGGATTTGGCCGCCACGG	XhoI
Shh-C	For	AGCGGAATTCTGTTTCCCGGGATCCGCCACCGT	EcoRI
	Rev	GCGCTCGAGTCAGCTGGACTTGACCGCCATTCCC	XhoI
Sort1	For	GCGCTCGAGGCGATGGAGCGGCCCCGGGGA	XhoI
	Rev	GCGAAGCTTTTTCCAGGAGGTCCTCATCTGA	HindIII
Gpc5	For	CCGCTCGAGAGCCTCGGCCGCTGT	XhoI
	Rev	GTAATCGATGTCATGATCTTTATAATCACCGTCATGGTCTTT GTAGTCGCAGGCTGACGCCCTCGCT	ClaI
	For	GACATCGATTACAAGGATGACGATGACAAGGAAGAAGTTC GGAAACTTTTCC	ClaI
	Rev	CTTGGTACCTTACCAAATCCCGGGAAGTAA	KpnI
Hip1	For	GCGGGATCCGCCACCATGGTGAAGATGCTCTCGTTT	BamHI
	Rev	GCGGGTACCTTACAATGTAAGTGTGAGATC	KpnI
ShhC (w/ SP)	For	GCGCTCGAGCATGTTTCCCGGGATCCGCCACC	BamHI
	Rev	GCGCTCGAGTCAGCTGGACTTGACCGCCATTCCC	XhoI
ShhC	For	GCGCTCGAGCATGTTTCCCGGGATCCGCCACC	XhoI
	Rev	GCGGAATTCGCTGGACTTGACCGCCATTC	EcoRI
SP	For	AGCGAAGCTTCCGCCACCATGCTGCTGCTGCTGGCCAGAT GT	HindIII
	Rev	AGCGGCCGGCCAGCCCCGGGGCACACCAGCAG	NaeI
ECFP	For	GCGGAATTCGGTGGCAGTGGGGGAATGGTGAGCAAGGGCG AGG	EcoRI
	Rev	GCGTCTAGATTACTTGTACAGCTCGTCCATGCC	XbaI

RE indicates restriction enzymes used to subclone amplicons into destination vectors. * indicates primers used for site-directed mutagenesis. For and Rev are sense and antisense primers, respectively. All Shh isoforms for expression analysis were subcloned with EcoRI and XhoI into pcDNA3. Shh-N and Shh-C were cloned into the GST-tagged bacterial expression vector pGEX-4T-1. Sort1 and Gpc5 were cloned into pcDNA3.1-myc-his(-)C.

7. APPENDIX II – DETAILED PROCEDURE AND SOLUTION SHEETS

A. Site-directed mutagenesis

Note: To design primers for site-directed mutagenesis, use Stratagene's QuikChange® Primer Design located at <http://www.stratagene.com/tradeshows/feature.aspx?fpId=118>.

1. Make two PCR tubes where one tube contains Pfx and the other lacks Pfx
 - 2.5 μ L 10X Pfx buffer
 - 2.5 μ L 10X Enhancer
 - μ L 50 mM MgSO₄
 - μ L 10 mM each dNTP
 - μ L 10 μ M Oligonucleotide 1
 - μ L 10 μ M Oligonucleotide 2
 - x μ L 5-20 ng Plasmid
 - μ L Platinum Pfx (Invitrogen)
 - up to 25 μ L ddH₂O
2. Perform PCR:
 - a. 95°C for 3 min
 - b. 18 cycles of:
 - i. 94°C for 20 sec
 - ii. 55°C for 1 min
 - iii. 68°C for 1-2 min/Kbp plasmid
 - c. 68°C for 10 min
 - d. Cool down the tubes on ice
3. Remove half of PCR reaction to a new PCR tube
4. To the other tubes, add 1 μ L DpnI
5. Incubate at 37°C for 2 hours
6. Run an 5-10 μ L on a gel to verify amplification of plasmid and completion of digest
7. Transform 1-5 μ L into supercompetent DH5 α *Escherichia coli* cells (Invitrogen) using standard heat shock procedures with SOC media
8. Miniprep and send 3-5 plasmids for sequencing

B. PC6 cell culture

1. Coat coverslips or plates with collagen
 - a. Coating coverslips with PDL
 - i. Boil coverslips in 100% EtOH for 20 min (after washing, can autoclave coverslips in glass Petri dish)
 - ii. Place coverslips in wells
 - iii. Air dry coverslips
 - iv. Immerse coverslips in 1 mg/mL PDL in borate buffer
 - v. Incubate for 1 hour to O/N at 37°C
 - vi. Wash 3X with ddH₂O and air dry
 - b. Add collagen to plates or coverslips (~ 1 mL for 12 well plate coverslips, 5 mL for 10 cm² dish, ~12 mL for 15 cm² dish)
 - c. Incubate at 37°C for 2 hours to O/N
 - d. Replace collagen into a Falcon tube
 - e. Wash 3X with ddH₂O and air dry
 - f. Store at 4°C
2. Thawing cells
 - a. Prewarm growth media to 37°C
 - b. Place cryogenic vial into 37°C water bath until ice has almost completely melted
 - c. Transfer cells to 5 mL prewarmed growth media in a 15 mL Falcon tube
 - d. Centrifuge at 300 x g for 5 min
 - e. Aspirate supernatant
 - f. Resuspend cells in 1 mL growth media and transfer to a collagen-coated 10 cm² dish containing 10 mL growth media
 - g. Culture at 37°C with 5% CO₂
 - h. Change media every 2-3 days
3. Passage: (upon 50-90% confluency)
 - a. Aspirate media
 - b. Wash cells with 5-10 mL HBSS or 2 mL 0.25% trypsin
 - c. Aspirate solution
 - d. Add 1 mL 0.25% trypsin
 - e. Incubate at RT for ~1 min
 - f. Tap plate until cells have detached
 - g. Add 5 mL growth media
 - h. Place 1-1.5 mL into a new 10 cm² dish
 - i. Discard cells into biohazard waste
4. Inducing differentiation into a sympathetic neuronal phenotype
 - a. Trypsinize cells from a 10 cm² dish as above and bring volume to 10 mL
 - b. 12-well plate: Place 25 µL of trypsinized cells per well containing 1 mL differentiation media (assuming 90% confluent 10 cm² dish)
 - c. Culture at 37°C with 5% CO₂
 - d. Change media every 2-3 days

