Using artificial substrates to define steps in nascent synapse development.

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ABSTRACT

In this thesis, I describe a unique system to study synaptic development *in vitro*. Our model is based on initial findings that presynaptic-like boutons can form if provided with an appropriately charged target. We report that complete presynaptic boutons can form *de novo* following adhesion to beads coated with poly-D-lysine (PDL), a positively charged artificial protein. We find that the effect is limited to only certain cationic substances, suggesting that the distribution of charge on the bead surface is critical. As demonstrated by a combination of atomic force, live imaging and confocal microscopy, we show that bead adhesion to the axonal surface triggers the recruitment of multiple presynaptic proteins, leading to the formation of functional presynaptic boutons within as little as 1h. Both GABAergic and glutamatergic-type boutons are equally capable of forming onto PDL-coated beads, suggesting that the critical requirements for presynapse formation are shared between inhibitory and excitatory neurons. We find that heparan sulfate proteoglycans, possibly adsorbed onto the bead or expressed on the axon surface, are required for assembly to proceed, as is the dynamic reorganization of F-actin. However, the postsynaptic membrane is not required.

Our results indicate that PDL can effectively bypass cognate and natural postsynaptic ligands to trigger presynaptic assembly. However, we find that postsynaptic compartment assembly can only proceed at bead sites in the presence of a formed presynaptic ending and never to the bead itself. This result suggests that postsynaptic development is comparatively restrictive, and requires factors derived from a natural presynaptic target.

Collectively, these findings suggest that the nascent presynapse is a critical player driving synaptogenesis. Furthermore, our observations underscore the lability with which axons can form presynaptic endings to unnatural targets, which may serve as a strategy *in situ* to ensure that sufficient numbers of synapses are formed during development. Finally, our observations that presynaptic endings can form in the absence of engagement

of any of the molecules shown to be synaptically inductive *in vitro* suggest that their natural role *in vivo* is most critical during later maturational stages of synaptogenesis.

RÉSUMÉ

Dans ce manuscrit nous présentons et caractérisons un système *in vitro* afin d'étudier le développement des synapses neuronales. Notre model se base sur des données antécédentes indiquant que des boutons présynaptiques peuvent se former sur un cible avec une charge électrostatique adéquate. Nous rapportons que des boutons présynaptiques entiers peuvent se former *de novo* suite à leur adhésion à des billes couvertes avec de poly-D-lysine (PDL), un mélange protéinique artificiel qui possède une charge électrostatique positive. Cet effet est observé avec certaines substances cationiques seulement, suggérant ainsi que la distribution des cations est importante pour l'effet. À l'aide de différentes techniques telles que la force atomique, l'imagerie de cultures vivantes, et l'imagerie par microscopie confocale, nous démontrons que l'adhésion des axones aux billes cause le recrutement de plusieurs protéines présynaptiques et que et cette adhésion donne naissance à des boutons présynaptiques fonctionnels en moins d'une heure. Ces boutons sont de type GABAergique et glutamatergique, laissant sous-entendre que les conditions nécessaires pour la formation de synapses inhibitrices et excitatrices sont communes. Nous constatons que les protéines de type héparine sulfate proteoglycan, probablement adhérées à la surface des billes ou exprimées par les axones, sont requises pour l'assemblage des boutons et la réorganisation de l'actine de forme F. Toutefois, la membrane postsynaptique n'est pas requise pour cet effet.

Nos résultats indiquent que le PDL peut promouvoir l'assemblage présynaptique en se substituant pour les ligands post-synaptiques. Par contre, nous observons que l'assemblage du compartiment post-synaptique ne peuvent prendre place que sur les billes possédant les facteurs pré-synaptiques. Cela indique que le développement postsynaptique est restrictif et tributaire de facteurs naturels provenant de l'élément présynaptique.

En somme, nos résultats démontrent que la présynapse naissante est cruciale pour promouvoir la synaptogénèse. De plus, nos observations indiquent que les axones sont aptes à former des liens pré-synaptiques avec des substrats artificiels; ce phénomène

pourrait être employé pour contrôler le nombre suffisant de synapses *in situ* durant le développement de l'organe. Finalement, le fait que les présynapses se forment en l'absence de molécules impliquées dans la synaptogénèse *in vitro* suggère que la fonction *in vivo* de ces molécules est plus importante durant les étapes ultérieures de la synaptogénèse.

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LIST OF ABBREVIATIONS

Abp: Actin binding protein
ACh: Acetylcholine
AMPA: a-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AP2: Accessory protein 2
AP: Action potential
ATP: Adenosine triphosphate
BDNF: Brain derived neurotrophic factor
CA1: Cornu ammonis 1
CAM: Cell adhesion molecule
CASK: Calcium/calmodulin-dependent serine protein kinase
CAST: CAZ-associated structural protein
CAZ: Cytomatrix at the active zone
C.elegans: Caenorhabditis elegans
CNS: Central nervous system
DAG: Diacylglycerol
DIV: Days in vitro
D.melanogaster: Drosophila melanogaster
E(17): Embryonic day (17)
ECM: Extracellular matrix
EM: Electron microscopy
ELKS: Glutamate (E)-leucine (L)-lysine (K)-serine (S)
EphB: EphrinB
ERC: ELKS-Rab6-interacting protein-CAST
F-actin: Filamentous actin
FERM: 4.1 (E)-ezrin (R)-radixin (M)-moesin
FITC: Fluorescein-conjugated
FGF: Fibroblast growth factor
FGFR: Fibroblast growth factor receptor
GABA: Gamma-aminobutyric acid
GAG: Glycosaminoglycan
GDNF: Glial-derived neurotrophic factor
GFRα: GDNF receptor α
GEF: Guanine nucleotide exchange factor
GFP: Green fluorescent protein
GIT1: G protein-coupled receptor kinase-interacting protein 1
GKAP: Guanylate kinase associated protein
GluR/GluR1-4: Glutamate receptor/AMPA-type glutamate receptor subunit 1-4
Gly: Glycine
GPI: Glycosyl-phosphatidylinositol
GRIP/ABP: GluR-interacting protein/AMPA-R binding protein
GTP: Guanosine triphosphate
hDlg: human Discs long
HBGAM: Heparin binding-growth associated molecule
HS: Heparan sulfate

HSPG: Heparan sulfate proteoglycan
Hsp70: Heat shock protein of 70kDa
Ig: Immunoglobulin
LAR: Leukocyte antigen related
LTP/LTD: Long-term potentiation/Long-term depression
MALS/Veli: Mammalian LIN-7/vertebrate homologue of LIN-7
MAGUK: Membrane-associated guanylate kinase
Mena/VASP: Mammalian enabled/vasodilator-stimulated phosphoprotein
mEPSC: mini excitatory post-synaptic current
mGluR: metabotropic glutamate receptor
Mint1: Munc-18 interacting protein-1
Munc: Mammalian uncoordinated (unc)
MuSK: Muscle-derived receptor tyrosine kinase
mRNA: messenger RNA
NCAM: Neural cell adhesion molecule
Necl: Nectin-like
NGL2: Netrin-G ligand 2
NMJ: Neuromuscular junction
NMDA: N-methyl-D-aspartate
NR1-2(A-D): NMDA-type glutamate receptor subunit 1-2(A-D)
NSF: N-maleimide sensitive protein
N-WASP: Neuronal Wiskott-Aldrich syndrome protein
P(5): Postnatal day (5)
PDZ: PSD-95, Discs large, Zona Occludens
PICK-1: Protein interacting with C kinase-1
PKA: Protein kinase A
PKC: Protein kinase C
Pra1: Prenylated rab3a receptor 1
ProSAP: Proline-rich synapse-associated protein-1
PSA: Polysialic acid
PSD: Postsynaptic density
PSD-95/93: Postsynaptic density protein-95/93
PTV: Piccolo-bassoon transport vesicle
RGC: Retinal ganglion cell
RIM: Rab3a-interacting molecule
RIMBP: RIM binding protein
RNA: Ribonucleic acid
RNAi: Interference RNA
SALM: Synapse adhesion-like molecule
SAP-90/97/102: Synapse-associated protein-90/97/102
SH3: Src homology region 3
SV: Synaptic vesicle
SNAP: Soluble NSF-attachment protein
SNARE: Soluble NSF attachment protein receptors
STV: Synaptic vesicle transport vesicle
SYD: Synapse-defective

SynCAM: Synapse cell adhesion molecule
Syt1: Synaptotagmin1
SV2: Synaptic vesicle-associated 2
TAG-1: Transient axonal glycoprotein-1
TARP: Transmembrane AMPA receptor-regulatory protein
TLSC: Tumor suppressor in lung carcinoma
UNC: Uncoordinated
VAMP: Vesicle-associated membrane protein
VGAT: Vesicular GABA transporter
VGlut: Vesicular Glutamate transporter

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AUTHOR CONTRIBUTIONS

RESULTS PART (A): MANUSCRIPT, "Rapid assembly of functional presynaptic boutons triggered by adhesive contacts."

Anna Lisa Lucido: Developed the rationale for the manuscript (with D.R.C.). Developed model system and prepared all hippocampal cultures for fixed immunocytochemistry (Figures 1B, 1D, 2A, 3B-E, 4C, 5A, 5G, 6E, 7A-D, S1, S2, S4) and electron microscopy (Figure 1C). Performed all immunofluorescence experiments as well as the processing of cultures for electron microscopy. Carried out all confocal and electron microscopic imaging. Performed all live imaging studies (Figures 2B, 5C-D, 6A-B, S5A,C,E,G, Supplemental Movies S3 and S4) except the AFM experiments (Figure 1A). Carried out all quantification and analysis. Prepared all figures (including the drawing of Figures 3A and 8). Wrote the manuscript.

Fernando Suarez Sanchez: Performed the atomic force microscopy (AFM) experiments (with P.T.)(Figure 1A, Supplemental Movies S1 and S2).

Peter Thostrup: Cultured and transfected all hippocampal cultures for the AFM experiments and carried out the AFM imaging (with F.S.S.)(Figure 1A, Supplemental Movies S1 and S2).

Adam V. Kwiatkowski: Created the β-actin lentiviral construct. Assisted A.L.L. with the live confocal imaging and analysis (Figures 5C-F, 6A-D).

Sergio Leal Ortiz: Prepared all lentiviral-infected hippocampal cultures for live imaging (Figures 5C-F, 6A-D, S5).

Gopakumar Gopalakrishnan: Prepared the cationic lipid-coated silica beads (Figure 3D).

Dalinda Liazoghli: Prepared the micropatterned substrates (with W.B.)(Figure 7A-B).

Wiam Belkaid: Prepared the micropatterned substrates (with D.L.)(Figure 7A-B).

Bruce Lennox: Edited the manuscript.

Peter Grutter: Assisted in devising the AFM experiments (with P.T. and F.S.S.).

Craig C. Garner: Assisted in devising the live imaging experiments (Figures 5C-F, 6A-D, S5), developing the rationale for the manuscript, and editing the manuscript.

David R. Colman: Developed the rationale and edited the manuscript.

RATIONALE AND OBJECTIVES

This thesis attempts to elucidate some of the precise steps in nascent synapse formation. Recognizing the synapse in its nascent form has represented a significant challenge, due to the difficulties in detecting *de novo* synaptogenesis *in vitro* and *in vivo* at a developmental stage when neurons are competent to form synapses. This has usually required random imaging of neuronal cells and tissues without assurance that a new synaptic junction would form during the imaging session. Other studies have relied on genetic and molecular techniques to study preexisting synaptic contacts; while important for dissecting the roles of synaptically localized proteins, this approach allows for no measurement of synaptic age and thus no temporal insight into the contribution of such proteins in the development process.

If it is possible to create a system to induce synaptic development in a temporally- and spatially-resolved manner, we reasoned that we could address at least some of the otherwise intractable questions in synaptic development. First, we wished to determine what is required to trigger synaptic induction- specifically, whether contact-mediated adhesion is truly a first step in synaptogenesis. Next, we wished to elucidate some of the early steps in the synaptogenic process, such as the recruitment of synaptic proteins, the relationship between protein recruitment and functionality, and the relationship between presynaptic and postsynaptic development. This second objective is predicated on a system that can truly induce the formation of a nascent synapse, one that can transition through all the typical assembly and maturational processes that normally occur but are so difficult to define *in situ* (Ahmari and Smith, 2002).

To address these questions, we decided to reinvigorate an old *in vitro* system of cultured neurons and beads coated with poly-cationic polymers, similar to that used by Burry, Peng and colleagues. These studies were highly intriguing and provided the pilot observations that initiated the work of this thesis, specifically, that positively charged substances could induce the formation of presynaptic-like specializations. Collectively, their most significant findings were as follows.

Burry and Peng showed that all cationic proteins they tested selectively induced "presynapses" to form, while no neutrally or negatively charged proteins could do so (Burry, 1980a; Burry, 1982b; Burry, 1985; Burry et al., 1985; Burry et al., 1986; Peng et al., 1987). Peng observed that muscle cells could cluster ACh receptors in response to polylysine-coated beads (Peng et al., 1981; Peng and Cheng, 1982), but neither investigated whether postsynaptic development occurred in neurons. These studies were almost exclusively performed using EM as a readout with the exception of two studies in which synaptic vesicle antigen 48 (SV48)(Burry et al., 1986; Peng et al., 1987) and synapsin I (Peng et al., 1987) was shown to accumulate at bead sites by immunofluorescence. Time course studies were performed (2h up to several days) at which the vesicle clustering phenomenon was assessed (Burry, 1982b; Burry, 1983). Finally, the vesicle-clustering phenomenon was determined to be protein synthesis independent (Burry, 1985), to be inhibited by glial proliferation (Burry et al., 1985), and could be observed using beads implanted into the cerebellum *in vivo* (Burry, 1983).

From these studies, it was speculated - not demonstrated - that electrostatic-based adhesion was a first step in synaptogenesis (Burry, 1980a; Peng et al., 1987). In their model, apposed axonal and dendritic membranes display complementary charges that facilitate the adhesion of neuronal membranes, leading to the subsequent clustering of pre- and postsynaptic molecules, and eventually, to the establishment of a functional synapse (Burry, 1980). The nature of these charges *in situ* was not described, and attempts to identify endogenous factors that facilitate presynapse formation did not progress beyond fractionating bead-presynaptic complexes by 2-D electrophoresis (Burry and Hayes, 1989). The role of the postsynaptic membrane was not clear, nor the extent to which the presynaptic ending could mature in response to the bead. These questions were never resolved, leaving this body of work highly intriguing but difficult to interpret and thereafter to place in context with the broader neurodevelopmental field.

In this thesis, we have attempted to pick up where these studies left off and in the process, to make fundamental insights into the way synapses are induced to form and assemble at their very earliest stages.

PREFACE TO THE INTRODUCTION

Our understanding of synaptic development and function has mostly relied on the study of synapses within two model regions of the nervous system: the cholinergic synapse of the peripheral neuromuscular junction, and within the central nervous system, the excitatory glutamatergic synapse of the hippocampus. A full consideration of the composition and function of all types of synapses in the nervous system, although worthwhile, will not be directly addressed. Rather, I focus attention on the advances made from studies using the hippocampus and the neuromuscular junction as model systems, including studies from other neuroanatomical regions where appropriate.

The emphasis of this thesis is on presynaptic development; therefore the introduction will contain a more extensive review of the components, function and formation of the presynaptic ending. Comparatively, the review of postsynaptic composition and function will be brief aside from a view of its major constituents and its role in synaptic development.

INTRODUCTION

PART I: Historical review of the synapse.

Neuroanatomy in its infancy: The earliest observations of nervous tissue.

The study of nervous tissue became a subject of science in the mid-1800s, following improvements to the compound microscope as well as techniques to stain nervous tissue, which together allowed for the detailed examination of neuronal connectivity (Bennett, 1999). It had already been established that individual cells of plants and animals were physically distinct from each other, each characterized by a nucleus, surrounded by cytoplasm, and bounded by a cell membrane, a conceptual framework known as the Cell Theory (Schleiden, 1838; Schwann, 1839). This concept was not immediately extended to nerve cells given their unique and complex morphology [reviewed in (Bennett, 1999)]. Instead, it was believed that for nerve cells to communicate, their terminations must be fused with their target cells. The ensuing syncytial-like continuity would be responsible for long- and short-range communication in the nervous system, forming the basis for the reticular theory of neural connectivity (Shepherd and Erulkar, 1997; Bennett, 1999; Guillery, 2007; Jones, 2007).