5. Freezing cells:
 - a. Trypsinize and collect cells into a 15 mL Falcon tube
 - b. Spin at 300 x g for 5 min
 - c. Aspirate supernatant
 - d. Resuspend pellet in 1 mL freezing media and transfer to a cryogenic vial
 - e. Place at -80°C overnight
 - f. Next day, transfer vial to liquid nitrogen tank

Solutions

Collagen

1. Add 57 µL Glacial acetic acid to 50 mL H₂O
2. Filter sterilize
3. Add 10 mL collagen solution (R&D #3440-100-01) to 40 mL diluted acetic acid
4. Store at 4°C

Growth media

85% RPMI 1640
10% Horse serum
5% FBS
1X Pen/Strep
1X L-glutamine

Differentiation media

90% RPMI 1640
10% Growth media
50 ng/mL NGF2.5S
1X L-glutamine

Freezing media

90% FBS
10% DMSO

Borate buffer

1.24 g boric acid
1.9 g Borax (sodium borate)
400 ml H₂O
Adjust to pH 8.5, filter sterilize and store at RT.

C. COS cell culture

1. Thawing cells
 - a. Prewarm growth media to 37°C
 - b. Place cryogenic vial into 37°C water bath until ice has almost completely melted
 - c. Transfer cells to 5 mL prewarmed growth media in a 15 mL Falcon tube
 - d. Centrifuge at 300 x g for 5 min
 - e. Aspirate supernatant
 - f. Resuspend cells in 1 mL growth media and transfer to a 10 cm² dish containing 10 mL growth media
 - g. Culture at 37°C with 5% CO₂
 - h. Change media every 2-3 days
2. Passage (upon 50-90% confluency):
 - a. Aspirate media
 - b. Wash cells with 5-10 mL HBSS or 2 mL 0.25% trypsin
 - c. Aspirate solution
 - d. Add 1-2 mL 0.25% trypsin
 - e. Place in incubator for ~2 min
 - f. Knock plate until cells have detached
 - g. Add 5 mL growth media
 - h. Place 0.5-1 mL into a new 10 cm² dish
 - i. Place rest of cells into biohazard waste
3. Freezing cells:
 - a. Make freezing media
 - b. Collect cells into a 15 mL Falcon tube
 - c. Spin at 300 x g for 5 min
 - d. Aspirate supernatant
 - e. Resuspend pellet in 1 mL freezing media and transfer to a cryogenic vial
 - f. Place at -80°C overnight
 - g. Next day, transfer vial to liquid nitrogen tank

Solutions

Growth media

90% DMEM (high glucose)

10% FBS

1X Pen/Strep

Freezing media

90% FBS

10% DMSO

D. Hippocampal neuron culture

1. Coat coverslips with PDL
 - a. Boil coverslips in 100% EtOH for 20 min (if desired, autoclave coverslips in glass Petri dish)
 - b. Place coverslips in wells
 - c. Air dry coverslips
 - d. Immerse coverslips in 1 mg/mL PDL in borate buffer
 - e. Incubate O/N at 37°C
 - f. Wash 3X with ddH₂O and air dry
2. Obtain P1 rat pups
3. Dissect brains and excise hippocampi in cold HBSS (without Ca and Mg) containing 10 mM HEPES (pH 7.4) and 1X Pen/Strep
4. Aspirate solution and add:
 - 9 mL HBSS (with Pen/Strep) plus 1 mL 2.5% trypsin solution (prewarmed)
 - 50 µL of DNaseI solution (prewarmed)
5. Incubate at 37°C for 15 min – mix 3 times by inversion
6. Remove solution by pipetting
7. Rinse 2X with NB+FBS by pipetting and allowing tissues to settle
8. Add <2 mL NB+FBS and 50 µL DNase
9. Triturate 10X with a 1 mL pipette tip
10. Triturate 10X with fire polished glass pipette
11. Filter through a 40 or 70 µm mesh filter
12. Centrifuge 300 x g for 5 min
13. Resuspend cells in 20 mL NB+FBS
14. Centrifuge 300 x g for 5 min
15. Resuspend cells in 5 mL NB+FBS
16. Count cells
17. Dilute cells with NB-FBS to ~100,000 viable cells per mL
18. Add 1 mL per well of a 12 well plate
19. Culture at 37°C with 5% CO₂
20. After 4-6 hours, replace NB+FBS with neuron media
21. After 24 hours, change half of neuron media containing 6 µM AraC (final concentration in plate is 3 µM)
22. Replace half volume of media every 3 days (remove 400 µL, add 500 µL)

Solutions

Borate buffer

1.24 g boric acid

1.9 g Borax (sodium borate)

400 ml H₂O

Adjust to pH 8.5, filter sterilize and store at RT.

Trypsin solution

2.5% trypsin in HBSS (0.2 g in 8 mL)

Filter sterilize and store at -80°C

PDL (100 mg/mL)
0.1 g Low molecular weight PDL
1 mL H₂O
Filter sterilize and store at -20°C

AraC (6 mM in H₂O)
64 mg
44 mL H₂O
Filter sterilize and store at -20°C

DNase solution (10 mg/mL)
100 mg DNase
10 mL HBSS
pH to 7.0-7.8
F/S and store at -20°C

Neurobasal/FBS
45 mL Neurobasal medium
5 mL FBS

Neuron media
20 mL Neurobasal A
0.4 mL B27
0.1 mL 200 mM glutamine
20 µL Gentamicin

E. Lipofectamine 2000 transfection

1. Day before transfection:
 - a. Seed cells such that cells are 90-95% confluent next day
 - b. Culture without antibiotics
2. Transfection
 - a. Mix pDNA and Neurobasal A or DMEM in 15 mL Falcon tubes or 1.5 mL eppendorfs and set aside at RT
 - b. Mix Lipofectamine 2000 and Neurobasal A or DMEM in 15 mL Falcon or 1.5 mL eppendorf tubes and incubate for 5 min at RT
 - c. Combine pDNA and Lipofectamine 2000 solutions
 - d. Incubate for at least 20 min
 - e. Change media depending on cell type (COS cells: Opti-MEM, without FBS; PC6 cells: normal growth or differentiation media; Hippocampal neurons: remove half of the conditioned media and store in the incubator)
 - f. Add pDNA-Lipofectamine complexes to cells
 - g. Incubate at 37°C with 5% CO₂ for at least 4 hours
 - h. Change media lacking antibiotics (for hippocampal neurons, replace with conditioned media)
3. Culture for 1-2 days

Size	Culture volume (mL)	DNA solution		Lipofectamine 2000 solution	
		DNA (µg)	Media (mL)	Lipofectamine 2000 (µL)	Media (mL)
96 well	0.1	0.2	0.025	0.5	0.025
24 well	0.5	0.8	0.05	2	0.05
12 well	1	1.6	0.1	4	0.1
6 well	2	4	0.25	10	0.25
6 cm ²	3-4	8	0.5	20	0.5
10 cm ²	10	24	1.5	60	1.5
15 cm ²	20	56	3.5	140	3.5

F. Calcium Phosphate transfection of primary hippocampal neurons

Note: use Clontech's CalPhos™ Mammalian Transfection Kit (Cat. No. 631312)

1. Culture neurons on coverslips in a 12 well plate
2. Day before transfection, change half media
3. Pre-equilibrate new media at 37°C with 10% CO₂
4. Transfer coverslips to a new 12 well plate containing fresh neuron media
5. Prepare mixtures in 15 mL tissue culture tubes:
 - a. Prepare DNA-CaCl₂ mixture
 - 3-5 µg endotoxinp-free pDNA
 - 12.4 µL 2M CaCl₂
 - x µL ddH₂O
 - b. Prepare 2X HBS mixture
 - 50 µL 2X HBS
 - 50 µL ddH₂O
6. Add DNA-CaCl₂ mixture dropwise to 2X HBS with 2-3 sec of gentle vortexing (600 rpm) with each drop (~1/8 volume at a time)
7. Incubate at RT for 15 min in the dark
8. Add mixture dropwise to each well and tilt dish to ensure even distribution
9. Incubate cells at 37°C with 5% CO₂ for 1-2 hours
10. Aspirate media
11. Add 2 mL pre-equilibrated to 10% CO₂
12. Incubate at 37°C with 5% for 15 min
13. Transfer coverslips to original wells
14. Incubate neurons at 37°C with 5% CO₂ for 24 hrs

Reference:

Jiang, M., and Chen G. (2006). High Ca²⁺-phosphate transfection efficiency in low-density neuronal cultures. *Nature Protocols* 1, 695-700.

G. Immunocytochemistry

1. Aspirate media
2. Wash coverslips 2X with PBS in a Petri dish
3. Fix with 4% PFA for 5 min at RT in a Petri dish
4. Wash 2X with TBS in a Petri dish
5. Permeabilize with 0.1% Triton X-100 in TBS for 5 min at RT
6. Wash 2X with TBS in a Petri dish
7. Block with 10% goat serum in TBLS for 20 min in a Petri dish
8. Incubate with 1° Ab in TBLS in a multiwell plate
9. Incubate at RT for 60 min
10. Wash 3X with TBS in a Petri dish
11. Incubate with 2° Ab in TBLS in a multiwell place
12. Incubate at RT for 60 min
13. Wash 3X with TBS in a Petri dish
14. Mount with DAKO Flourescent Mounting Medium

Solutions

PBS

137 mM NaCl
2.7 mM KCl
8.1 mM Na₂HPO₄
1.5 mM KH₂PO₄
pH 7.4

TBS

50 mM Tris-HCl, pH 7.4
150 mM NaCl

TBLS

50 mM Tris-HCl, pH 7.4
150 mM NaCl
1% BSA (Sigma A2153)
100 mM L-Lysine
0.04% Sodium Azide