However, the reticular theory was inconsistent with reports that such cellular continuity could not be observed microscopically (Kolliker, 1867; His, 1886; Kuhne, 1888; His, 1889). It fell further from favour following studies of stained tissues after lesioning, in which it was observed that the resulting atrophy of nerve fibres did not extend to their functionally connected cells (Waller, 1850; Forel, 1887; Bennett, 1999). Finally, it was the histological staining method developed by Camillo Golgi, and refined by Santiago Ramon y Cajal, that provided a definitive breakthrough. In the 1870s, Golgi developed a method to stain nervous tissue with silver salts, producing a black impregnation of neurons that revealed their entire structure under the microscope. Cajal then used infant and small animals in which myelination had not yet commenced or was sparse, and subjected incompletely stained tissue to repeated infiltration of the silver staining reagents, resulting in a more consistent and reliable staining than was achieved by Golgi

[reviewed in (Jones, 2007)]. Using his refined Golgi method, Cajal reported that the terminations of neurons ended without any sign of physical continuity (Cajal, 1888). These observations led to his firm and steadfast conclusion that these points of discontinuity were not the result of experimental artefacts or limits in imaging resolution, but indeed reflected the true nature of neural connectivity and represented the critical point of communication between nerve cells (Cajal, 1891a, b, 1909-1911).

Although groundbreaking and ultimately correct, Cajal's hypothesis lacked functional corroboration. Functional studies of neurotransmission began with the work of Claude Bernard, who showed that the arrow-tip poison curare induced muscle paralysis by paralyzing the nerve, implying the existence of a nerve-muscle junction within which the poison acted (Bernard, 1856). Almost 50 years later, Charles Sherrington focused on the reflex arc in the spinal cord, and found that the transmission of neural impulses between connected cells did not exhibit the same kinetic activity as observed during conduction of action potentials along nerve fibres (Shepherd and Erulkar, 1997; Bennett, 1999; Levine, 2007). He then reasoned that if sensory nerve arbors terminate in free endings, these represent the sites through which impulses from sensory to motor nerve must take place (Shepherd and Erulkar, 1997; Bennett, 1999; Levine, 2007). This proposal united the neuroanatomical and physiological evidence into a single term that Sherrington will forever be credited for, the *synapse,* which officially came into being in 1897 with the updated publication of Foster's 'Textbook of Physiology' (Sherrington and Foster, 1897):

"So far as our present knowledge goes, we are led to think that the tip of a twig of the arborescence is not continuous with but merely in contact with the substance of the dendrite or cell body on which it impinges. Such a special connection of one nerve cell with another might be called a synapse."

Within several decades, advanced studies in neurophysiology led to the discovery of chemical neurotransmission (Dale, 1914; Loewi, 1921; Fatt and Katz, 1951, 1952). This was shortly followed by the characterization of synaptic ultrastructure by electron

microscopy, revealing synaptic vesicles within axon terminations to be separated from their contacting dendrites by a cleft space (De Robertis and Bennett, 1954; Palade and Palay, 1954; De Robertis, 1955; Palay, 1956). Together, these discoveries dispelled any remaining doubt of the synapse being the critical anatomical and physiological site responsible for neural communication.

Model systems for the study of synaptogenesis.

Determining how synapses are wired from all vantage points- anatomical, physiological, molecular and genetic- is critical if we are to understand how synaptic function translates into behavioural phenotypes (Munno and Syed, 2003). It is therefore worthwhile to comment on the model systems that have yielded the most significant advances in our understanding of synapse biology. The focus in this thesis is on the mammalian NMJ and the CNS hippocampal synapse, which are the most heavily utilized systems in contemporary synapse biology and have yielded significant advances in our understanding of the molecular constituents, function and plasticity of the synapse itself. However, the sheer numbers of neurons combined with the astonishing rate at which synapses are formed in the mammalian nervous system complicates all attempts to define the mechanisms of synaptic connectivity. For the study of synaptic circuitry, neurobiologists have instead turned to a number of other model organisms whose more tractable nervous systems offer various advantages to the mammalian CNS.

Among the best-studied invertebrate model systems are those of the soil nematode *Caenorhabditis elegans* and the marine invertebrate *Aplysia californica.* Each of these organisms has a defined and limited number of neurons- exactly 302 for *C.elegans* and approximately 20,000 for *A.californica* which are clustered into 10 discrete ganglia- whose positions are fixed, thus allowing for the identification and characterization of specific neurons and their circuits (Bargmann, 1993; Schafer, 2005; Hawkins et al., 2006). Each organism has several well-defined behaviours that have been shown to depend on specific synapses, thus validating these organisms for such studies (Bargmann, 1993; Sattelle and Buckingham, 2006). Moreover, the nervous system of

Aplysia has proven to be an excellent model for the study of memory, as these organisms exhibit reflex behaviours that can be shaped by learning (Hawkins et al., 2006; Sattelle and Buckingham, 2006).

Along with *Aplysia*, other invertebrate mollusks including *Lymnaea stagnalis* and *Helisoma* have proven valuable for the study of synaptic circuitry using electrophysiological approaches (Camardo, 1983; Haydon and Zoran, 1989; Syed et al., 1990; Zoran et al., 1991). In this context, the advantage relates to size- these systems have very large and identifiable neurons that can be isolated from the organism and cultured *in vitro*, where they appear to recapitulate their patterns of development with high accuracy (Munno and Syed, 2003).

C.elegans belongs to a group of model genetic organisms that include *Drosophila melanogaster* as well as the vertebrate species *Danio rerio* (commonly known as zebrafish). These are models with a shared advantage in that they are highly amenable to germline transformation, and are thus valuable systems for studying the contribution of specific genes to synaptic connectivity (Bargmann, 1993; Rohrbough et al., 2003; Gahtan and Baier, 2004; Margeta et al., 2008). This, combined with their rapid developmental growth patterns, vastly improves the efficiency in which the genetic contributions of specific circuits can be defined (Bargmann, 1993; Rohrbough et al., 2003; Gahtan and Baier, 2004; Margeta et al., 2008). Finally, several of these organisms- including *c.elegans*, zebrafish as well as the tadpoles of the aquatic frog *Xenopus laevis,* are invaluable models for the study of synaptic circuitry using optical approaches. This advantage is served by the fact that their bodies are transparent, allowing for the direct visualization of their development *in vivo*. When combined with various genetic approaches, including transgenic expression of reporter genes, high-resolution imaging can serve as a powerful readout for the effects of different genetic manipulations on synaptic connectivity (Haas et al., 2002; Gahtan and Baier, 2004; Schafer, 2005; Scott et al., 2007; Hewapathirane and Haas, 2008). Taken together, it is clear that there are a variety of model systems to study synaptic development, each with their own distinct advantages depending on the desired techniques to be used.

The Ultrastructural Synapse: Compartments as defined by EM.

Ultrastructurally, the synapse is defined as having 3 distinct compartments: the presynaptic bouton, the postsynaptic density, and a synaptic cleft that separates the two by a short distance of approximately 20nm (see figures below). Within the presynaptic bouton (*Pre*, Figure 1) resides hundreds of closely packed, clear-centered vesicles approximately 50nm in diameter, which contain the neurotransmitter responsible for chemical neurotransmission. Where the pre and postsynaptic plasma membranes align, synaptic vesicles found immediately adjacent to the presynaptic membrane are embedded within an electron dense material which, viewed *en face,* appears as a highly ordered array (Bloom and Aghajanian, 1968; Pfenninger et al., 1972; Peters et al., 1991; Phillips et al., 2001)(black arrows, Figure 1 bottom). This presynaptic membrane region is known as the active zone, and is responsible for the regulated fusion of neurotransmitter-containing synaptic vesicles with the plasma membrane.

The postsynaptic density (PSD)(black arrowheads, Figure 1) is the electron dense region of the plasma membrane that apposes the presynaptic bouton. By EM, the PSD is not as morphologically complex although the electron dense material lining the membrane surface is visibly thicker compared to the presynaptic side. Finally, the synaptic cleft separating the pre and postsynaptic compartments also contains a dense filamentous plaque of intercellular material (Pfenninger, 1971; Cotman and Taylor, 1972; Peters et al., 1991). The electron dense synaptic cleft, together with the pre and postsynaptic densities, form a junctional complex that is both functional and remarkably resistant to degradation (Pfenninger, 1971; Cotman and Taylor, 1972; Peters et al., 1991; Phillips et al., 2001). Thus, the synapse is a construct whose neuroanatomy and structural integrity work together to subserve its physiology.

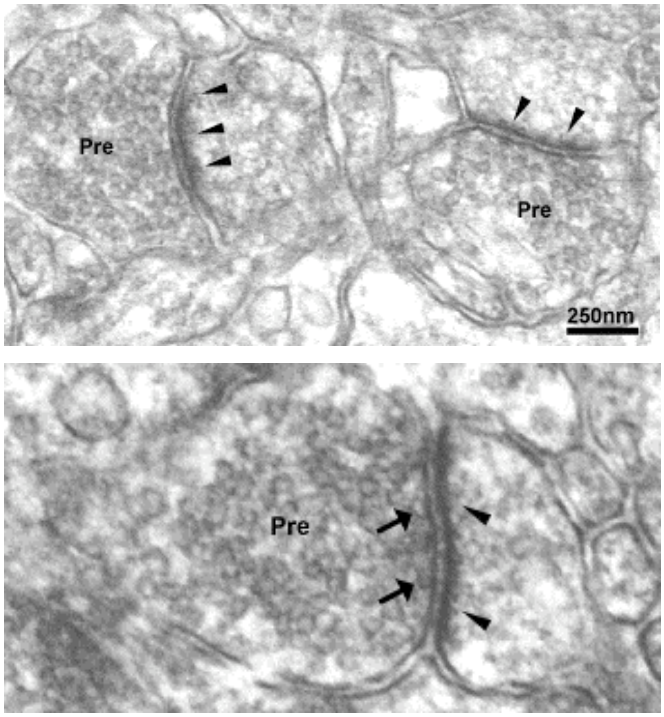

Figure 1. Electron microscopic images of synapses in the rodent CNS. Both top and bottom images are taken from adult rodent cortex. Pre, presynaptic bouton. Black arrowheads delineate the postsynaptic density. Black arrows (bottom) highlight docked synaptic vesicles within the presynaptic active zone. Images acquired and processed by A.L.L.

In recent decades, the widespread use of molecular techniques has revealed the electron densities observed ultrastructurally to represent a variety of synaptic molecules arranged with incredible sophistication and complexity. These individual molecules may be classified into functional classes according to their specific role(s) in synaptic function, including the regulated release of neurotransmitter, receptor signalling and transduction, intracellular scaffolding, and transsynaptic adhesion. Many hundreds of proteins have been reported to be expressed at synapses, although only a subset that have been thoroughly characterized will be described next as per their role within each functional class.

PART II: Structure of the presynaptic ending.

(I) Synaptic vesicle proteins (integral and vesicle-associated).

The synaptic vesicle is an organelle whose contents, namely the transmitter molecules packaged within them, are responsible for postsynaptic activation. On their membrane surface, synaptic vesicles (SVs) express a variety of integral proteins that regulate essential presynaptic tasks including the uptake, storage and Ca++-dependent release of neurotransmitter as well as adaptor molecules responsible for the regulated fusion and retrieval of synaptic vesicles.

SVs are filled with neurotransmitter by vesicular transporter proteins whose expression varies depending on the type of transmitter they are designed to load. In the hippocampus, these include vesicular glutamate (vGlut1-3, with vGlut1 being the dominant transporter in the hippocampus) and GABA (VGAT) transporters, along with trimeric GTPases and vacuolar H+-ATPase that fuels the transport (Ahnert-Hilger et al., 2004).

Within the presynaptic bouton, the functional organization of synaptic vesicles is hypothesized to take the form of discrete pools differing in their size (% of total vesicle pool), location, mobility and stimulation requirements for release; these pools are known as the readily releasable, recycling, and reserve pools (Heuser and Reese, 1973; Rosenmund and Stevens, 1996; Richards et al., 2000; Richards et al., 2003; Rizzoli and Betz, 2005). Actin is a major component of the presynaptic scaffold whose filaments interact with SVs, and is widely thought to behave as the molecular track that SVs travel along between and within their respective pools (Fifkova and Delay, 1982; Drenckhahn et al., 1984; Landis et al., 1988; Dillon and Goda, 2005; Cingolani and Goda, 2008). Synapsin proteins, expressed on SVs themselves, link SVs to F-actin and are thought to behave as the ‘glue’ that binds SV clusters together, particularly within the reserve pool (Cingolani and Goda, 2008). Given that the activity of synapsin proteins is controlled by phosphorylation suggests a plausible mechanism whereby activity induces

phosphorylation-dependent changes in synapsin activity, thus mobilizing vesicles to traffic between pools as needed for efficient neurotransmission (Chi et al., 2001, 2003).

Proteins regulating synaptic vesicle exocytosis

Regulated SV fusion is a tightly regulated form of membrane trafficking that in its final steps is overseen by a set of highly conserved proteins collectively referred to as SNARE *(soluble NSF-attachment protein (SNAP) receptors)* proteins. It is thought that synaptic vesicles within the active zone are docked immediately beneath the plasma membrane through the interaction of the vesicle SNARE synaptobrevin with the transmembrane SNAREs syntaxin-1 and SNAP-25, to form helical core complexes of extraordinary stability (Sutton et al., 1998; Jahn and Scheller, 2006). Upon action potential depolarization of the presynaptic terminal and Ca++ influx, these coiled coil bundles undergo conformational changes that orient the complex parallel to the plasma membrane, thereby exerting a mechanical force on the vesicle and active zone membranes and promoting membrane fusion (Hanson et al., 1997; Lin and Scheller, 1997; Jahn and Scheller, 2006).

The SV protein synaptotagmin-1 (Syt1) is a member of a family of Ca++ binding proteins thought to act as the nerve terminal 'Ca++ sensor'. The binding of Ca++ to Syt1 promotes its physical interaction with SNARE complex proteins (Chapman et al., 1995; Davis et al., 1999), with phospholipids (Chapman and Jahn, 1994; Fernandez et al., 2001) and with itself, forming homo-oligomers (Chapman et al., 1996; Sugita et al., 1996; Yoshihara and Littleton, 2002). Following several years of studies, the emerging consensus is that Syt1-SNARE interactions couple Ca++ entry with fast exocytosis while Syt1 oligomerization and phospholipid binding triggers the rapid fusion event (Brose et al., 1992; Yoshihara and Littleton, 2002; Yoshihara and Montana, 2004; Lynch et al., 2008; Paddock et al., 2008).

Several other integral SV proteins are thought to participate in vesicle exocytosis by direct physical or functional interactions with SNAREs; these include synaptophysin,

synaptogyrin, and synaptic vesicle-associated protein 2 (SV2). Synaptophysin was the first synaptic vesicle protein to be discovered, and despite its abundant expression and demonstrated interactions with synaptobrevin, genetic studies revealed that it is not critical for synaptic transmission (Calakos and Scheller, 1994; Washbourne et al., 1995; McMahon et al., 1996; Valtorta et al., 2004). However studies using synaptophysin/synaptogyrin double knockout mice revealed profound defects in synaptic transmission, suggesting a redundant function for these proteins in exocytosis (Janz et al., 1999; Valtorta et al., 2004). By contrast, genetic ablation of SV2 (SV2A/B) leads to an increase in release probability and a significant increase in seizures, suggesting that SV2 regulates SV exocytosis (Custer et al., 2006; Chang and Sudhof, 2009).

Proteins regulating synaptic vesicle endocytosis and recycling

Retrieval of SVs following fusion takes place by clathrin-mediated endocytosis, followed by vesicle recycling via endosomal sorting. This was first observed by the seminal studies of Ceccarrelli and Heuser, who showed by microscopic tracing as well as electrophysiological methods that nerve terminals contain clathrin-coated vesicles exclusively following neuronal stimulation (Ceccarelli et al., 1973; Heuser and Reese, 1973). Furthermore, SV fusion with the plasma membrane was found to only briefly deplete the total vesicle pool, which was correctly hypothesized to replenish via reformation of the vesicle membrane (Ceccarelli et al., 1973; Heuser and Reese, 1973).

The regulated reuptake of SVs begins with the binding of soluble N-maleimide sensitive fusion protein (NSF) followed by soluble NSF attachment protein (SNAP), which together bind the surface of SNARE complexes and promote their disassembly (Jahn and Scheller, 2006). Next, assembly of the clathrin triskelion leads to invagination of the synaptic vesicle membrane, a process promoted by a number of clathrin accessory proteins [reviewed in (Brodin et al., 2000; Murthy and Camilli, 2003; Ungewickell and Hinrichsen, 2007)]. The invaginated ‘pit’ is cleaved from the plasma membrane by the action of dynamin, a GTPase that promotes membrane fission (Koenig and Ikeda, 1983; Roux et al., 2006; Ferguson et al., 2007). Once in the cytosol, the clathrin coat is rapidly

removed from the vesicle via interaction with proteins that bind to clathrin and disrupt the triskelion (Cremona et al., 1999; Massol et al., 2006; Perera et al., 2006; Ungewickell and Hinrichsen, 2007). Finally Rab GTPases, a subset of which are expressed on synaptic vesicles (Rab3a/5a), are thought to facilitate the proper endosomal sorting of vesicles, while actin is thought to mobilize sorted vesicles toward their appropriate pools (Fischer von Mollard et al., 1994; Zerial and McBride, 2001; Shupliakov et al., 2002; Sankaranarayanan et al., 2003b; Star et al., 2005; Cingolani and Goda, 2008).