H. Sucrose gradient

Note: keep everything on ice

1. Maintain PC12 cells in a 15 cm² dish
2. Induce differentiation for 7-10 days with NGF2.5S
3. Make a 0.6-1.8M sucrose gradient using peristaltic pump and gradient mixer in a SW41 tube
 - a. 1.8M – 5.5 mL
 - b. 0.6M – 5.5 mL
 - c. 2 mL/min
 - d. Keep tube on ice
4. Wash dish 2X with PBS
5. Scrape cells in PBS
6. Transfer cells to a 1.5 mL eppendorf
7. Pellet at 380 x g for 5 min
8. Resuspend cells in 1.1 mL sucrose solution containing protease inhibitors with a 1 mL tip
9. Pass cells 4X through a 1 mL syringe fitted with a 28-gage needle
10. Dounce homogenize with tight fitting pestle 40X (7 mL capacity)
11. Spin at 1000 x g for 10 min
12. Place supernatant to new tube
13. Apply 1 mL to continuous gradient
14. Spin at 41,000 rpm for 3 hours in a SW41 rotor
15. Collect 850 µL fractions from the bottom (puncture bottom of tube with a hot needle)

Solutions

Sucrose solution

- 0.32 M Sucrose
- 10 mM HEPES-KOH, pH 7.4 (7.2 or 7.0)
- 5 mM EDTA
- 1M HEPES, pH 7.4
- 23.83 g
- ~11.5 KOH pellets
- Top to 100 mL
- 2 M Sucrose
- 34.23 g
- 28 mL
- Make fresh and keep on ice
- 1.8 M Sucrose
- 18 mL 2M Sucrose
- 0.2 mL 1M HEPES
- 0.2 mL 0.5M EDTA
- 1.6 mL H₂O

0.6 M Sucrose

- 6 mL 2M Sucrose
- 0.2 mL 1M HEPES
- 0.2 mL 0.5M EDTA
- 13.6 mL H₂O

PBS

- 137 mM NaCl
- 2.7 mM KCl
- 8.1 mM Na₂HPO₄
- 1.5 mM KH₂PO₄
- pH 7.4

I. Glycerol gradient

Note: keep everything on ice

1. Maintain PC12 cells in a 10 cm² dish
2. Induce differentiation for 7-10 days with NGF2.5S
3. Wash cells 2X with PBS
4. Scrape cells in PBS and transfer cells to a 1.5 mL eppendorf tube
5. Spin cells at 380 x g for 5 min
6. Resuspend cells with 0.75 mL HBSE
7. Pass cells 4X through a 1 mL syringe fitted with a 22-gage needle
8. Dounce homogenize cells 70X (7 mL capacity, tight fitting pestle)
9. Spin lysate at 1000 x g for 10 min
10. Transfer supernatant to a new tube
11. Make a 5-25% gradient in a SW55 tube
 - a. 2.3 mL 5%
 - b. 2.3 mL 25%
 - c. Use gradient maker with a peristaltic pump at 2 mL/min
12. Apply ~0.5 mL lysate to top of gradient
13. Spin at 55,000 rpm for 45 min
14. Collect fractions from the top and resuspend pellet in 1% SDS

Solutions

HBSE

- 10 mM HEPES-KOH, pH 7.4
 - 0.15 M NaCl
 - 5 mM EDTA
 - Protease inhibitors
- ### 40% Glycerol
- 16 mL Glycerol
 - 24 mL H₂O
- ### 25% Glycerol
- 12.5 mL 40% Glycerol
 - 0.2 mL 1M HEPES, pH 7.4
 - 0.2 mL 0.5M EDTA
 - 7.1 mL H₂O

5% Glycerol

- 2.5 mL 40% Glycerol
- 0.2 mL 1M HEPES, pH 7.4
- 0.2 mL 0.5M EDTA
- 17.1 mL H₂O

PBS

- 137 mM NaCl
- 2.7 mM KCl
- 8.1 mM Na₂HPO₄
- 1.5 mM KH₂PO₄
- pH 7.4

J. Detergent-resistant membrane analysis

NOTES:

Do everything in the cold room and keep everything on ice.

All spins are at 4°C

Precool rotor and ultracentrifuge

1. Grow PC6 cells in a 15 cm² dish
2. Stimulate with NGF2.5S for 4 days
3. Wash 1X with PBS
4. Wash 2X with TNE
5. Scrape cells with 1 mL TNE
6. Centrifuge at 380 x g for 5 min
7. Resuspend pellet in 1.6 mL TNE containing 1% Triton X-100 and protease inhibitors
8. Incubate on ice for 10 min
9. Dounce homogenize 10X with loose fitting pestle (7 mL capacity)
10. Incubate on ice for 20 min
11. Mix 1.5 mL lysate with 1.5 mL 80% sucrose in TNE
12. Place 3 mL mixture in a SW41 tube
13. Overlay with 6 mL 35% sucrose
14. Overlay with 3 mL 5% sucrose
15. Centrifuge at 39,000 rpm for ~20 hours in a SW41 rotor
16. Draw fractions from the top
17. Resuspend pellet with in TNE with 1% SDS

Solutions

5X TNE

43.8 g NaCl

30.3 g Tris

20 mL 0.5 M EDTA

pH to 7.4

Top to 1 L

80% sucrose

8 g sucrose

2 mL 5X TNE

3 mL ddH₂O

35% sucrose

40.29 g sucrose

100 mL TNE

5% sucrose

5.1 g sucrose

100 mL TNE

PBS

137 mM NaCl

2.7 mM KCl

8.1 mM Na₂HPO₄

1.5 mM KH₂PO₄

pH 7.4

K. Detergent-free lipid raft fractionation

1. Grow cells in 2 15 cm² dishes
2. Stimulate PC12 cells with NGF2.5S for 7-10 days
3. Wash 2X with BCM
4. Scrape cells in BCM and transfer cells to a 1.5 mL eppendorf tube
5. Pellet cells at 380 x g for 5 min
6. Resuspend cells in 1 mL BCM plus protease inhibitors
7. Dounce homogenize 50X (7 mL capacity, tight fitting pestle)
8. Centrifuge at 1000 x g for 10 min
9. Transfer supernatant to a new tube
10. Resuspend pellet with 1 mL BCM plus protease inhibitors
11. Homogenize 50X
12. Centrifuge at 1000 x g for 10 min
13. Pool supernatants
14. Mix 2mL supernatant with 2 mL 50% OptiPrep in base buffer
15. Place mixture in bottom of SW41 tube
16. Overlay with
 - 1.6 mL 20% OptiPrep
 - 1.6 mL 15% OptiPrep
 - 1.6 mL 10% OptiPrep
 - 1.6 mL 5% OptiPrep
 - 1.6 mL 0% buffer
17. Centrifuge for 90 min at 20,500 rpm
18. Collect fractions from the top

Solutions

Base buffer

20 mM Tris-HCl pH 7.8
250 mM Sucrose

	BCM	0%	5%	10%	15%	20%	50%
60% OptiPrep	0	0	0.83	1.67	2.5	3.33	8.33
2M Sucrose	1.25	1.25	1.25	1.25	1.25	1.25	1.25
0.5 M Tris-HCl, pH 7.8	0.4	0.4	0.4	0.4	0.4	0.4	0.4
1 M CaCl ₂	0.01	0	0	0	0	0	0
1 M MgCl ₂	0.01	0	0	0	0	0	0
ddH ₂ O	8.33	8.35	7.52	6.68	5.85	5.02	0.02

Reference:

Macdonald J.L. and Pike L.J. (2005). A simplified method for the preparation of detergent-free lipid rafts. J Lipid Res. 46, 1061-1067.

L. Induced secretion

1. Treat PC6 cells with NGF for 7-10 days in 6-well dishes
2. Prewarm basal and stimulating media
3. Aspirate media
4. Wash cells 2X with 2 mL basal media
5. Add 1 mL basal or stimulating media
6. Incubate at 37°C for 20 min
7. Place dishes on ice
8. Transfer cell culture supernatant to 1.5 mL eppendorf tubes and proceed to ELISA
9. Add 1000 μ L lysis buffer to dishes
 - a. Incubate on ice for at least 20 min
 - b. Pipette 5X
 - c. Transfer supernatant to a new 1.5 mL eppendorf
 - d. Centrifuge at 10,000 x g for 10 min
 - e. Transfer supernatant to a new tube
 - f. Read A_{280}
10. ELISA using DuoSet ELISA Development System (R&D Systems DY461)
 - a. Day before:
 - i. Dilute Capture antibody to 4 μ g/mL (Stock: 720 μ g/mL)
 - ii. Add 100 μ L to each well of a 96 well plate
 - iii. Seal plate with parafilm
 - iv. Incubate O/N at RT in the dark
 - b. Aspirate solution
 - c. Wash 3X with $\sim$ 400 μ L Wash buffer
 - d. Add 300 μ L Reagent diluent to each well
 - e. Incubate at RT for at least 1 hour in the dark
 - f. Wash 3X with $\sim$ 400 μ L Wash buffer
 - g. Add 100 μ L of sample/standards per well
 - h. Seal with parafilm
 - i. Incubate for 2 hours at RT in the dark
 - j. Wash 3X with $\sim$ 400 μ L Wash buffer
 - k. Add 100 μ L working dilution of Streptavidin-HRP to each well
 - l. Incubate for 20 min at RT in the dark
 - m. Wash 3X with $\sim$ 400 μ L Wash buffer
 - n. Add 100 μ L Substrate solution
 - o. Incubate for 20 min at RT in the dark
 - p. Add 50 μ L Stop Solution to each well and mix by tapping
 - q. Measure A_{450} with wavelength correction set to A_{540} or A_{570}

Solutions

Resting solution

5.6 mM KCl
145 mM NaCl
2.2 mM CaCl₂
0.5 mM MgCl₂
5.6 mM glucose
15 mM HEPES-KOH, pH 7.4
0.1 mg/mL BSA

Stimulating solution

56 mM KCl
95 mM NaCl
2.2 mM CaCl₂
0.5 mM MgCl₂
5.6 mM glucose
15 mM HEPES-KOH, pH 7.4
0.1 mg/mL BSA

10 mg/mL BSA

0.1 g
10 mL H₂O

Lysis buffer

1% NP-40 or Triton X-100
20 mM Tris-HCl, pH 7.4

PBS

137 mM NaCl
2.7 mM KCl
8.1 mM Na₂HPO₄
1.5 mM KH₂PO₄
pH 7.4

Wash buffer

0.05% Tween-20
PBS

Reagent diluent

1% BSA
PBS

Substrate solution (R&D Systems DY999)

1:1 mixture of Colour reagent A and Colour reagent B

Stop solution (R&D Systems D994)