Taken together, it is clear that the synaptic vesicle is a highly complex organelle whose function is dependent on the expression of a variety of integral vesicle proteins, along with transiently associated proteins which attach and detach during the vesicle cycle depending on the discrete vesicle localization and excitatory state of the bouton. For more details beyond this short review, please consult the following reviews (and references therein) on SNARE complex proteins and synaptic vesicle cycling (Sudhof, 2004; Jahn and Scheller, 2006; Kavalali, 2006; Voglmaier and Edwards, 2007).

(II) Proteins of the presynaptic active zone

The active zone is the restricted site for regulated transmitter release in the presynaptic terminal. It is composed of a tightly packed complex of channels, receptors, scaffolds, cytoskeletal proteins and signalling molecules all closely coupled in space so as to promote efficient and regulated synaptic transmission.

Calcium channels

Fast neurotransmitter release by SNARE-mediated fusion of SVs requires Ca++ entry into the presynaptic terminal; this is accomplished by the expression of voltage-gated Ca++ channels within the presynaptic active zone membrane. Voltage-gated calcium channels are composed of multiple subunits and are classified according to their distinct currents, pharmacological properties and physiological roles (Dunlap et al., 1995; Catterall and Few, 2008). Most conventional synapses express N-type and P/Q type Ca++ channels, which require strong depolarization for activation, such as that observed

during action potential (AP)-mediated depolarization of the nerve terminal. These channels open shortly following AP arrival (within 200μs), resulting in an influx of Ca++ ions and the formation of a concentrated Ca++ microdomain within the nerve terminal (up to 200-300μM)(Llinas et al., 1992; Catterall and Few, 2008; Neher and Sakaba, 2008). Termination of the Ca++ current is brought about by direct binding of trimeric G-protein subunits to the Ca++ channel, liberated from G-protein coupled autoreceptors expressed in the nerve terminal (Strock and Diverse-Pierluissi, 2004; Catterall and Few, 2008). Thus, during activity-dependent neurotransmission, Ca++ influx is highly regulated spatially and temporally to facilitate the demands of efficient neurotransmission while maintaining the functional integrity of the synapse.

Active zone cytomatrix proteins

The exocytosis machinery within the active zone is embedded in a rich protein lattice, the active zone cytomatrix, whose individual constituents display multiple physical and functional interactions. Although many of these proteins have been studied using biochemical, genetic and cell biological techniques, the extent to (and mechanisms by) which all these complexes interact remains unclear. However, what is clear is that active zone proteins do not act in isolation but rather are all constituents of highly sophisticated modular complexes which together participate in the maintenance of active zone architecture, synaptic vesicle tethering, calcium channel anchoring, and organization of the exo- and endocytosis machineries (Zhen and Jin, 2004; Schoch and Gundelfinger, 2006; Toonen and Verhage, 2007).

Bassoon and piccolo

Bassoon and piccolo are two structurally related active zone-specific proteins expressed selectively in vertebrates (Wang et al., 1999b; Fenster et al., 2000; Altrock et al., 2003). Both proteins are large (420kDa and 530kDa, respectively), with overlapping expression at both excitatory and inhibitory synapses in most regions of the brain (Zhen and Jin, 2004; Schoch and Gundelfinger, 2006). Bassoon and piccolo are thought to function as modular scaffolds via physical interaction with proteins linked to synaptic vesicles

(Pra1), with other modular scaffolds that complex with proteins involved in synaptic vesicle priming (CAST, RIM1α), and in the case of piccolo, with various actin binding proteins (Abp1, profilin, GIT)(Wang et al., 1999b; Fenster et al., 2000; Fenster et al., 2003; Kim et al., 2003; Takao-Rikitsu et al., 2004). By live imaging studies, it has also been revealed that bassoon and piccolo are among the first proteins to accumulate at nascent synapses, suggesting a role in presynaptic assembly (Friedman et al., 2000; Zhai et al., 2001; Shapira et al., 2003). However, parsing out their detailed functions has been a challenge.

First, bassoon and piccolo are not present in worms and flies, suggesting that these proteins are not necessary for the formation or function of the more conserved active zone complexes with which they are demonstrated to interact (Zhen and Jin, 2004; Schoch and Gundelfinger, 2006). Addressing these issues using genetic approaches has been hampered by their very large gene sizes and to date there are no known transgenic models for piccolo. However, using an interference RNA (RNAi) approach in cultured hippocampal neurons, Leal-Ortiz *et al* have shown that piccolo is important for actin-dependent synaptic vesicle recycling, consistent with the demonstrated interactions of piccolo with actin-binding proteins, while normal synapse number and ultrastructure is maintained (Leal-Ortiz et al., 2008). A model for bassoon has been developed whereby mutant mice express a truncated version of bassoon that is non-functional but retains the synaptic localization of the remaining fragments. Phenotypically, these mice have normal presynaptic ultrastructure in the hippocampus and cerebellum, but display a significant proportion of functionally inactive (silent) synapses as well as an enhanced behavioural propensity to undergo epileptic seizure (Altrock et al., 2003). In contrast, at the photoreceptor synapse in the retina, the loss of functional bassoon leads to a disordered presynaptic architecture as well as impaired functional transmission, suggesting that its expression is critical for assembly and function selectively at this synapse (Dick et al., 2003).

M(unc)-13 and -18: Regulators of the fusion machinery

Sec1/Munc18s are highly conserved proteins critical for fusion in all species from yeast to humans, and an emerging view is that Munc18 forms an integral part of the SNARE complex. Munc18 binds to monomeric syntaxin-1 as well as assembled SNARE complexes (Dulubova et al., 2007; Khvotchev et al., 2007; Toonen and Verhage, 2007), and links to other protein complexes via Mint1 (Okamoto and Sudhof, 1997; Butz et al., 1998). Munc13 is the mammalian homolog of the *C.elegans* UNC13 protein, originally identified in a classic genetic screen for *uncoordinated* movements in worms (Maruyama and Brenner, 1991). Munc13 is a component of a ternary complex of RIM1α and Rab3, whose primary function is thought to be the close spatial coupling of synaptic vesicles to the exocytosis machinery (Betz et al., 2001; Schoch et al., 2002; Dulubova et al., 2005). This function, known as *priming*, is critical for the generation of fusion-competent vesicles within the active zone. Genetic studies reveal that loss of either Munc13 or Munc18 in mice results in a similar phenotype: a complete loss of stimulus-evoked neurotransmission and early postnatal death (Augustin et al., 1999; Verhage et al., 2000; Varoqueaux et al., 2002). Interestingly, synaptic ultrastructure is largely normal in these mutants, suggesting that neither Munc proteins nor transmitter release itself are critical for synapse formation.

Rab3a-interacting molecule (RIM)

RIMs constitute a family of multidomain scaffolding proteins initially discovered as effectors for the integral synaptic vesicle protein Rab3a (Wang et al., 1997). In vertebrates, 4 RIM genes encode six principal isoforms, of which RIM1α is the most abundantly expressed (Schoch et al., 2002). RIM1α is proposed to have physical and functional interactions with more proteins than any other constituent of the active zone. In addition to its function in vesicle priming with Munc13 and Rab3a, RIM1α has been shown to physically interact with other scaffolding proteins including CAST, piccolo, bassoon, and liprin-α (Ohtsuka et al., 2002; Wang et al., 2002; Ko et al., 2003; Takao-Rikitsu et al., 2004). RIM has also been shown to interact directly with members of the exocytosis machinery including synaptotagmin1, SNAP25, and N-type calcium channels, although these latter interactions are controversial (Schoch and Gundelfinger, 2006). The

interaction of RIM with Ca++ channels may alternatively be regulated by RIM binding proteins (RIMBPs)(Wang et al., 2000; Hibino et al., 2002). Finally, RIMs are substrates for cAMP-dependent protein kinase A (PKA), suggesting that RIM proteins are involved in plasticity-related signalling (Lonart et al., 2003).

Knockout models for the RIM/Unc-10 proteins exhibit an array of phenotypes consistent with their diverse functions. In mice, loss of RIM1α caused defects in both short and long-term plasticity, while overall synapse size and density, vesicle pool size, and number of docked vesicles within a given active zone appeared normal (Castillo et al., 2002; Schoch et al., 2002; Lonart et al., 2003). Mutant mice exhibit behavioural impairments including associative and spatial learning deficits, suggesting that RIM1α-mediated synaptic plasticity is important for memory formation (Powell et al., 2004). These alterations are not due to loss of other RIM-interacting proteins in the active zone, as except for Munc 13 (whose levels fall by half in RIM1α KO mice) all other RIM-interacting proteins appear to localize normally to the active zone (Schoch et al., 2002; Schoch and Gundelfinger, 2006).

Liprin-α

Liprin proteins were originally identified as interaction partners for leukocyte common antigen-related (LAR) protein tyrosine phosphatases (Serra-Pages et al., 1995). Vertebrates have four liprin-α genes each with high homology and broad expression patterns, while invertebrates contain a single liprin-α ortholog that is responsible for active zone formation in SYD-2 (for *synapse-defective*) mutants in *C.elegans* as well as in *D. melanogaster (Dliprin)*(Zhen and Jin, 1999; Kaufmann et al., 2002). Genetic deletion of liprin-α results in increases in the length and number of vesicles within the active zone as well as defects in synaptic transmission, suggesting that liprin–α proteins are important for regulating active zone formation and morphology (Zhen and Jin, 1999; Kaufmann et al., 2002; Zhen and Jin, 2004).

Liprins are multidomain proteins that contribute to the cytomatrix scaffold via direct interactions with several other active zone proteins, including RIM1α (Schoch et al., 2002), the small GTPase-activating protein GIT1 (Kim et al., 2003), and CAST (Wang et al., 2002; Ko et al., 2003), each of which are components of many of the ternary complexes mentioned above. In addition, Liprin–α proteins bind to the CASK-Veli/MALS-Mint1 ternary complex that is found on both sides of the synapse; presynaptically, this complex appears to anchor the transmembrane adhesion molecule neurexin to the plasma membrane (Hata et al., 1996; Butz et al., 1998; Olsen et al., 2005).

ELKS/CAST/ERC Proteins

ELKS/CAST/ERC proteins are the most recent proteins found to be enriched at the presynaptic active zone. Mammals contain two ELKS genes which code for multiple splice variants of which one, CAST/ERC2, is active-zone specific while another, ERC1b, is brain-specific but expressed more broadly in the cytosol as well as the presynaptic active zone (Wang et al., 2002; Schoch and Gundelfinger, 2006). CAST is thought to behave primarily as a cytomatrix scaffold through its interactions with other multidomain scaffolds, and some studies point to a role for CAST in the targeting of other proteins to the active zone. This concept is supported by studies in *Drosophila* whereby deletion of *Bruchpilot*, a protein with homology to ELKS, display alterations in active zone morphology and a reduction in the density of Ca++ channels (Kittel et al., 2006; Wagh et al., 2006). Interference with CAST function in cultured hippocampal neurons has been shown to affect the localization of RIM at the active zone and to diminish synaptic transmission (Ohtsuka et al., 2002; Takao-Rikitsu et al., 2004). However, deletion of the *C.elegans* ortholog results in no gross defects in synaptic transmission or behaviour, with RIM being targeted to the active zone at normal levels (Deken et al., 2005). Thus, the role of CAST in the formation and maintenance of the active zone will likely remain the subject of future studies.

Taken together, it is clear that the presynaptic active zone is tightly packed with proteins whose interactions are diverse each of which never appear to be separated from any other by more than a few degrees. And yet, the presynaptic active zone is not a deck of cards that collapses when one card is removed- it is interesting to note how many of these proteins form integral components of the presynaptic active zone and yet isolated deletion of many of them does not affect presynaptic ultrastructure. This suggests some functional redundancy among many of the individual constituents, whereby the presence of all of these scaffolds promotes sophisticated plasticity-related neurotransmission but only a subset is truly required for transmitter release. Alternatively, perhaps all of these proteins depend on a master scaffold that is most profoundly critical for assembly, morphology and function, and an excellent candidate is actin.

(III) Actin and its multifunctional role at the presynapse

Actin is a fundamental component of the cytoskeletal network in all eukaryotic cells, and together with microtubules and intermediate filaments regulates many critical cellular functions, from cell division and motility to intracellular trafficking. In the nervous system, the cytoskeleton drives the formation of axons and dendrites during development and propels outgrowing processes toward their target cells, thereby helping neurons to develop their exquisite morphology and circuitry. Within the synapse, actin is the main cytoskeletal protein which postsynaptically acts as an active scaffold to correctly position the receptor adaptor and signalling proteins within the postsynaptic matrix, and to maintain postsynaptic morphology [reviewed in (Bonhoeffer and Yuste, 2002; Boeckers, 2006)]. Presynaptically, actin critically regulates a number of functions within the active zone as well as the extended bouton by virtue of its ability to reorganize dynamically and rapidly to meet the needs of the terminal, from development to mature synaptic transmission.

Cellular Actin Dynamics: A brief overview

Actin exists in two states: as polymerized two-stranded helical filaments (filamentous, or F-actin) or as monomers (globose, or G-actin) that provide the building blocks for F-

actin assembly. Actin remodelling is regulated by a diverse family of actin-binding proteins, which can influence its assembly and disassembly in a variety of ways. Some act to bundle or cross-link F-actin filaments (for example, Arp2/3 and spectrin), others to sequester actin monomers (profilin), to sever F-actin (ADF/cofilin), to cap the ends to prevent G-actin incorporation, or to accelerate disassembly (Pollard and Borisy, 2003). These actin-binding proteins are, in turn, targets for a host of signal transduction cascades whose signals can derive from a variety of extracellular and intracellular stimuli. Within the nervous system, actin binding and regulatory proteins are particularly well understood for their role in growth cone dynamics and axon guidance (Huber et al., 2003; Heasman and Ridley, 2008). However, many of the same signalling pathways appear to regulate actin dynamics at the synapse (Luo, 2002; Meyer and Feldman, 2002).

Subcellular localization of actin in the presynaptic bouton

The detailed arrangement of the presynaptic cytoskeleton was first revealed in pioneering experiments using freeze-etched electron microscopy. In these experiments, a dense and varied meshwork of actin filaments was found throughout the presynaptic terminal cytoplasm, linked to synaptic vesicles by short linking strands (30nm), and to the plasma membrane by longer strands approximately 100nm in length (Landis et al., 1988; Hirokawa et al., 1989; Siksou et al., 2007). The short actin-SV linking strands were shown to be filaments of the phosphoprotein synapsin, which by immunoelectron microscopy were observed to richly decorate the pool of synaptic vesicles >50nm distance from the presynaptic active zone (Shupliakov et al., 2002; Bloom et al., 2003; Evergren et al., 2007; Siksou et al., 2007). More recent studies show that F-actin, while present within the core of the SV pool, is most abundant at its surrounding edges (Dunaevsky and Connor, 2000; Shupliakov et al., 2002; Bloom et al., 2003; Sankaranarayanan et al., 2003b). This subcellular distribution is observed almost irrespective of the preparation used, from the lamprey reticulospinal synapse to the rodent hippocampus, suggesting that this basic appearance of presynaptic ultrastructure is highly conserved throughout evolution.

Actin is also highly enriched within active zone itself (Hirokawa et al., 1989; Gotow et al., 1991; Phillips et al., 2001; Siksou et al., 2007). Biochemically, actin is consistently found in isolated fractions of active zone membranes (Phillips et al., 2001; Khanna et al., 2007), along with spectrin proteins, a family of cytoskeletal matrix proteins that localize to the submembrane region of the active zone as well as the synaptic vesicle pool (Zagon et al., 1986). Spectrins contain distinct binding sites for both actin and synapsin proteins, and have long been thought to represent the longer strands extending from the active zone membrane to link SVs as well as cytoplasmic actin filaments (Landis et al., 1988; Hirokawa et al., 1989; Sikorski et al., 1991). Spectrins have been shown to regulate basal synaptic transmission through their interactions with synapsin proteins (Sikorski et al., 2000). Moreover, deletion of spectrin expression at the mature drosophila NMJ results in synapse disassembly (Pielage et al., 2005), and at the newly formed NMJ causes a decrease in expression of multiple components of the exocytosis machinery (Featherstone et al., 2001). Taken together, these studies strongly implicate the actin-spectrin network as critical for the maintenance of the presynaptic cytoskeletal matrix, as well as for regulated transmitter release.

The many functions of actin at the presynaptic ending

By virtue of its extensive distribution throughout the presynaptic terminal and intimate association with synaptic vesicles, actin is poised to regulate all aspects of presynaptic function. The study of actin and its many roles have relied on pharmacological tools that inhibit its dynamic activity, as well as genetic knockout studies of its interacting partners rather than genetic deletion of actin itself, as actin is critical for cell survival. These studies have illuminated multiple roles for actin throughout the synaptic vesicle cycle, and are beginning to shed new light on the role of actin in synaptogenesis.

In the absence of stimulation, actin depolymerization experiments reveal very little structural change, particularly at the level of the reserve synaptic vesicle pool which remains fully intact (Sankaranarayanan et al., 2003b; Richards et al., 2004). In contrast, interference with the synapsin proteins, whether by the use of function blocking

antibodies or by genetic deletion, results in a significant reduction in the size of the reserve pool and a dispersion of vesicles throughout the terminal (Li et al., 1995; Gitler et al., 2004; Evergren et al., 2007; Siksou et al., 2007). These experiments suggest that synapsin rather than actin is the main structural "glue" holding the reserve pool together and that actin is a secondary scaffold in the resting terminal.

Acute and prolonged treatment with actin disrupting agents reveals multiple discrete effects on synaptic vesicle exocytosis. Brief exposure to LatA results in a transient increase in both spontaneous and evoked neurotransmitter release that is blocked by pre-treatment with jasplakinolide, a toxin that stabilizes F-actin (Morales et al., 2000; Sankaranarayanan et al., 2003b). These studies suggest that actin acts as a negative regulator of synaptic vesicle exocytosis within the active zone. In addition, acute exposure to LatA leads to a lengthening in the synaptic active zone following stimulation (Morales et al., 2000), a phenotype similar to that observed in liprin-α knockout models (Zhen and Jin, 1999; Kaufmann et al., 2002), suggesting an additional role for actin in the maintenance of active zone morphology during neurotransmission.

A number of endocytosis defects have also been observed in response to interference with actin dynamics. Following high frequency stimulation, F-actin accumulates at sites of clathrin-mediated endocytosis, termed the endocytic zone, which typically surrounds the active zone. The same high frequency stimulation paradigm performed after prolonged exposure to LatA leads to an increase in the number clathrin-coated invaginations (or 'pits') along the plasma membrane along with a significant reduction in the size of the reserve pool. This suggests that actin participates in two sequential endocytic processes, beginning with (i) cleavage of clathrin-coated pits from the plasma membrane and followed by (ii) transport of recycled vesicles back to the reserve pool (Shupliakov et al., 2002; Bloom et al., 2003; Bourne et al., 2006).

Finally, a number of experimental observations support the concept that actin remodelling is an important first step for synaptogenesis, both pre and postsynaptically. Evidence to support this assertion include the following studies: (a) promoting actin

polymerization on axons leads to the formation of actin-rich clusters at sites which go on to form functional presynaptic boutons (Colicos et al., 2001), while exposure to actin-blocking agents results in (b) a reduction in the number of presynaptic clusters in immature cultures (Zhang and Benson, 2001), (c) the inhibition of activity-dependent synaptogenesis (Antonova et al., 2001; Wang et al., 2005), and (d) defects in the conversion of silent to functional presynapses, a phenomenon called "awakening" (Shen et al., 2006; Yao et al., 2006).

Many of these studies have relied on stimulation-induced protocols rather than observations of spontaneous bouton formation; whether there is a differential dependency for actin between these two forms of synaptogenesis is unclear. Furthermore, in studies of immature cultures it is unclear whether the loss of actin activity results in a true defect in synaptogenesis or is secondary to other defects in maturation. And yet, these data are strongly suggestive of a role for dynamic actin reorganization during nascent synaptogenesis and will likely remain a subject of future studies.

PART III: Structure of the postsynaptic ending.

Postsynaptic densities were originally classified into two types, Type I and Type II, based on their electron microscopic ultrastructure: Type I PSDs had thicker electron densities lining the plasma membrane surface than did Type II PSDs (Gray, 1959). We now know that Type I and Type II synapses represent, respectively, the glutamatergic and GABA/glycinergic synapses which together carry out most excitatory and inhibitory neurotransmission in the brain. The molecular architecture of both types of PSDs is one of multiple layers of receptor, receptor targeting, scaffold and signalling proteins organized in a laminar fashion beginning at the plasma membrane and extending to the actin-rich cytoskeleton found at the core of the PSD. Many of these individual proteins contain sequence motifs known as PDZ domains, named after the proteins in which these motifs were originally identified (PSD-95-Discs-large (DLG)-Zona occludens-1). PDZ domains represent the sites of protein-protein interactions shown to bind and link most proteins of the PSD into their highly organized lattice.

Postsynaptic transmembrane receptors

Glutamate mediates most excitatory neurotransmission in the nervous system through the actions of three main types of receptors expressed at the postsynaptic plasma membrane. Fast neurotransmission is carried out mainly through ionotropic AMPA-type glutamate receptors, formed by the association of four GluR subunits (GluR1-4) whose functional properties are defined based on subtype composition. Ionotropic NMDA receptors are also heteromultimers, formed by the association of an obligatory NR1 along with a complement of NR2 subunits (NR2A-D). These receptors, while also fast-acting, require a higher threshold for activation and are implicated in processes related to synaptic plasticity. Finally, metabotropic glutamate (mGluR) receptors belong to the 7-transmembrane domain spanning superfamily of receptors, are comparatively slow acting and activate trimeric G protein-mediated signalling cascades in the postsynaptic domain (Hollmann and Heinemann, 1994; Hall and Ghosh, 2008; Kerchner and Nicoll, 2008).

Receptor anchoring proteins

The cytoplasmic domains of AMPA, NMDA and mGluR receptors interact with a variety of proteins critical for their correct targeting, insertion and localization at synapses. These proteins may be grouped into several families that preferentially interact with certain subtypes of receptors, creating AMPA, NMDA, and mGluR receptor complexes.

PSD-95 (also known as SAP-90) was the first scaffolding protein identified to be enriched in the PSD (Cho et al., 1992) and was later discovered to physically associate with the NR2 subunit of NMDA receptors (Kornau et al., 1995). PSD-95 and its related family members SAP97/hDlg, SAP102, and PSD-93/chapsyn-110, all contain multiple modular domains for distinct protein interactions including the aforementioned PDZ domains along with an SH3 and a guanylyl kinase-like domain. These proteins are members of the Membrane-Associated GUanylyl Kinase, or MAGUK, family of postsynaptic receptor scaffolding proteins that interact primarily with NMDA receptors, forming NMDA receptor-scaffold complexes which can in turn interact with both AMPA and mGluR complexes by way of their PDZ domains (Scannevin and Huganir, 2000; Boeckers, 2006; Okabe, 2007).

The correct targeting and insertion of AMPA receptors to PSDs depends on the expression of a family of auxiliary transmembrane proteins known as TARPs, for transmembrane AMPA receptor regulatory proteins. The first TARP gene was discovered as the genetic target of a mutation leading to spontaneous epileptic seizures and ataxia in mice (Letts et al., 1998). This gene, named *stargazin*, codes for a protein with sequence similarity to the Ca++ channel gamma (γ) subunit, and its deletion was soon discovered to result in a lack of functional AMPA receptors in cerebellar granule cells (Letts et al., 1998; Chen et al., 2000). Since then, several stargazin/TARP family members have been discovered which show distinct expression patterns in the brain and are responsible for the clustering of AMPA receptors at synapses (Okabe, 2007).

Unlike NMDA or mGluR receptors, AMPA receptors are subject to rapid trafficking between plasma membrane and subplasmalemmal compartments depending on the

activation state of the PSD. The trafficking of AMPA receptors involves another set of interacting proteins, among which include GRIP/ABP, PICK-1, as well as the endocytic accessory proteins NSF and AP-2 (Dong et al., 1997; Song et al., 1998; Noel et al., 1999; Xia et al., 1999; Boeckers, 2006). These studies have relied in part on biochemical approaches to define physical interactions, and it should be noted that the opposite, whereby AMPA receptors immunoprecipitate only with TARPs and not any of these other proteins, has been described (Fukata et al., 2005). This discrepancy suggests that the interaction between AMPA receptor-TARP complexes with GRIP, PICK-1, NSF and/or AP-2 is a functional rather than a lasting physical one. Finally, AMPA receptors have been demonstrated to physically interact with SAP97, thus connecting the AMPA and NMDA receptor complexes at the synapse via MAGUK family proteins (Leonard et al., 1998; Cai et al., 2002).

Homer proteins are a family of PSD-enriched cytoplasmic scaffolds that target Type I mGluRs (mGluR1 or mGluR5) to synaptic sites (Xiao et al., 1998; Ango et al., 2000; Ulrich, 2002). Homer proteins exhibit multiple complex interactions, both with themselves and with a variety of other cytoplasmic scaffolds in the PSD, especially members of the Shank/ProSAP family (Xiao et al., 1998; Tu et al., 1999). Homer also physically binds to intracellular IP3 receptors, ligand-gated Ca++ channels expressed on ER membranes which are downstream effectors of mGluR signalling (Tu et al., 1998).

The "Master Scaffolds"- Shank/ProSAPs and GKAP/SAPAPs

Glutamate receptor complexes at the synaptic plasma membrane are connected to each other within the postsynaptic cytomatrix. This is accomplished by proteins of the Shank/ProSAP family (Lim et al., 1999), which interact with all these complexes through various protein-protein interactions, and interact with a variety of signalling molecules linked to the actin-rich cytoskeleton [reviewed in (Boeckers, 2006; Okabe, 2007)]. This arrangement sets up the distinct laminar organization characteristic of the postsynaptic cytoplasm.

GKAPs1-4 (Kim et al., 1997; Takeuchi et al., 1997) comprise another scaffolding protein family which can physically cross-link MAGUK family proteins to the Shank scaffold. This interaction is exemplified by the demonstrated binding of PSD95 to GKAP, which can simultaneously bind to shanks by PDZ domain interactions (Naisbitt et al., 1999). In turn, shank can bind to Homer proteins, thus cross-linking the mGluR and NMDA receptor complexes together (Tu et al., 1999). Furthermore, shanks are known to bind to various regulators of actin filaments including cortactin (Naisbitt et al., 1999), spectrin (Bockers et al., 2001), Abp1 (Qualmann et al., 2004), and βPIX, a guanine nucleotide exchange factor for Cdc42/Rac1 Rho GTPases (Park et al., 2003)[reviewed in (Boeckers, 2006; Okabe, 2007)]. Thus, the Shank scaffold has the potential to regulate the dynamic activity of actin, which in turn can promote structural changes in postsynaptic morphology as well as protein trafficking events within the postsynaptic density.

Actin and the PSD

Actin itself, as with the presynaptic ending, has a number of important functions in the PSD. Actin is mainly concentrated in the cytoplasmic core of the PSD, and through its interactions with postsynaptic scaffolds can affect receptor anchoring and trafficking processes at the postsynaptic membrane. Dynamic actin remodelling is also a mechanism for promoting structural as well as functional changes during activity-dependent synaptic plasticity. This is accomplished by a diverse group of signalling molecules which regulate the remodelling of actin through an equally diverse family of actin binding proteins embedded within the cytoplasmic scaffold [reviewed in (Dillon and Goda, 2005; Schubert and Dotti, 2007; Sekino et al., 2007; Cingolani and Goda, 2008)].

The inhibitory postsynaptic density: unique receptors and scaffolds

Compared to glutamatergic synapses, comparatively little is known about the molecular composition of inhibitory GABAergic/glycinergic synapses. It is likely that many of the PSD proteins discussed above have the same expression and function irrespective of the neurotransmitter used, but a definitive picture is hindered by the challenge of isolating Type II synapses biochemically. That being said, there are notable constituents of

GABAergic/glycinergic synapses that are unique and this list is likely to grow in the future.

Ionotropic receptors at the inhibitory postsynapse: $GABA_A$ & glycine

At inhibitory synapses in the adult CNS, two types of ligand-gated chloride channels carry out fast neurotransmission. Both $GABA_A$ and glycine receptors are heteromeric pentamers composed of a mixture of subunits, generating receptors of varying stoichiometry depending on the nervous system region (McKernan and Whiting, 1996; Lynch, 2004). Inhibitory synapses may be composed of only $GABA_A$ or glycine-type receptors or a mixture of the two, and these receptors constitute important therapeutic targets- in the case of $GABA_A$, for a range of sedative and anxiolytic agents, while glycine is the main target for the nervous system toxin strychnine (McKernan and Whiting, 1996; Lynch, 2004; Michels and Moss, 2007).

Gephyrin: A glycine and GABA receptor scaffold at inhibitory postsynapses

A major cytoplasmic component of $GABA_A$/glycinergic synapses is the scaffolding protein gephyrin. Initially discovered in preparations of affinity-purified glycine receptors (Prior et al., 1992), deletion of gephyrin either by antisense knockdown *in vitro* or genetic deletion *in vivo* prevents the synaptic clustering of glycine receptors (Kirsch et al., 1993; Fischer et al., 2000; Levi et al., 2004), pointing to a critical role for gephyrin in the establishment of glycinergic postsynapses. Gephyrin is also localized to GABAergic PSDs although in contrast, loss of gephyrin expression significantly reduces but does not abolish the synaptic expression of $GABA_A$ receptors (Kneussel et al., 1999; Fischer et al., 2000; Levi et al., 2004; Yu et al., 2007). Conversely, the genetic deletion of certain $GABA_A$ receptor subunits reduces the synaptic expression of gephyrin clusters, suggesting some interdependence for their colocalization rather than gephyrin driving the expression of $GABA_A$ receptors (Essrich et al., 1998). The targeting of $GABA_A$ receptor subunits may instead depend on another protein, $GABA_A$ receptor binding protein (Wang et al., 1999a), although this is not confirmed (Fritschy et al., 2008). Taken together, these findings point to a critical role for gephyrin in the development of glycinergic

postsynapses, while at GABAergic synapses gephyrin likely participates in, but does not critically orchestrate the targeting of $GABA_A$ receptors to the PSD membrane (Kneussel and Betz, 2000; Fritschy et al., 2008).

Gephyrin, like most of its scaffolding counterparts in the excitatory PSD, physically interacts with a number of proteins in the inhibitory postsynaptic cytoplasm. In addition to the glycine receptor β subunit, binding partners for gephyrin include tubulin (Prior et al., 1992), collybistin, a Cdc42 Rho GEF (Kins et al., 2000), actin regulatory proteins including Mena/VASP and profilin (Giesemann et al., 2003), and Raft1, a regulator of protein translation (Sabatini et al., 1999). The expression of collybistin is shown to be critical for the normal synaptic clustering of gephyrin as well as both glycine and $GABA_A$ receptor subunits, implicating collybistin in the regulation of inhibitory synapse development and maintenance (Kins et al., 2000; Harvey et al., 2004; Papadopoulos et al., 2007; Papadopoulos et al., 2008). The interaction of gephyrin with tubulin likely underscores the mechanism of dendritic transport to the PSD, while its interactions with components of the actin cytoskeleton suggests a role in assembly and maintenance of the submembraneous scaffold (Kirsch and Betz, 1995; Fuhrmann et al., 2002; Charrier et al., 2006; Fritschy et al., 2008). Finally the localization of Raft1 in the gephyrin postsynaptic scaffold likely promotes the local synthesis of dendritic mRNAs at inhibitory PSDs, a critical component of late LTP-related synaptic plasticity (Sabatini et al., 1999; Fritschy et al., 2008)[for reviews on local protein synthesis at the synapse, please refer to (Steward and Schuman, 2001; Klann and Dever, 2004)]. Therefore, gephyrin appears to be a multifunctional constituent of the inhibitory PSD.

PART IV: Transsynaptic and synaptic cleft proteins.