M. GST pulldown

A. GST-fusion expression

1. Inoculate a single colony into 50 mL LB media O/N with 0.1 mg/mL ampicillin
2. Reinnoculate at 1:50 dilution with 0.05 mg/mL ampicillin
3. Incubate at 37°C for ~2-3 hours
4. Add IPTG to 0.4 mM when OD₆₀₀ is ~0.4
5. Incubate at 37°C for 4 hours
6. Place on ice
7. Aliquot into 15 or 50 mL tubes (GST:ShhN: 14 mL, GST:ShhC: 40 mL, GST: 6 mL)
8. Spin at 3500 rpm for 15 min
9. Completely aspirate LB media
10. Either:
 - a. Proceed with GST purification
 - b. Flash freeze on liquid nitrogen (or dry ice) and store at -80°C

B. GST preparation

1. GST:ShhN or GST
 - a. Resuspend culture with 2 mL lysis solution A
 - b. Transfer to an eppendorf
 - c. Ice 10-15 min
 - d. Sonicate ~6X at 30% amplitude for 9 sec
 - e. Centrifuge at 16,000 x g for at least 10 min
2. GST:ShhC
 - a. Resuspend mL culture with 4 mL lysis solution B
 - b. Ice 10-15 min
 - c. Add 10 µL 1M DTT and 250 µL 10% sarkosyl to each tube
 - d. Sonicate ~6X at 30% amplitude for 9 sec
 - e. Add 600 µL 10% Triton X100 to each tube
 - f. Rotate at 4°C for 30-60 min
 - g. Centrifuge at 16,000 x g for at least 10 min

C. Bead preparation

1. Add 133 µL of glutathione-sepharose beads to an eppendorf tube (bed volume 100 µL) per GST prep
2. Add 1.5 mL lysis solution A or B
3. Mix by rotation
4. Centrifuge at 1000 x g for 3 min at 4°C
5. Discard supernatant
6. Repeat 2X

D. Immobilizing GST fusion to glutathione-sepharose beads

1. Apply GST proteins to washed GST beads
2. Rotate at 4°C for 1.5 hours
3. Wash 4X with Wash 1 (high salt wash)
4. Wash 3X with Wash 2 (Triton X wash)
5. Wash 3X with Wash 3 (normal salt wash)
6. Resuspend beads with 100 µL Wash 3
7. Measure protein concentration
 - i. Remove 10 µL of resuspended beads

- ii. Add 1.5 mL of 50 mM Tris-HCl, pH 8.0
 - iii. Spin at 1000 x g for 3 min at 4°C
 - iv. Remove supernatant
 - v. Resuspend beads in 20 µL elution buffer
 - vi. Measure A_{260} assuming 90% purity (GST: 1 A_{280} = 0.62; ShhN:GST: 1 A_{280} = 0.68; ShhC:GST: 1 A_{280} = 0.78)
- 8. Split beads into 2 tubes in equimolar amounts, topping with empty beads if necessary (bed volume 50 µL per tube) – this will represent one tube that received protein prep and the other tube will just be a control
- 9. Store on ice
- E. Tissue preparation
 - 1. Obtain 2 litters of rat pups (P1-P3)
 - 2. Extract brains and store in cold DPBS with Ca^{2+} and Mg^{2+}
 - 3. Wash brains in DPBS until blood is removed
 - 4. Rinse brains in SIM solution
 - 5. Mince brains in a Petri dish
 - 6. Weigh brains
 - 7. Add 2X volume (weight over volume) of SIM buffer + protease inhibitors
 - 8. Dounce homogenize brains 20X (tight fitting pestle)
 - 9. Spin at 10,000 x g for 30 min at 4°C
 - 10. Recover supernatant into SW28 ultracentrifuge tubes
 - 11. Spin at 100,000 x g for 60 min at 4°C
 - 12. Resuspend pellet in SIM buffer + protease inhibitors with Dounce homogenizer (~5X)
 - 13. Spin at 100,000 x g for 60 min at 4°C in a SW41 rotor
 - 14. Discard supernatant
 - 15. Homogenize pellet in ~4 mL HNM (to be used immediately)
 - 16. Check protein concentrations
 - 17. Solubilize pellet with CHAPS or NP-40
 - a. Add detergent to 0.5% final concentration
 - b. Incubate with rotation for 1 hour
 - c. Centrifuge at 20,000 x g at 4°C for 30 min
 - d. Transfer supernatant to a new tube
 - e. Check protein concentration
- F. GST-pulldown
 - 1. Preclear protein extract
 - a. Transfer supernatants to eppendorfs containing 20 µL bed volume of glutathione sepharose beads (beads prewashed 2X with Wash 3 buffer)
 - b. Rotate at 4°C for 30 min
 - c. Pellet beads at 1000 x g for 3 min at 4°C
 - d. Repeat 2X
 - 2. Apply precleared tissue rat brain protein extractions to GST preps
 - 3. Rotate at 4°C for 4 hours
 - 4. Spin at 1000 x g at 4°C for 3 min
 - 5. Wash 3X with 1.5 mL Wash 4 (buffer wash)
 - 6. Wash 3X with 1.5 mL Wash 5 (high salt wash)