The synaptic junction is approximately 20nm thick, whose borders are defined by the length of the active zone and postsynaptic density membranes with which it is associated. However, this definition is rather passive and undermines the complexity of this junctional "space". Since the earliest ultrastructural descriptions of the synapse, it was observed that the junction between adjoining pre and postsynaptic compartments was highly complex, rich in an electron-dense heterogeneous matrix comprised of structures orienting in various ways depending on the staining and fixation procedure used (Bloom and Aghajanian, 1966; Bondareff and Sjostrand, 1969; Pfenninger, 1971). This matrix of "fuzz" (Pfenninger, 1971), interspersed with regularly-spaced "pegs", "bristles", "fibrils" and "knobs" (van der Loos, 1963; Cotman and Taylor, 1972; Landis and Reese, 1983; Ichimura and Hashimoto, 1988) were thought early on to confer the incredible structural integrity of the transsynaptic junction such that only the harshest proteolytic treatments could result in their dissolution (De Robertis, 1967; Pfenninger, 1971; Cotman and Taylor, 1972; Phillips et al., 2001). Since then, much has been learned about the molecular nature of the synaptic junction; this "space" is full of a variety of classes of extracellular and transmembrane molecules which together constitute a structural and signalling scaffold important in the formation and maintenance of the synapse.

(I) Extracellular matrix molecules

The extracellular matrix (ECM) is the diverse mixture of collagens, proteoglycans and glycoproteins that surrounds all organized groups of cells in the body. Rather than behaving simply as a passive structural support, the ECM activates signalling cascades by virtue of its ability to embed within itself both soluble and transmembrane molecules that may, in turn, be presented to cell-surface receptors. These signalling cascades can facilitate a number of cellular processes including migration, proliferation, differentiation, and adhesion depending on the tissue and complement of matrix molecules expressed. Within the nervous system, the ECM can participate in all of these processes and evidence points to an additional role for the ECM at the synapse.

Pioneering studies of the ECM within the synaptic cleft

Some of the earliest studies of the synaptic cleft contents were published in the 1960s, shortly following the ultrastructural characterization of the synapse itself. Using differential staining methods, it was shown that a variety of glycoproteins typically found in the extracellular matrix are enriched in the synaptic cleft. Notably, the staining methods used varied according to, among other things, the charge of the staining solutions themselves, most of which were acidic, others basic (Bloom and Aghajanian, 1966; Bondareff, 1967; Rambourg and Leblond, 1967; Bondareff and Sjostrand, 1969; Pfenninger, 1971; Marx et al., 1973). In turn, these charged staining methods revealed the presence of complementarily charged glycoproteins, including acidic mucopolysaccharides (Bondareff, 1967) and carbohydrates (Rambourg & Leblond, 1967), sialic acid- and sulfate-containing glycoproteins (Bondareff & Sjostrand, 1969; Marx et al., 1973; Pfenninger, 1971), and concanavalin A receptors (Cotman and Taylor, 1974). This material was sensitive to enzymatic digestion by neuraminidase (Marx et al., 1973; Bondareff and Sjostrand, 1969; Pfenninger, 1971), as well as to proteolytic digestion using pepsin and trypsin (Bloom and Aghajanian, 1966; Cotman and Taylor, 1972) although this treatment must be harsh. In contrast, the cleft material was resistant to detergent extraction (De Robertis, 1967). It was hypothesized from these studies that the enrichment of ECM material within the cleft functioned to limit diffusion of ions and neurotransmitter while adding to structural integrity of the synapse itself.

It is now possible to reflect on several decades of research highlighting the role of specific ECM molecules in nervous system development, including synaptic development. It is likely that many of the ECM molecules characterized recently for their role at the synapse are constituents of the glycoprotein-rich cleft space described in these early studies. An overview of the most notable molecules demonstrated to have roles in synaptic formation, transmission and plasticity follows.

ECM Molecules and the Synapse

Collagens

Collagens are triple helical ECM molecules assembled from three separate polypeptide (α) chains containing Gly-X-Y sequence repeats. They have been most extensively studied outside of the nervous system for their ability to self-assemble from individual helices into elongated fibres, which adds tensile strength and structure to peripheral tissues (Vakonakis and Campbell, 2007; Fox, 2008). Although collagen fibres are not present in the nervous system, non-fibril-forming collagens and collagen-like molecules are widely expressed. At the neuromuscular junction, it was recently discovered that specific collagen IV α-chains have distinct and sequential roles in the organization of this synapse, whereby collagen IV α1/2 chains act alongside FGF to direct the initial differentiation of nerve terminals, while collagen IV α3–6 chains are required to maintain them (Fox et al., 2007).

Glycoproteins: Laminins and Integrins

Glycoproteins are composed of a polypeptide backbone onto which monosaccharide chains (glycans) are covalently attached by posttranslational modification. The length and composition of the monosaccharide chains, structure of the backbone and location of the polypeptide-sugar linkages can vary significantly, resulting in ternary structures that are astonishingly varied.

Laminins are a family of glycoproteins with demonstrated roles in synaptic differentiation at the NMJ (Dityatev and Schachner, 2003; Dityatev and Schachner, 2006). They are expressed as heterotrimers composed of individual α, β and γ subunits, whose diversity arises from variations in chain combinations, but together are major components of the basal lamina in most tissues of the body (Patton, 2003; Fox and Umemori, 2006). In vertebrates, β2-containing laminin trimers were among the first identified molecules present in the basal lamina of the NMJ (Hunter et al., 1989), and were later discovered to promote the stabilization of axons and discourage further outgrowth, and to promote presynaptic differentiation (Porter et al., 1995; Patton, 2003; Fox et al., 2007). Conversely, mice lacking laminin β2 form few active zones and fail to

correctly cluster SVs (Noakes et al., 1995; Fox et al., 2007). Thus, at the NMJ synapse, laminins are critical synaptic organizing molecules within the extracellular milieu.

At central synapses, the role of laminins is largely defined through studies of their main cell-surface receptors, the integrins. Integrins are heterodimers of noncovalently-linked α and β subunits, each of which contain an extracellular binding domain and a cytoplasmic tail for interactions with the actin cytoskeleton and intracellular signalling network (Hynes, 2002; Morgan et al., 2007). As with the laminins, there are multiple types of α and β subunits leading to diversity in the types of αβ heterodimers that can be expressed (Morgan, 2007). It is known that many of these subunits are expressed in the CNS and some, such as the β1 and β3 subunits, are enriched at synapses (Chan et al., 2003; Cingolani et al., 2008). Perturbation of integrin-mediated signalling, either by function-blocking peptides or snake toxins (disintegrins) which inhibit the binding of integrins to ECM ligands, have been shown to block the stabilization of early LTP (Staubli et al., 1998; Chan et al., 2003), to inhibit pre and postsynaptic maturation (Chavis and Westbrook, 2001), and to regulate the cell-surface expression of synaptic AMPA receptors (Cingolani et al., 2008). Direct evidence that the effects of integrins on synaptic transmission are mediated through laminins is largely lacking at this time and thus, the specific role for laminins at central synapses awaits future study.

Proteoglycans

Proteoglycans compose a special class of glycoproteins consisting of a core protein to which one or more glycosaminoglycan (GAG) side chain(s) are covalently attached. GAG chains are long, unbranched polymers composed of anywhere from 20-200 repeating disaccharide units, which are posttranslationally modified by enzymes that attach one or more sulfate groups to each side chain. These sulfated GAG chains are attached to the core protein via serine residues and characteristic carbohydrate linkage sites (Bandtlow and Zimmermann, 2000; Schwartz and Domowicz, 2004; Van Vactor et al., 2006). Not surprisingly, there is great structural diversity within this family, although it is possible to group its members into six distinct classes based on differences in

monosaccharide composition, sulfation and epimerization of the individual GAG chains [for a comprehensive review, see (Bandtlow and Zimmermann, 2000; Schwartz and Domowicz, 2004)]. Of these classes, the heparan sulfate proteoglycan (HSPG) family is particularly interesting in the context of synaptogenesis. Heparan sulfate proteoglycans contain GAG chains composed of repeating uronic acid and glucosamine disaccharide subunits, arranged in elongated polymers and subsequently modified through variable epimerization, de-acetylation and sulfation reactions (Esko and Selleck, 2002; Van Vactor et al., 2006). The HSPG core proteins fall into four major families: the transmembrane syndecans, GPI-linked glypicans, and the secreted perlecans and agrin. Although typically linked to the cell surface, the syndecans and glypicans both contain cleavage sites within their extracellular domains and thus may be released into the extracellular space, thereby making all HSPGs part of the extracellular matrix (Esko and Selleck, 2002; Van Vactor et al., 2006).

At the synapse, there is a large body of evidence to suggest that HSPGs participate in synaptogenic processes, both individually and through their complex interactions within the matrix. The best-studied individual member of this family would be the secreted HSPG agrin, which is critical for NMJ formation by inducing the clustering of acetylcholine (ACh) receptors on the postsynaptic muscle membrane (Yamaguchi, 2002; Fox and Umemori, 2006). Agrin may be secreted from both muscle cells and motor neurons (Yamaguchi, 2002; Fox and Umemori, 2006), and facilitates postsynaptic receptor clustering by activating signalling cascades mediated through the muscle-derived receptor tyrosine kinase MuSK (Fox and Umemori, 2006; Witzemann, 2006; Kim et al., 2008). In the absence of agrin the NMJ fails to form (Gautam et al., 1996), pointing to a critical role for agrin in synaptogenesis within the peripheral nervous system (Sanes and Lichtman, 2001).

In contrast to the NMJ, agrin is not a critical organizer of synapses within the central nervous system. However, other HSPGs have been demonstrated to coordinate synaptic functions in a number of complex ways. Although the presence of sulfate-like carbohydrate substances in the cleft of CNS synapses has been known for many decades

(Marx et al., 1973; Pfenninger, 1971), the first specific HSPG to be localized to the synaptic cleft was reported only in the late 1990s, the transmembrane HSPG syndecan-2 (Ethell and Yamaguchi, 1999; Hsueh and Sheng, 1999; Hsueh et al., 1998; Yamaguchi, 2002). *In vitro*, syndecan-2 was shown to be enriched along the membranes of dendritic spines and its overexpression alters dendritic spine morphology (Ethell and Yamaguchi, 1999; Lin et al., 2007). Syndecan-2 has also been shown to interact with CASK, an intracellular scaffolding molecule present on both sides of the synapse (Hsueh et al., 1998; Hsueh and Sheng, 1999). Finally, syndecan-2 is subject to phosphorylation, and it has been shown that EphB receptors on the postsynaptic membrane can directly influence the clustering of syndecan-2 and its subsequent ability to influence spine morphology (Ethell et al., 2001; Yamaguchi, 2002).

Pharmacological approaches have revealed a role for HSPGs in synaptic plasticity. In vitro, exposure to heparin has been shown to increase the open probability of AMPA receptors (Sinnarajah et al., 1999). Furthermore, injection of heparinase, an enzyme that cleaves heparan sulfates, has been shown to inhibit LTP (Lauri et al., 1999). These effects are thought to be mediated through another member of the syndecan family, N-syndecan (syndecan-3) (Dityatev and Schachner, 2003; Dityatev and Schachner, 2006).

An additional mechanism by which transmembrane and/or secreted HSPGs could influence synaptic processes is by creating extracellular scaffolds that bind and concentrate other secreted extracellular molecules (Yamaguchi, 2002). This is an established mechanism for the formation and stabilization of receptor-ligand signalling complexes for various growth factors, including FGF-FGF receptors (Faham et al., 1996; Lin et al., 1999; Ornitz, 2000; Berry et al., 2001; Kwan et al., 2001), a family of receptor-ligand pairs of which a subset are known synaptic organizers (Dai and Peng, 1995, 1996a; Umemori et al., 2004; Fox et al., 2007). Furthermore, HS chains have been shown to bind a wide variety of extracellular and cell-surface proteins, including secreted morphogens (Jackson et al., 1997; Lin and Perrimon, 1999; The et al., 1999; Lin, 2004), cell adhesion molecules (Cole and Akeson, 1989; Storms, 1998; Dityatev et al., 2004), and axon guidance factors (de Wit and Verhaagen, 2007; Matsumoto et al., 2007).

In summary, although the ECM is unequivocally critical for the organization of all tissues of the body, it is difficult to assign specific roles to ECM molecules in the context of neuronal development and in particular, of synaptic development. ECM molecules display such diversity in form, function and distribution that assessing the role of specific candidates is a challenge, especially when trying to consider their actions within the broader milieu in which they are expressed. That being said, their enrichment within the synaptic cleft and ability to interact with synaptogenic molecules suggests that at synapses, the ECM serves not only to limit ionic and neurotransmitter diffusion but to impact the form and function of the synapse itself.

(II) Transsynaptic adhesion molecules

EM studies revealing the enrichment of various glycoproteins within the synaptic cleft also noted the presence of fibrillar or peglike structures (De Robertis et al., 1961; van der Loos, 1963). These observations were later confirmed by freeze-etch electron microscopy, which revealed the presence of several structurally distinct elements oriented perpendicular to the cleft space and extending from the pre- and postsynaptic plasma membranes (Landis and Reese, 1983; Ichimura and Hashimoto, 1988). In addition, these fibrils appeared to have intracellular anchor sites, thereby forming a contiguous network running along the cytoplasmic face (Ichimura and Hashimoto, 1988; Landis and Reese, 1983). Although their precise functional significance was not known at the time, the authors speculated that this fibrillar network, along with the rich glycoprotein matrix within the cleft space, functioned to mechanically support synaptic contacts, while their connections with the subplasmalemmal matrix suggested additional roles within intracellular compartments.

A number of transsynaptic adhesion molecules have since been discovered which satisfy these physical characteristics and indeed, are critically important for the structural and functional integrity of the synapse. Transsynaptic molecules that promote *bona fide* adhesion include neural cell adhesion molecule (NCAM), nectins, and cadherins. Other

transsynaptic molecules primarily defined as receptor-ligand signalling complexes are also considered within this group given that they too can promote cell adhesion, these include the neurexin-neuroligin, ephrin-Eph, and GDNF-GFRα receptor families. Furthermore, a variety of transmembrane molecules have been discovered in recent years that display a synaptic localization and an adhesive function, including synCAM, SALM proteins, and NGL2. Finally, the syndecans (see preceding section) should also be included as adhesion-promoting molecules given their heterophilic binding affinities for the extracellular matrix as well as other transmembrane adhesion molecules. Together, these molecules link the extracellular environment to intracellular signal transduction processes through the vast cytoskeletal and signalling network with which they are associated. In so doing, synaptic CAMs can profoundly affect a number of synaptic processes including target recognition, induction of synaptogenesis, maintenance of dendritic spine morphology and modulation of synaptic plasticity. The following section will highlight synaptic CAMs most relevant for this thesis; for a more comprehensive discussion please see any of the following excellent reviews (Obst-Pernberg and Redies, 1999; Redies, 2000; Frank and Kemler, 2002; Takai et al., 2003; Yamagata et al., 2003; Washbourne et al., 2004b; Sakisaka and Takai, 2005; Gerrow and El-Husseini, 2006; Piechotta, 2006; Dalva et al., 2007; Gascon et al., 2007; Shapiro et al., 2007; Biederer and Stagi, 2008; Takai et al., 2008).

Review: Bona fide adhesion molecules expressed at the synapse

Within the nervous system, a number of recently identified molecules have been classified as cell adhesion molecules; however, only a subset has been rigorously shown to promote *bona fide* adhesion. Criteria used to define transmembrane molecules as true cell adhesion molecules (CAMs) include the following: promotion of cell aggregates and/or spontaneous segregation of subtype-selective CAMs when expressed *in vitro*, cell dispersion or tissue disruption following addition of blocking peptides or antibodies, the promotion of axon outgrowth and tissue morphogenesis, as well as structural studies of specific adhesive domains within the core proteins which promote adhesion between apposed membranes by cis/trans interactions (Edelman, 1984; Takeichi, 1990; Shapiro et

al., 2007; Takeichi, 2007). The major classes of CAMs expressed at synapses that satisfy all of these criteria are neural cell adhesion molecule (NCAM), cadherins, and nectins (Edelman, 1984; Piechotta, 2006; Gascon et al., 2007; Shapiro et al., 2007). These molecules may be found either within the cleft itself (NCAM), or in the case of nectins and N-Cadherin are localized to the puncta adherentia flanking the synaptic cleft space. Their roles go beyond mere synaptic adhesion but feature prominently throughout neural development and appear to function importantly in synaptogenesis as well as synaptic transmission and plasticity-related processes.

The Cadherin superfamily: Focus on N-Cadherin

Cadherins are a large and diverse family of transmembrane molecules that promote Ca++ dependent intercellular adhesion. They are classified according to the presence of a varying number of ~100 amino acid-long repeats (termed cadherin repeats) within their extracellular domains, which contain sequence motifs that promote dimerization and binding specificity between cadherin pairs (Takeichi, 1990; Obst-Pernberg and Redies, 1999; Takeichi, 2007). Cadherins are thereafter grouped into multiple subfamilies, including the classical cadherins, desmosomal cadherins, protocadherins, and cadherin-related molecules. Classical cadherins (of which N-Cadherin is a member) contain five extracellular cadherin repeats (EC1-EC5), a transmembrane domain, and a conserved cytoplasmic domain (Obst-Pernberg and Redies, 1999; Takeichi, 1990; Takeichi, 2007).

Within the plasma membrane, cadherins typically exist in two forms: as weakly adhesive monomers or strongly adhesive *cis* strand dimers, whose stiffness is aided by the binding of Ca++ ions between the cadherin repeat domains (Pokutta et al., 1994; Shapiro et al., 1995; Brieher et al., 1996; Nagar et al., 1996; Colman, 1997; Redies, 2000). Intercellular adhesion proceeds following homophilic *trans* interaction of the *cis* strand dimers with their counterparts on the opposing plasma membrane (Shapiro et al., 1995; Tamura et al., 1998). The formation of these "double dimers" leads to the stable locking in of juxtaposed membranes, resulting in a strongly adhesive complex (Tanaka et al., 2000; Phillips et al., 2001).

The cytoplasmic domain of classical cadherins is connected to the cytoskeletal network through their intracellular binding partners, the catenins. Catenins lend structural support to intercellular junctions by physically linking cadherin surface molecules to the actin cytoskeleton, and act as signalling mediators which bind multiple actin-binding proteins that in turn promote actin-based cytoskeletal remodelling (Kwiatkowski et al., 2007a; Yamada and Nelson, 2007). Thus, cadherin-catenin complexes are not simply passive adhesion sites that reinforce intercellular adhesion, but can propagate broad changes in synaptic structure and function through their involvement in diverse signalling pathways.

N-Cadherin and CNS synapses

Although several cadherins have been shown to have a synaptic expression (Obst-Pernberg and Redies, 1999; Redies, 2000; Junghans et al., 2005), neural (N-) cadherin (Ncad) is the best-studied cadherin subtype and arguably the best understood adhesion molecule at CNS synapses. Ncad was first observed to have a synaptic localization at the neuromuscular junction of the chick embryo (Cifuentes-Diaz et al., 1994), while biochemical approaches revealed the expression of Ncad as a major glycoprotein in isolated PSD fractions (Beesley et al., 1995). Subsequently, the ultrastructural localization of Ncad was determined by immunogold electron microscopy in mature CNS tissue. In the hippocampus and cerebellum, Ncad expression was sharply limited to the borders of the synaptic cleft, forming what appeared to be adhesive sites akin to the adherens junctions seen between epithelial cells (Fannon and Colman, 1996; Uchida et al., 1996). Parallel studies of α- and β-catenins revealed that these molecules are localized within the cytoplasmic face of the synaptic cleft immediately subadjacent to the cadherins (Uchida et al., 1996), consistent with their structural and functional relationship. Developmental studies *in vitro* revealed that Ncad expression is expressed at all synapses in immature neurons, but is absent from mature GABAergic synapses, becoming restricted to a subpopulation of excitatory synapses in adult cells (Benson and Tanaka, 1998). This finding supports a model whereby cadherins behave as synaptic "specifiers", whereby their subtype selectivity and restricted expression profiles is

thought to provide a molecular basis for the formation of correct synaptic connections (Shapiro and Colman, 1999; Redies, 2000; Benson et al., 2001).

More recently, a number of studies have pointed to a role for Ncad during early synapse development. Ncad is a constituent of the dense-cored vesicles that appear at nascent presynaptic endings, known as piccolo-bassoon transport vesicles (PTVs). As the name implies, these vesicles contain the presynaptic scaffolding molecules piccolo and bassoon, along with other constituents of the vertebrate active zone scaffold (Zhai et al., 2001; Shapira et al., 2003). This finding is supported by studies *in vivo*, whereby zebrafish expressing GFP-tagged Ncad reveal the extensive trafficking of Ncad-GFP-containing transport packets in outgrowing axons, forming stable puncta in the wake of migrating growth cones (Jontes et al., 2004). Furthermore, blockade of Ncad expression in developing hippocampal cultures has been shown to disrupt dendritic spine architecture as well as the distribution of pre and postsynaptic proteins (Togashi et al., 2002; Tanabe et al., 2006). However, it has also been shown that Ncad cannot induce synapses to form *in vitro* (Sara et al., 2005), and its genetic deletion in zebrafish leads to lamination defects in the retina but does not affect normal synaptic ultrastructure (Erdmann et al., 2003). Furthermore, differentiated embryonic stem cells from Ncad knockout mice also reveal normal synapse number and ultrastructure (Jungling et al., 2006; Kadowaki et al., 2007). These discrepancies have not been fully resolved.

Given that Ncad is widely expressed throughout development (Hatta and Takeichi, 1986), perhaps it has an important role in supporting adhesivity between apposed membranes prior to synaptogenesis, and/or nascent synaptic contacts following synaptic induction, but leaves the task of triggering synapse assembly to other receptor-ligand or adhesion molecules. In later stages of synaptic development, it is known that despite normal synaptic ultrastructure, the absence of Ncad expression leads to defects in synaptic transmission. These include defects in high frequency stimulation (Jungling et al., 2006), reduced mEPSC frequency and synaptic vesicle recycling (Bozdagi et al., 2004; Saglietti, 2007), and the absence of late phase LTP (Bozdagi et al., 2000), which together suggest an important role for Ncad-mediated adhesion and signalling in basal as

well as plasticity-related transmission. Thus, from the broad and extensive literature on N-cadherin, one can only conclude that its role is complex and multifaceted, appearing to take part in multiple distinct stages within the life of the synapse.

(III) Receptor-ligand pairs that promote cell adhesion and synapse formation

The synapse is highly enriched in a variety of transmembrane molecules that interact across the cleft space to promote synaptogenesis as well as synaptic adhesion. Unlike the classic cell adhesion molecules, which critically mediate nervous system development at multiple stages, these molecules are best understood for their ability to specifically promote synaptogenic processes. These include the ability to trigger synapses to form, an event known as *synaptic induction*, their ability to promote the formation of discriminating synaptic contacts, known as *synapse selectivity*, as well as their function in later stages of stabilization and maturation.

Neurexins and Neuroligins: Heterotypic adhesion partners at the synapse

Neurexins and neuroligins are currently among the most intensively studied of all the synaptic adhesion molecules. Neurexins were first discovered as the receptors for α-latrotoxin, a toxin found in the venom of black widow spiders that causes massive synaptic vesicle release (Ushkaryov et al., 1992), while the neuroligins were identified soon after as their endogenous binding partners (Ichtchenko et al., 1995; Ichtchenko et al., 1996). Three independent genes code for the neurexins, each of which contains two promoter sites to generate either the longer α or the shorter β version, producing 6 basic variants. In turn, each variant is subject to alternative splicing at multiple sites, thus potentially generating a large variety of neurexin isoforms (Ullrich et al., 1995). Four main subtypes of neuroligins (numbered 1-4) exist in vertebrates, each of which are coded by separate genes; neuroligins 1-3 are expressed predominantly in the brain while neuroligin 4 has a more widespread distribution (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Bolliger et al., 2001). Like the neurexins, the neuroligins are also subject to alternative splicing, therefore adding to the diversity in potential pairings between different neuroligin and neurexin variants (Dalva et al., 2007).

Neurexins and neuroligins mediate heterotypic adhesion in a Ca++-dependent manner (Nguyen and Sudhof, 1997). At synapses, the neuroligins are localized to the postsynaptic membrane while the neurexins are expressed along the presynaptic active zone membrane (Ushkaryov et al., 1992; Song et al., 1999; Dean et al., 2003; Dresbach et al., 2004; Graf et al., 2004; Varoqueaux et al., 2004; Rosales et al., 2005). Intracellularly, these molecules interact with various scaffolding proteins via their PDZ domains; for the neuroligins these include the NMDA receptor-binding protein PSD-95 whereas neurexins directly interact with the active zone scaffolds CASK and Mint (Hata et al., 1996; Irie et al., 1997; Butz et al., 1998; Biederer and Sudhof, 2000; Bolliger et al., 2001). Neurexin-neuroligin pairings are therefore poised to promote the structural integrity of the synapse by both transsynaptic adhesion as well as through physical links to their respective intracellular synaptic scaffolds (Dalva et al., 2007).

Neurexin-neuroligin complexes have been widely hypothesized to behave as "synaptic specifiers", able to induce the formation of discriminate synaptic contacts based on their structure, alternative splicing and localization patterns. Evidence to support this concept comes from studies showing that (i) neuroligins- 1, 3 and 4 are localized exclusively to excitatory postsynaptic endings while neuroligin-2 is expressed at inhibitory PSDs (Song et al., 1999; Varoqueaux et al., 2004), (ii) this differential neuroligin expression pattern can drive excitatory versus inhibitory postsynaptic development, respectively (Scheiffele et al., 2000; Graf et al., 2004), and that (iii) different splice variants induce differential binding affinities for certain neurexin-neuroligin pairs (Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006). Neurexins and neuroligins are among the few synaptic adhesion pairs with the capacity to directly trigger *de novo* synapse assembly. This has been shown *in vitro* using the so-called "reconstituted synapse" system, whereby cultured neurons are combined with neurexin- or neuroligin-expressing fibroblasts or beads to induce postsynaptic or presynaptic assembly at contact sites (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004). Finally, altering the expression levels of neuroligin *in vitro* affects synapse number as well as the balance between excitatory versus inhibitory synaptic contacts (Prange et al., 2004; Chih et al., 2005).

Collectively, the aforementioned studies suggest that neuroligin-neurexin pairs act early in the life of a synapse, to not only trigger synapse formation but to promote specificity between pre- and postsynaptic contacts (Dalva et al., 2007). However, this conclusion is complicated by genetic deletion studies of neurexin and neuroligin function *in vivo*. Mice lacking neuroligins 1-3 or α-neurexins die shortly after birth, demonstrating that they are essential genes. However, at the time of death, synapse number is not significantly reduced and synaptic ultrastructure appears normal (Missler et al., 2003; Varoqueaux et al., 2006; Dudanova et al., 2007), suggesting that while neurexins and neuroligins can drive synapse development *in vitro* they are not essential for synaptogenesis *in vivo*. The absence of neuroligins does lead to defects in GABAergic and glutamatergic neurotransmission, while the absence of α-neurexins causes dysfunctions in synaptic vesicle exocytosis and a selective decrease in inhibitory synapse density (Missler et al., 2003; Varoqueaux et al., 2006; Chubykin et al., 2007; Dudanova et al., 2007), findings which suggest that *in vivo*, these molecules function most critically during synaptic maturation and in activity-dependent neurotransmission. Furthermore, mutations in human neuroligin genes have been linked to autism spectrum disorders (Jamain et al., 2003; Sudhof, 2008) and these mutations have been shown in mouse models to cause increased inhibitory synaptic transmission (Tabuchi et al., 2007). This latter finding lends support to the concept that neurexin-neuroligin pairs, while not critical for synapse formation *per se*, do critically maintain the balance between excitatory and inhibitory synaptic transmission both *in vitro* and *in vivo* (Dalva et al., 2007; Sudhof, 2008).

The Ephrin family: Bidirectional signaling molecules at the synapse

Ephrin-B (EphB) receptors are transmembrane receptor tyrosine kinases whose membrane-bound activating partners, the ephrin-B ligands, permit adhesion by contact-mediated signaling (Piechotta, 2006; Dalva et al., 2007). The cytoplasmic domains of EphB receptors and ephrin-B ligands contain multiple sites for protein-protein interactions including PDZ binding domains (EphB and ephrin-B), tyrosine phosphorylation sites (ephrin-B) as well as the intracellular kinase domains (EphB)

critical for their activation. Through their intracellular domains, EphB-ephrin-B complexes can activate a variety of signaling cascades on both sides of the complex; those driven by the activation of the EphB kinase domains are known as forward cascades while reverse signaling cascades are those driven by the phosphorylation of tyrosine residues on ephrin-B and subsequent recruitment of other signaling effectors (Klein, 2009).

Investigations into their role at synapses began with the discovery that EphB and ephrin-Bs were present at hippocampal synapses both *in vitro* and *in situ* (Torres et al., 1998; Buchert et al., 1999). Subcellular localization studies reveal EphB receptors to be present selectively on postsynaptic membranes (Buchert et al., 1999), while ephrinB ligands are expressed on both sides of the synapse (Dalva et al., 2007). *In vitro*, exposure to cells/beads heterologously expressing EphB or ephrinB can induce excitatory presynaptic or postsynaptic development, respectively, on contacting neurites (Dalva et al., 2000; Kayser et al., 2006). Conversely, genetic deletion of EphBs *in vivo* (EphB1/2/3) causes a significant reduction in the number of synapses within the cortex of early postnatal mice (Henkemeyer et al., 2003b). Interestingly, mice lacking EphB1/2/3 are viable and able to breed, suggesting that only a subset of synapses is critically affected by the loss of all three subtypes (Henkemeyer et al., 2003b).

Several lines of evidence point to a strong postsynaptic role for this complex. Blockade of EphB receptor function negatively affects the formation of dendritic spines *in vitro* (Ethell et al., 2001; Henkemeyer et al., 2003b; Kayser et al., 2008) and genetic deletion of EphBs lead to abnormal spine morphology *in vivo* (Henkemeyer et al., 2003), while in contrast, presynaptic boutons are still able to form on dendritic shafts in normal numbers (Kayser et al., 2006; Dalva et al., 2007). These results suggest that the loss of transsynaptic signaling through EphB-ephrin-B complexes affects presynapse assembly secondary to the effects on dendritic spine morphogenesis.

Glial-derived neurotrophic factor and synapses

The nervous system expresses many soluble growth factors, known as neurotrophins, which promote the survival, development, and function of neurons. Certain neurotrophin family members have been shown to promote activity-dependent synaptic plasticity and memory formation, including brain derived neurotrophic factor (BDNF) [reviewed in (Tyler et al., 2002; Bramham and Messaoudi, 2005)]. More recently, another member of the neurotrophin family, glial-derived neurotrophic factor (GDNF) has been reported to directly influence synapse formation by ligand-induced cell adhesion.

GDNF was initially characterized as a growth factor that promotes the survival of midbrain dopaminergic neurons [reviewed in (Paratcha and Ledda, 2008)]. The classical signaling pathway for GDNF is through two receptor subunits, the transmembrane receptor tyrosine kinase Ret and the ligand-binding GDNF receptor GFRα, which is GPI-linked to the plasma membrane (Paratcha and Ledda, 2008). However, a recent study has shown that GDNF can promote trans-homophilic interactions between GFRα receptors and that this can trigger synapse formation in a Ret-independent manner (Ledda et al., 2007). In this study, it was shown that (i) GFRα was endogenously expressed along both axonal and dendritic membranes, (ii) GDNF could induce the clustering of cells heterologously expressing GFRα *in vitro*, (iii) beads coated with GFRα could trigger both excitatory or inhibitory presynaptic development along cultured hippocampal axons in the presence of GDNF and (iv) the presynaptic clustering effect was partially dependent on the expression of NCAM. This study reveals a novel form of cell adhesion in which a classic ligand-receptor system is the mediator of the contact which activates signaling responses to promote synapse formation (Paratcha and Ledda, 2008). It is interesting that the phenomenon is partially dependent on a classic cell adhesion molecule, indicating that even highly selective homophilic adhesion molecules can interact with novel partners to promote various aspects of neuronal development.

(IV) Other synapse-inducing adhesion molecules: NGL2, SALMs & SynCAM

Recent efforts to identify novel synaptogenic adhesion molecules have led to the discovery of several new Ig superfamily members able to trigger *de novo* synapse assembly (Biederer et al., 2002; Kim et al., 2006; Ko et al., 2006). By yeast two-hybrid screen, netrin-G ligand 2 (NGL2) was selected as a candidate synaptogenic molecule for the presence of PDZ domains for PSD95 (Kim et al., 2006). NGL2 belongs to a family of cell adhesion molecules that share several domain structures, including leucine-rich N-terminal repeats, a single extracellular Ig domain, a transmembrane domain and a C-terminal PDZ domain-binding motif. Their binding partners are netrin-Gs, GPI-anchored membrane molecules that interact with NGLs in an isoform-specific manner to promote Ca++-independent adhesion (Kim et al., 2006).

NGL2 localizes to the postsynaptic membrane of excitatory synapses, and biochemically fractionates with NMDA receptors and PSD-95, indicating a physical interaction (Kim et al., 2006). Heterologous cells or beads expressing NGL2 trigger presynaptic assembly on contacting hippocampal axons in culture, as well as the clustering of postsynaptic proteins on contacting dendrites. Finally, overexpression of NGL2 promotes spine formation while knockdown reduces excitatory synapse number selectively (Kim et al., 2006). Collectively, these findings point to a role for NGL2 in excitatory synapse development; however, the axonal binding partner mediating these effects is unclear. While the authors show that NGL2 binds to and clusters netrin-G2 along axonal membranes, the clustering of netrin-G2 alone does not induce the formation of presynaptic specializations (Kim et al., 2006). This suggests other presynaptic targets critically mediate the effects of NGL2.