7. Wash 3X with 1.5 mL Wash 6 (high salt plus detergent wash)
 8. Wash 3X with 1.5 mL PBS
 9. Rotate with 100 μ L Thrombin solution O/N at 4°C
 10. Spin at 1000 x g for 3 min at °C
 11. Transfer supernatant to a new tube
 12. Extract solution from beads with 200 μ L PBS 2X, pooling supernatants
 13. Concentrate thrombin-cleaved supernatants with a Micocon YM-10 prep for 45 min at 14,000 x g (4°C)
 14. Add ~10 μ L 5X SDS-PAGE loading buffer
 15. Add 2X SDS-PAGE loading buffer to leftover beads
 16. Boil samples for 3-5 min
 17. Load onto BioRad precast gradient gels (4-16%)
 18. Run overnight at 70-80V
- G. Silver staining
1. Briefly rinse gel in deionised water
 2. Fixative solution for at least 30 min
 3. 10 min in wash solution
 4. Deionised water for 10 min
 5. Deionised water for 10 min
 6. Sensitizer solution for 2 min
 7. Deionised water for 3 min
 8. Deionised water for 3 min
 9. Stain solution for 30 min
 10. Deionised water for 1 min
 11. 50:50 developer solution for ~10 sec (brief rinse)
 12. Developer solution until bands appear
 13. Dilute with deionised water (to slow the reaction)
 14. Stop solution for 5 min
 15. Store in storage solution
 16. Excise bands for mass spectrometry analysis

Solutions

LB broth	1M DTT
10 g/L Tryptone	154 mg
5 g/ L Yeast extract	1 mL 10 mM NaOAc, pH 5.2
10 g/L NaCl	Aliquot and store at -20°C
pH 7.0-7.4 with NaOH	STE
Ampicillin	10 mM Tris-HCl, pH 8
0.1 g	150 mM NaCl
1 mL ddH ₂ O	1 mM EDTA
IPTG	50 mg/mL lysozyme
1 g	500 mg lysozyme
42 mL H ₂ O	10 mL H ₂ O
Aliquot and store at -20°C	Aliquot at store at -20°C

PMSF
 174.1 mg
 1 mL isopropanol
 Store at 4°C
 10% Sarkosyl
 1 g sarkosyl
 10 mL STE
 Lysis buffer A
 1X PBS
 1 mM EDTA
 1 mM DTT
 0.5 mg/mL lysozyme
 1 mM PMSF
 5% Glycerol
 Lysis buffer B
 150 mM NaCl
 20 mM Tris-HCl, pH 8.0
 1 mM EDTA
 1 mM PMSF
 0.5 mg/mL Lysozyme
 5% Glycerol
 Elution buffer
 50 mM Tris-HCl, pH 8.0
 20 mM reduced glutathione
 SIM

250 mM sucrose
 5 mM imadazole
 1 mM MgCl₂
 pH 7.4
 HNM
 20 mM HEPES-NaOH, pH 7.4
 150 mM NaCl
 1 mM MgCl₂
 10X PBS
 140 mM NaCl
 2.7 mM KCl
 10 mM Na₂HPO₄
 1.8 mM KH₂PO₄
 pH 7.3
 Thrombin
 5 µL 5 U Thrombin/µL PBS (Aliquot &
 store at -80°C)
 45 µL 1X PBS
 Silver stain solution
 250 µL 37% formaldehyde
 15 g Na₂CO₃
 500 mL H₂O

	Wash 1	Wash 2	Wash 3	Wash 4	Wash 5	Wash 6
0.5 M HEPES	1.6	1.6	3.2	1.6	1.6	1.6
0.1 M PMSF	0.4	0	0	0	0	0
5 M NaCl	4	1.2	2.4	0	2.4	2.4
Glycerol	2	0	0	0	0	0
MgCl ₂	0	0	0.16	0	0	0
25% Triton X-100	0	1.6	0	0	0	0
10X CHAPS/NP-40	0	0	0	0	0	0.8
H ₂ O	32	35.6	74.2	38.4	36	35.2
Final Volume	40 mL	40 mL	80 mL	40 mL	40 mL	40 mL

	Fixative	Wash	Sensitize	Stain	Develop	Stop	Storage
Ethanol	100 mL	100 mL					
Acetic acid	10 mL					10 mL	2 mL
Sodium thiosulfate			40 mg				
Silver nitrate				200 mg			
Formalin					0.27 mL		
Sodium carbonate					5 g		
ddH ₂ O	90 mL	100 mL	200 mL	200 mL	250 mL	190 mL	198 mL

N. Coimmunoprecipitation

1. Preparation of lysates
 - a. Cotransfect COS cells in 6 cm² dishes
 - b. 48 hours later, place dishes on ice
 - c. Wash cells 2X with cold PBS
 - d. Add 1 mL lysis solution with protease inhibitors
 - e. Incubate on ice for 10 min
 - f. Scrape and place into a chilled 1.5 mL eppendorf
 - g. Pipette ~5X
 - h. Incubate on ice for 20 min
 - i. Spin at 10,000 x g for 15 min
 - j. Transfer supernatant to a new eppendorf tube (keep 20 µL volume for loading control – add 5-10 µL 5X SDS-PAGE loading buffer and store at -80°C)
2. Preparation of beads
 - a. Take an aliquot of beads into an 1.5 mL eppendorf tube
 - b. Add 1 mL lysis buffer and mix
 - c. Centrifuge at 5000 x g for 30 sec
 - d. Magnetic beads:
 - i. Place on magnet for 2-3 min
 - ii. Pipette out solution
 - e. Repeat washes 2X
 - f. Add original volume of lysis buffer to beads
3. Immunoprecipitation
 - a. Either add
 - i. Fc
 1. Add 30 µL Protein A beads to clarified cell lysates
 2. Incubate for 2 hours at 4°C
 - ii. Indirect IP
 1. Add 1 µg Ab to sample to clarified cell lysates
 2. Incubate for 2 hours at 4°C
 3. Add 20 µL anti-mouse/rabbit IgG beads
 4. Incubate for 1 hour at 4°C
 - iii. Direct IP
 1. 20 µL Flag-M2 beads to clarified cell lysates
 2. Incubate for 2 hours at 4°C
 - b. Wash 4X with 1 mL wash buffer
 - c. Add 30 µL 2X SDS-PAGE loading buffer
 - d. Boil for 5 min
 - e. Pellet beads at 13,000 rpm for ~10 sec
 - f. Load supernatant onto SDS-PAGE

Solutions

PBS

137 mM NaCl
2.7 mM KCl
8.1 mM Na₂HPO₄
1.5 mM KH₂PO₄
pH 7.4

Lysis solution

20 mM HEPES-NaOH, pH 7.4
100 mM NaCl
1% NP-40
10% Glycerol

Wash buffer

20 mM HEPES-NaOH, pH 7.4
300 mM NaCl
0.1% NP-40
5% Glycerol

8. CURRICULUM VITAE

Shawn Beug

Education

- 2004 to present: PhD: Biochemistry (Human and Molecular Genetics), University of Ottawa.
2001 to 2004: MSc: Cellular and Molecular Medicine (Development), University of Ottawa.
1997 to 2001: BSc: Biology with Honours (Animal Physiology), University of Regina.

Research Experience

- 2004 to present: PhD candidate, Department of Biochemistry, University of Ottawa.
2003 to 2004: Research technician, University of Ottawa Eye Institute.
2001 to 2004: MSc student, Department of Cellular and Molecular Medicine, University of Ottawa.
2000 to 2001: Honours student, Department of Biology, University of Regina.

Publications

- *McNeill B, *Wall DS, ***Beug ST** and Wallace VA. (2008). Probing the Hedgehog pathway in retina development. *In* Fliesler SJ. and Kisselev OG. *Signal transduction in the retina*. pp 299-328. New York: CRC Press.
- Dakubo GD, **Beug ST**, Mazerolle CJ, Thurig S, Wang Y and Wallace VA. (2008). Control of glial precursor cell development in the mammalian optic nerve by sonic hedgehog from retinal ganglion cells. *Brain Res.* 1228: 27-42.
- Beug ST**, Vascotto SG and Tsilfidis C. (2006). The newt orthologue of *Growth Arrest-Specific 6* is implicated in stress response during newt forelimb regeneration. *Dev. Dyn.* 235: 711-722.
- Vascotto SG, **Beug ST**, Liversage RA and Tsilfidis C. (2006). Expression profiles of *Eastase1* (*NvElastaseI*) and *secretory leukocyte protease inhibitor* (*NvSLPI*) during forelimb regeneration in adult *Notophthalmus viridescens* suggest a role in epithelial remodelling and delamination. *Dev. Genes Evol.* 216: 499-509.
- *Vascotto SG, ***Beug ST** and Tsilfidis C. (2005). *Nvβ-actin* and *NvGAPDH* as normalization factors for gene expression analysis in limb regenerates and cultured blastema cells of the adult newt, *Notophthalmus viridescens*. *Int. J. Dev. Biol.* 49: 833-842.
- Vascotto SG, **Beug ST** and Tsilfidis C. (2005). Identification of cDNAs associated with late dedifferentiation in adult newt forelimb regeneration. *Dev. Dyn.* 233: 347-355.

Cameron C, **Beug ST** and Tsilfidis C. (2004). Captive breeding of *Notophthalmus viridescens* through hormonal manipulation. Herpetol. Rev. 35: 257–259.

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Manuscripts in Preparation

Beug ST, Parks RJ, McBride H, Wallace VA. Trafficking of the Sonic Hedgehog morphogen to the regulated secretory pathway in mammalian neurons.

Beug ST, McBride HM and Wallace VA. Identification of Sonic hedgehog interacting proteins in the central nervous system.

Honours and Awards

2005 to 2009: Research Studentship, Multiple Sclerosis Society of Canada.
2004 to 2008: Ph.D. Entrance Scholarship, University of Ottawa.
2002 to 2003: Let's Talk Science Excellency Award, University of Ottawa.
2001 to 2002: Let's Talk Science Excellency Award, University of Ottawa.
2001 to 2002: M.Sc. Entrance Scholarship, University of Ottawa.
2000 to 2001: Dean's Honour List, University of Regina.
2000 to 2001: Research Grant, Saskatchewan Environment and Resource Management.

Selected Presentations at Major Conferences

Beug ST, Parks R, McBride HM and Wallace VA. (December 2007). Molecular regulation of Shh trafficking in neurons. American Society for Cell Biology 47th Annual Meeting. Washington, DC.

Beug ST, Parks R, McBride HM and Wallace VA. (December 2007). Molecular regulation of Shh trafficking in neurons. The Canadian EndMS Research Conference. Banff, AB.

Beug ST, Dakubo GD, McMahon AP and Wallace VA. (July 2006). Sonic hedgehog regulates gliogenesis in the rodent optic nerve. Glia in Health and Disease, Cold Springs Harbor. NY.

Beug ST, Dakubo GD, McMahon AP and Wallace VA. (May 2005). Sonic hedgehog from retinal ganglion cells regulates gliogenesis in the mammalian optic nerve. ARVO, Fort Lauderdale, FL.

Beug ST, Dakubo GD, McMahon AP and Wallace VA. (March 2005). RGC-derived Sonic hedgehog is required for normal gliogenesis in the rodent optic nerve. 25th Great Lakes Mammalian Development Meeting, Toronto, ON.