Another family of excitatory postsynapse-inducing molecules are the SALM (synapse adhesion-like molecule) proteins. SALMs are a family of five single-pass transmembrane proteins (1-5) that, like NGL2, were first discovered using the PDZ domains of PSD-95 as bait in yeast two-hybrid screens (Ko et al., 2006; Wang et al., 2006). SALMs are multidomain structures that, on the extracellular side, contain six leucine-rich repeats, an Ig-like and a fibronectin-like domain, a transmembrane region and an intracellular PDZ

binding domain (present in SALMs 1-3 but absent in SALMs 4 and 5)(Ko and Kim, 2007; Wang et al., 2008).

SALM1 and SALM2 are thought to participate in the organization of postsynaptic specializations. SALM1 has been shown to colocalize with NMDA receptors and to promote the surface targeting of PSD95 (Wang et al., 2006). In the case of SALM2, Ko and colleagues show that SALM2 selectively promotes excitatory postsynaptic development, whereby *in vitro* (i) the overexpression or inhibition of SALM2 leads to an increase or decrease, respectively, of excitatory postsynaptic specializations, (ii) beads coated with SALM2 induce the clustering of excitatory postsynaptic molecules on contacting dendrites but (iii) SALM2 fails to promote presynapse formation *in vitro*, and does not appear to mediate its effects on postsynaptic development through homophilic adhesion (Ko et al., 2006). Although much remains to be learned about the mechanism of SALM-induced synaptic differentiation, these early studies are intriguing and will likely be the subject of future study *in vitro* and *in vivo*.

SynCAMs, (for *synapse cell adhesion molecules*), are another family of newly discovered adhesion molecules. Four synCAM subtypes exist (1-4), each of which are coded by separate genes (CADM1-4), and are endowed with several names due to their parallel identification in different tissues including *tumor suppressor in lung carcinoma* (TLSC) and *nectin-like* (Necl) molecules (Biederer, 2006). Although SynCAM1 is found in certain peripheral tissues, its expression is most abundant in the nervous system while SynCAMs 2, 3 and 4 are exclusively found in neural tissue (Biederer, 2006; Fogel et al., 2007). SynCAM proteins contain three extracellular Ig-like domains that mediate adhesive interactions, a single transmembrane domain and a short cytoplasmic sequence containing binding sites for both PDZ domain and FERM domain proteins, the latter of which typically promote binding to cytoskeletal adaptors (Biederer, 2006; Biederer and Stagi, 2008).

The first identified SynCAM, known as SynCAM1, was shown *in vitro* to promote synaptogenesis by (i) overexpression of full-length SynCAM, which results in enhanced

spontaneous synaptic activity, (ii) expression of a dominant-negative version of SynCAM, leading to a decrease in synapse number, and (iii) co-culture of neurons with SynCAM-expressing fibroblasts, which induces the formation of presynaptic specializations on contacting axons (Biederer et al., 2002; Sara et al., 2005). SynCAM1 is localized to both pre and postsynaptic membranes and can mediate homophilic adhesion (Biederer et al., 2002), although heterophilic complexes of different SynCAM subtypes also appear to form *in vitro* (Fogel et al., 2007) and *in situ* (Thomas et al., 2008). Interestingly, studies examining the distribution of all SynCAM subtypes *in situ* revealed that SynCAM proteins are expressed on both inhibitory and excitatory neurons, but are divergently expressed between different neuronal populations (Thomas et al., 2008). As has been suggested for the neurexins-neuroligins, it could be that the distinct spatial patterning of SynCAMs can promote synaptic specificity during the establishment of local neuronal circuits (Biederer, 2006; Thomas et al., 2008).

The only knockout mouse model reported for SynCAM family proteins is for SynCAM1, whose genetic deletion did not result in any central nervous system defects (Fugita et al., 2006; van der Weyden et al., 2006). This suggests that SynCAM1, while sufficient, is not necessary for synapse formation. However, compensatory up-regulation of other SynCAM subtypes may explain the lack of phenotype; embryonic null or conditional knockdown studies of multiple subtypes *in vivo* could help to resolve this issue.

(V) Just in: New transmembrane molecules identified as synaptic organizers

The list of synaptic organizing molecules is a work in progress that will continue to grow for some time. In a recent study by Linhoff and colleagues, the primary aim was to discover novel synapse-inducing molecules (Linhoff et al., 2009). The approach that the authors used was an unbiased expression screen using cDNAs isolated from rat brains during the peak rate of synaptogenesis *in situ* (P11). The authors tested over 10^5 cDNAs by expressing them in fibroblasts for co-culture with hippocampal neurons, and then selected those clones that triggered the clustering of presynaptic proteins along contacting axons. In so doing, they uncovered not only established synaptic inducers

including neuroligins (thus validating the approach) but a family of transmembrane molecules known as leucine rich repeat transmembrane molecules, or LRRTMs.

The LRRTM family of proteins (LRRTMs1-4) were originally discovered in a search for proteins bearing sequence similarity to the Slit family of axon guidance molecules (Lauren et al., 2003). They contain 10 leucine-rich repeat sequences in the extracellular region and a short C-terminal domain that ends in a PDZ-binding motif. All 4 LRRTMs possessed synaptogenic activity in the fibroblast co-culture assay, although LRRTMs1 and 2 appear to be the most potent inducers and were the most heavily investigated. LRRTM1s 1 and 2 could both instruct the formation of functional excitatory presynaptic endings using the fibroblast co-culture assay (Linhoff et al., 2009). Furthermore, the authors suggest that LRRTM1 can drive excitatory postsynaptic differentiation following experiments where neurons were transfected with a GFP-tagged version of LRRTM1 and stimulated to form clusters using GFP-coated beads, which led to the co-clustering of the NMDA receptor subunit NR1 as well as PSD95 (Linhoff et al., 2009).

As with the neuroligins, it appears that deletion of LRRTM family members does not prevent synapse formation *in situ*. In LRRTM1$^{-/-}$ mice, the only phenotype was an increase in the size of VGlut1 clusters in some strata of the hippocampal CA1 region, with synapse number and active zone integrity appearing normal. Clearly, simultaneous deletion of all 4 LRRTMs will be necessary to determine if this family of proteins is truly critical for the induction of synapse assembly. In parallel, a better assessment of their precise subcellular localization, as well as the determination of their axonal binding partners, will improve our understanding of how these proteins function in synapse development. Lastly, it is worth noting that their screening approach has uncovered a number of other molecules, some known for roles in different neurobiological processes and others whose function is entirely unknown, that could trigger presynaptic protein clustering (Linhoff et al., 2009). Thus, this comprehensive study has undoubtedly isolated other unknown synaptic organizers that are sure to be the subject of future study.

PART V: The development and formation of the synapse

It is clear that several decades of research into the composition of synapses has yielded a dizzying number of synaptically localized molecules. The role of several of these molecules is well understood, having benefited from the availability of molecular and genetic tools that allow them to be studied individually. However, our higher-order understanding of how these molecules function in concert with each other remains rudimentary. This applies not only for how individual molecules accumulate, organize, and assemble within their respective synaptic compartments, but also extends to our understanding of how the respective compartments interact with each other to form a functional synaptic junction. Efforts to study nascent synaptic development are inevitably hampered by the challenge of observing newly forming synaptic contacts at a developmental stage where neurons are competent to form them. Therefore, most of those discrete steps remain mysterious.

Equally fundamental, and also incompletely understood, are the instructive cues which signal two neuronal membranes to initiate the formation of a synapse. Much has been described to date about the role of adhesion molecules, which function primarily to lock in pre and postsynaptic membranes. Due to their adhesive properties, the highly selective nature of their interaction, and their ability to trigger intracellular signaling cascades, the hypothesis that adhesion molecules are the early inducers of synaptic formation has gained much critical support and indeed, is supported by many *in vitro* studies. However, one reaches an impasse when comparing the effects of these molecules *in vitro* with knockout studies *in vivo*, whereby genetic loss of many of them does not dissolve synaptogenesis. It seems likely that synaptogenesis is far too critical during development to depend on a single class or even family of molecules to proceed. If this is true, then perhaps the formation of a mature synapse proceeds based on a hierarchy of inductive cues which function at discreet stages during the synaptogenic process, a concept that adds tremendous complexity to the mystery of how synapses form *in situ*. Although many questions remain, our understanding of the process of synapse formation has advanced significantly in recent years thanks to the advent of sophisticated new

techniques combined with high-resolution imaging, which allow one to follow synaptogenesis with unprecedented precision.

Arrival of proteins at nascent sites: Studies in vitro

Early studies focused on the recruitment of individual synaptic proteins to synapses using fixed samples of differentiating hippocampal neurons. These studies gave an impression that synapse formation was a slow process occurring over a period of days to weeks, as the expression of individual proteins in these cultures increased as maturation progressed (Fletcher et al., 1991; Mammen et al., 1997). However, more recent studies addressing this issue show that protein recruitment is far more dynamic and rapid than previously predicted by these early studies. Furthermore, synaptic proteins appear to be transported to synaptic sites in different ways, some accumulating alone while others arriving in preformed packets containing multiple individual components.

Presynaptic protein recruitment and the role of preformed packets

Various components of the synaptic machinery appear to be presynthesized in advance of nascent synaptic contact (Munno and Syed, 2003; McAllister, 2007). Among the earliest studies revealing this phenomenon is by Ahmari and colleagues, who transfected hippocampal neurons with a GFP-tagged version of the synaptic vesicle-bound SNARE VAMP (synaptobrevin) (Ahmari et al., 2000). Using live time-lapse imaging, they found that highly mobile VAMP-GFP clusters could be observed along axons whose diameter was substantially larger than what would be expected of a single synaptic vesicle. By retrospective immunocytochemistry, these VAMP-GFP clusters were found to contain a variety of characteristic presynaptic proteins, including voltage-dependent Ca++ channel subunits, SV2, synapsin I, as well as amphiphysin I, a component of the endocytosis machinery (Ahmari et al., 2000). Furthermore, these clusters could be rapidly stabilized following axo-dendritic contact and could recycle transmitter within 4h of axo-dendritic contact (Ahmari et al., 2000). These packets have since become known as STVs, for *synaptic vesicle protein transport vesicles*, and are thought to represent the precursors of an active presynapse (McAllister, 2007).

Several studies have shown that isolated SV clusters can also recycle transmitter prior to axo-dendritic contact (Matteoli et al., 1992; Kraszewski et al., 1995; Dai and Peng, 1996a; Krueger et al., 2003; Sabo et al., 2006). This type of presynaptic activity can exhibit unusual features, including increased brefeldin-A sensitivity and reduced sensitivity to tetanus toxin (Verderio et al., 1999; Zakharenko et al., 1999). Furthermore, these clusters tend to be highly mobile (Krueger et al., 2003). These features suggest that a proportion of STVs represent highly dynamic release sites with the potential to become active zone precursors depending on the availability of a native postsynaptic target. This complex is therefore highly useful when synapses need to be assembled in short order.

Another type of precursor vesicle contains multiple components of the presynaptic active zone. Piccolo-bassoon transport vesicles (PTVs) are 80-nm dense-cored vesicles that move rapidly within axons of young CNS neurons (Zhai et al., 2001; Shapira et al., 2003), and are thought to represent the dense-cored vesicles observed ultrastructurally at nascent presynaptic sites. Using a combination of biochemistry and retrospective immunocytochemistry, PTVs were found to contain the active zone scaffolds piccolo and bassoon, proteins that mediate exocytosis including Munc13, Munc18, syntaxin and SNAP25, as well as N-cadherin. Like STVs, the stabilization of PTVs along axons was shortly followed by the capacity of these sites to undergo activity-dependent SV cycling (Zhai et al., 2001; Shapira et al., 2003), suggesting that PTVs are constituents of nascent presynaptic boutons. Furthermore, the total amount of piccolo or bassoon protein at a mature synapse can be derived from just 2-3 PTVs (Shapira et al., 2003). However, they do not contain synaptic vesicles and do not appear to contain any of the proteins in STVs, suggesting that while both accumulate early in the life of a nascent presynapse their synthesis and transport is physically distinct.

Postsynaptic protein recruitment: packets vs. diffuse transport

Postsynaptically, the accumulation of proteins at nascent sites appears more contentious, as several studies have shown the same candidate molecules to accumulate by mutually

distinct mechanisms. An example would be PSD95, widely considered to be one of the earliest markers of the nascent postsynapse (Waites et al., 2005). PSD95 clusters were demonstrated to be recruited from a diffuse cytoplasmic pool, forming clusters at new sites of axo-dendritic contact within 20-60min which became presynaptically functional within 1-2h (Bresler et al., 2001). However, PSD95 has also been shown to traffic in discrete puncta, and recruitment of these clusters to filopodia-spines resulted in their stabilization (Prange and Murphy, 2001). PSD-95 was also shown to be a component of a newly-discovered postsynaptic packet composed of PSD95, GKAP and Shank, which the authors describe as a precursor vesicle involved in nascent postsynapse assembly (Gerrow et al., 2006).

The trafficking of postsynaptic receptors also appears to be rather confusing, as NMDA receptors have been shown to accumulate at nascent sites both as discrete puncta (Washbourne et al., 2002; Washbourne et al., 2004a) as well as from diffuse pools (Bresler et al., 2004). An explanation for these discrepancies has not been adequately described. One clear difference is that the papers describing the trafficking of postsynaptic proteins in packets tended to use young cultured neurons (DIV3-7)(Washbourne et al., 2002; Washbourne et al., 2004a; Gerrow et al., 2006), a developmental stage *in vitro* characterized by prolific neurite outgrowth but very little synaptogenesis. In contrast, the diffuse recruitment of postsynaptic proteins was observed in older cultures during the typically synaptogenic phase of *in vitro* development (DIV8-12)(Bresler et al., 2001; Bresler et al., 2004). Therefore, it is possible that the preferred mode of postsynaptic protein recruitment changes with neuronal development, although this is a hypothesis that has not been directly tested.

Furthermore, determining the time point at which stable postsynaptic puncta become functional, at least *in vitro*, has largely relied on measures that assess presynaptic functionality, and have not directly addressed postsynaptic functionality using electrophysiological techniques. For example, it is possible in many of these studies that the stabilization of postsynaptic packets occurs on previously functional presynaptic sites. Determining the extent to which the nascent PSD is functionally silent following

the stabilization of postsynaptic proteins, and the reliance on the presence of the presynaptic ending remains an open question in these studies.

Time course of synaptogenesis in vitro

When axodendritic contact is made, when and in what order do synaptic proteins accumulate? A number of *in vitro* studies have combined live imaging with retrospective immunocytochemistry to study the time course of synaptic protein accumulation at nascent sites. The first of these studies was by Friedman and colleagues, who used a repetitive stimulation protocol along with the vital amphipathic dye FM4-64 to label newly recycling presynaptic boutons in DIV11-14 hippocampal cultures (Friedman et al., 2000). Once a new actively recycling site was found, these boutons were repeatedly stimulated and then fixed at defined time points. By retrospective immunocytochemistry, they found that these new sites almost always contained bassoon (95%) beginning at the earliest time points of fixation (<45min after new bouton detection)(Friedman et al., 2000). However, PSD95 and the NMDA receptor subunit NR1 were present at a significantly smaller fraction of sites for boutons fixed within 45min of detection (9-18%) but this increased with time, until after 2h nearly every new bouton also contained apposed clusters of PSD95 and NR1 (Friedman et al., 2000). These results suggest that presynaptic proteins tend to be recruited before postsynaptic proteins to nascent sites, with components of PTVs being among the earliest constituents of the presynaptic bouton. This conclusion has since been supported by other studies (Okabe et al., 2001).

With respect to these initial reports, other studies have demonstrated conflicting timelines of synaptic protein accumulation at nascent sites. Washbourne (2002) defined a nascent site as the visible stabilization of a contact between an axonal growth cone with a dendritic shaft during a live imaging session, using cultures that were 3-5DIV (Washbourne et al., 2002). Using these criteria, they observed that NMDA receptor packets along with axonal STVs could be recruited to nascent synaptic sites within 10min of each other, while PSD95 accumulation was highly variable, sometimes not appearing even after 1h post-NMDA receptor recruitment. AMPA receptors were

recruited after NMDA receptors, suggesting that sites were postsynaptically silent for a period of time, while these nascent sites were presynaptically active within 1h following contact (Washbourne et al., 2002). Finally, Gerrow *et al.* used the stabilization of postsynaptic transport packets (containing PSD95, GKAP and Shank) as a "time zero" to measure the development of a functional pre-postsynaptic pair (Gerrow et al., 2006). They found that stabilized clusters became apposed to presynaptically functional boutons within 2h, suggesting that these postsynaptic clusters drove the assembly of a presynaptic terminal (Gerrow et al., 2006).

Again, it is difficult to attempt direct explanations of these discrepancies given the differences in neuronal age *in vitro* and criteria for defining new sites. However, it is reasonable to assume that these studies have all uncovered that the temporal order of assembly may proceed by multiple mechanisms depending on the age of the contact, age of the culture, or other as-yet undefined mechanism. Furthermore, these studies have been performed with a very small set of candidate synaptogenic molecules and therefore recruitment of the remaining majority is mostly a mystery. It is thus equally plausible that the temporal order and functionality of nascent sites is critically affected by the presence of other untested, synaptically localized molecules. Widening the pool of candidate molecules and performing live time-lapse imaging of multiple proteins simultaneously will help clarify these issues.

What kinds of contacts initiate synaptogenesis? Studies in vitro and in situ

During development, most contacts between axons and dendrites are fleeting, persisting for mere seconds before retracting their respective processes and rerouting themselves to search for other targets (McAllister, 2007). However, a subset of contacts will go on to form stable synapses, and studies *in vitro* and *in situ* seem to suggest that nearly every type of contact, whether initiated by axons or by dendrites, has the potential to lead to the establishment of a *bona fide* synapse.

Initiation of synaptic contacts by dendritic filopodia

Many studies suggest that dendritic filopodia, the highly motile protrusions along dendrites, are the precursors for dendritic spines, the mature postsynaptic specializations of the synaptic junction. Dendritic spines are typically classified by their morphology, with mature spines having a bulbous head (the spine head) and a thin neck that connects the head of the spine to the shaft of the dendrite, while immature spines have no distinct head and neck and appear long and thin (Yuste and Bonhoeffer, 2004). Several studies have revealed a developmental transition in the protrusions along dendritic shafts whereby young neurons are rich in highly mobile dendritic filopodia; with development, the density of these filopodial protrusions decrease while the density of mature spines along dendritic shafts increases (Dailey and Smith, 1996; Ziv and Smith, 1996; Fiala et al., 1998; Portera-Cailliau et al., 2003). In young neurons, many of these filopodia serve as postsynaptic specializations at sites of axonal contact, and these sites have been shown to be presynaptically functional (Ziv and Smith, 1996; Ahmari et al., 2000). Taken together, these studies suggest that filopodia, given their high motility and density in young neurons, serve to initiate synaptic contact with axonal targets and thereafter evolve morphologically into mature spines (Ziv and Smith, 1996; Fiala et al., 1998; Yuste and Bonhoeffer, 2004).

Initiation of synaptogenesis by growth cones, preexisting contacts, or activity

En passant synapses are typically defined as synaptic contacts initiated by the terminal growth cone of an axon or dendrite that contacts its target along the shaft. These are most often observed for axonal growth cones contacting dendritic shafts (Ahmari et al., 2000; Jontes et al., 2000) although dendritic growth cones have also been observed to trigger presynaptic terminal differentiation (Ahmari et al., 2000; Sabo et al., 2006; McAllister, 2007). In addition, preexisting contacts between axonal and dendritic shafts can also be triggered to form synapses, even long after they have made their initial contact (Friedman et al., 2000; Gerrow et al., 2006; Wierenga et al., 2008).

The bulk of synaptogenesis occurs during early postnatal development and does not appear to require activity (Verhage et al., 2000). However, a number of studies show that neuronal activity can sculpt neuronal growth and synapse formation in both young and mature CNS by promoting the formation of filopodial protrusions (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Tashiro et al., 2003; Knott et al., 2006). This has been shown using various electrophysiogical paradigms as well as behavioural paradigms, and could represent an additional mechanism to promote synaptogenesis which could serve throughout the life of the organism (Waites et al., 2005).

Taken together, these studies clearly show that synaptogenesis does not depend on a certain type of axo-dendritic contact to proceed, given that the entire length of axons and dendrites appear equally well-suited to become cognate pre or postsynaptic specializations, respectively. However, how can one explain this seemingly random process given the specificity with which synaptic circuits are established during development? If the type of contacts made between axons and dendrites does not endow synaptic specificity, then what does?

Synaptogenesis and Sperry's hypothesis

Since the earliest days of neuronal tract tracing, it has been known that the mature nervous system is composed of highly organized connections between neurons. However, the notion that the orderly connection of neurons was responsible for behaviour was not widely accepted in the earliest days of neuroanatomy (1930s). Instead, it was believed that all neurons were equally capable of connecting with any target and that connectivity ultimately arose not from specified connections between neurons, but from patterns of activity shaped by learning (Meyer, 1998). This "functionalist" view was widely supported prior to the work of Roger Sperry, who is since credited for establishing the conceptual framework that led to enormous advances in our understanding of neuronal connectivity.

Sperry performed his groundbreaking experiments using the retinotectal circuit of adult frogs, which allowed for the study of both neuronal circuitry as well as behaviour. He found that if he severed the optic nerve, the axons would spontaneously grow back to the contralateral tectum, their original target destination (Sperry, 1963). In other experiments, the optic nerves were simultaneously uncrossed and severed, thereby encouraging the axons to regenerate towards the ipsilateral tectum and leading to a left-right reversal of the animal's visual field. In this case, the animals behaved as though their visual field was left-right reversed; they performed permanent errors when attempting to localize objects within their visual fields and never correctly adapted to their new situation (Sperry, 1945). These experiments clearly show that these behaviours are not mediated by learning, but arise from orderly connections between neurons (Meyer, 1998). Sperry hypothesized that in order for this wiring to be established, that 1) axonal growth was directed by chemical markers, and 2) axons and target cells expressed differentiating markers that allowed them to find each other during development. These are the central tenets of what is now known as Sperry's chemoaffinity hypothesis (Sperry, 1963; Meyer, 1998).

Since Sperry's groundbreaking hypothesis, a number of diffusible chemotactic molecules have been identified in the past several decades, including the netrins, semaphorins, and a variety of extracellular matrix (ECM) proteins to which axons grow towards or away from en route to their final destination [reviewed in (Tessier-Lavigne and Goodman, 1996)]. In the context of synapse formation, a growing consensus in the field is that cell adhesion molecules (CAMs), expressed at the neuronal surface, act as the molecular tags which correctly identify synaptic partners to each other, and provide the adhesive substrate to lock in pre and postsynaptic membranes (Dalva et al., 2000; Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003; Graf et al., 2004; Craig, 2006; Ko et al., 2006; Dalva, 2007). The molecular diversity and selective interaction of these synaptic adhesion molecules satisfies the specificity that is required in accordance with Sperry's hypothesis. However, this scheme is complicated by a number of considerations.

First, a variety of classes of molecules other than transmembrane adhesion proteins have been identified *in vitro* for their ability to induce the *de novo* clustering of synaptic proteins. These include diffusible molecules such as Wnt, FGF22 and GDNF, extracellular matrix molecules, as well as glial-derived secreted factors (Hall et al., 2000; Umemori, 2002; Christopherson et al., 2005; Dityatev and Schachner, 2006; Ledda et al., 2007). Second, a number of studies *in vivo* have revealed that genetic ablation of some classes of adhesion molecules had profound effects on synaptic stabilization and maturation, but not on synapse formation (Fugita et al., 2006; Piechotta, 2006; van der Weyden et al., 2006; Varoqueaux et al., 2006), despite being identified as synaptic inducers *in vitro* (Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003; Graf et al., 2004). Furthermore, when assayed in combination, some of the soluble synapse-inducing factors have been shown *in vivo* to act not as synaptogenic triggers but rather influence later stages of synaptogenesis (Fox et al., 2007). Taken together, these results suggest that certain molecules that are sufficiently inductive *in vitro* do not necessarily have an inductive function *in vivo*. As it seems that no one synapse-inducing molecule is critical for the establishment of all synapses, perhaps it is the case that certain, unique combinations contribute to the establishment of specific neural circuits. Understanding how these molecules work in relation to each other to help establish neuronal circuitry will likely be an important future area of investigation in the field of synapse biology.

Formation of ectopic synapses in vitro and in situ

Another issue relates to the formation of ectopic synapses. It is certainly true that the vast majority of axons navigate over very long distances and complex terrains, restraining themselves along the way, until they reach their desired neighbourhood to form synapses with their appropriate targets. However, even though axons generally do not form inappropriate contacts does not mean that they cannot, and a number of *in vitro* and *in vivo* paradigms have revealed the flexibility with which axons can form presynaptic endings.

The "heterochronic culture" approach *in vitro* entails combining neurons of different developmental age in the same dish. Using these cultures, Fletcher *et al.* demonstrated that hippocampal neurons are competent to form presynaptic specializations before a critical stage of dendritic maturation has been reached, such that immature axons contacting mature dendrites displayed enhanced synaptophysin clustering, whereas contact with immature dendrites did not (Fletcher et al., 1994). This finding has also been shown using cultured embryonic retinal ganglion cells (RGCs), which at E17 normally do not contact their postsynaptic targets and therefore do not have any synapses. However, if E17 RGCs are engineered to contact both mature (P5) RGCs as well as astrocytes, they become presynaptically receptive well in advance of the natural developmental program, but never became postsynaptically receptive at this immature stage (Barker et al., 2008). Taken together, these studies reveal that axons are precocious, able to form presynaptic endings before dendrites and able to form rapidly if given an appropriately mature contact.

These aforementioned studies combine cells that ultimately would become natural pre and postsynaptic targets (Fletcher et al., 1994; Barker et al., 2008). However, axons do not appear limited to their natural partners. Rather, there are many examples, from invertebrates to mammalian organisms, of ectopic synapses being formed between axons and unnatural targets. *In vitro*, studies of cholinergic Helisoma neurons B5 and B19 show that B19 will only form presynaptic elements to its appropriate muscle target, while neuron B5 presynthesizes its synaptic machinery and forms synapses promiscuously with all neurons it contacts (Haydon and Zoran, 1989). A similar phenomenon is found in Aplysia neurons, where cholinergic neuron R2 can form presynaptic endings when contacting target neurons not normally encountered *in vivo* (Schacher et al., 1985). Even in mammalian neurons, rerouted RGC axons can form presynaptic-like endings to incorrect targets in both the cerebellum (Zwimpfer et al., 1992) and medial geniculate nucleus (Sur et al., 1988).

It is interesting that in each of these studies, it is the presynaptic ending that is particularly promiscuous in its target selection. An extreme version of this conclusion

may be found in a series of experiments in which presynaptic-like specializations could be induced to form by substrates coated with positive charge alone. Specifically, it was observed that cultured cerebellar neurons formed bouton-like swellings containing synaptic vesicles when contacting beads coated with positively charged proteins, including poly-L-lysine and arginine-rich histones, but not with beads coated with negative or neutral charge (Burry, 1980a; Burry, 1982b). These findings were replicated at the NMJ, whereby co-culture with polylysine-coated beads induced the formation of either presynaptic-type boutons or acetylcholine receptor clusters when contacting spinal cord neurons (Peng et al., 1987) or muscle cells (Peng et al., 1981), respectively. Poly-lysine and its derivatives are nothing more than single amino acid polymers of lysine residues that are positively charged at neutral pH. Solutions of poly-lysine are typically used to coat substrates for cell adhesion *in vitro*, and in the case of neurons, to promote neurite outgrowth (Yavin and Yavin, 1974). And yet, Burry, Peng and colleagues uncovered a mysterious property of this artificial substance to induce the formation of presynaptic-like specializations.

How can one reconcile these findings with the specificity required in accordance with Sperry's hypothesis? What promotes the formation of a presynaptic ending to non-cognate, and even entirely artificial targets? A unifying theme in all of these experiments is physical contact, suggesting that axons, with their high motility during development and abundance of presynthesized packets of proteins, are intrinsically competent to assemble presynaptic endings and simply depend on an appropriately adhesive target to trigger assembly. This very early stage in the life of a synapse may therefore not absolutely depend on the presence of highly selective cytochemical markers. What then endows synaptic contacts with the specificity that is observed in the mature CNS?

Chemoaffinity vs. selective stabilization

One consideration that Sperry's hypothesis left out was the role of activity in shaping circuits, an aspect of synaptic development that is particularly relevant within the visual system in which Sperry made his groundbreaking observations [reviewed in (Shatz,

1990)]. Although it remains to be determined whether activity influences the connectivity of all neuronal circuits, other mechanisms that shape connectivity such as target recognition (by adhesion molecules or receptor-ligand pairings, for example) may play a similar temporal role during refinement of neural circuits rather than synapse formation *per se*. Therefore, perhaps chemoaffinity might be responsible for guiding fibers to their correct neighbourhoods, within which synapses form by rather generalized requirements and are later refined by activity, recognition, or a combination of both. Such a hypothesis, known as the "selective stabilization hypothesis" (Changeux and Danchin, 1976; Katz and Shatz, 1996; Jontes and Phillips, 2006), could accommodate these observations of ectopic synapse formation, as well as provide a reasonable explanation as to why so many synapses are formed during development only to be pruned back during later stages, long after assembly is complete. This suggests that specificity is more an issue of which synapses are maintained than one of which synapses are allowed to form in the first place (Jontes and Phillips, 2006). However, these assertions have not been complemented by current *in vitro* models.

RESULTS PART (A): MANUSCRIPT

PREFACE

The following section comprises the bulk of the thesis experiments, which are contained within a manuscript that has been submitted to the Journal of Neuroscience (but has not yet been accepted for publication at the time of thesis submission). All other thesis experiments will be described in Results Part (B): Supplemental thesis results.

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ABSTRACT

CNS synapse assembly typically follows after stable contacts between "appropriate" axonal and dendritic membranes are made. We show that presynaptic boutons selectively form *de novo* following neuronal fiber adhesion to beads coated with poly-D-lysine (PDL), an artificial cationic polypeptide. As demonstrated by atomic force and live confocal microscopy, functional presynaptic boutons self-assemble as rapidly as 1h post-bead contact, and are found to contain a variety of proteins characteristic of presynaptic endings. Interestingly, presynaptic compartment assembly does not depend on the presence of a biological postsynaptic membrane surface. Rather, heparan sulfate proteoglycans including syndecan-2, as well as others possibly adsorbed onto the bead matrix or expressed on the axon surface, are required for assembly to proceed by a mechanism dependent on the dynamic reorganization of F-actin. Our results indicate that certain (but not all) nonspecific cationic molecules like PDL, with presumably electrostatically-mediated adhesive properties, can effectively bypass cognate and natural postsynaptic ligands to trigger presynaptic assembly in the absence of specific target recognition. In contrast, we find that postsynaptic compartment assembly depends on the prior presence of a mature presynaptic ending.

INTRODUCTION

Synapses are asymmetric sites of cell-cell contact that mediate the vectorial transfer of information between neuronal cells and their targets. In recent years, numerous proteins comprising the molecular architecture of the synaptic junction have been identified (Kim and Sheng, 2004; Okabe, 2007; Jin and Garner, 2008). Many of these proteins, particularly those of the presynaptic ending, can assemble rapidly following nascent axo-dendritic contact (Ahmari et al., 2000; Friedman et al., 2000) and this process can be driven artificially by transmembrane cleft-spanning molecular arrays such as neurexin-neuroligin pairs, NGL-2 and/or SynCAM (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004; Kim et al., 2006). Eventually, the synaptic junctional complex becomes remarkably resistant to degradation such that only membrane-disruptive treatments will lead to dissolution of the complex (Phillips et al., 2001). Collectively, these data reveal that constituents of the synapse can integrate, dissociate and reassemble quite readily, and disclose properties that would likely be essential features for synapse formation, pruning and re-formation *in situ*. Of course, many molecular details of synaptogenesis are lacking, due to the rarity of observing these rapid events *in vitro* and *in vivo* and the difficulty in temporally controlling nascent synapse formation during neuronal development.

In this study, we examined CNS synapse formation utilizing an artificial matrix to induce the assembly of a presynaptic ending that appears strikingly similar to one produced *in situ.* The paradigm is based on earlier observations showing that cultured cerebellar neurons form presynaptic-like specializations when placed in contact with beads coated with positively-charged proteins including poly-L-lysine and arginine-rich histones, but not with beads coated with negative or neutral charge (Burry, 1980b, 1982a; Peng et al., 1987). These studies imply that target adhesion alone can trigger presynaptic endings to form in the absence of any specialized cues. However, it was never clear from these early studies whether these synaptic vesicle-filled varicosities were functional, whether the requirements for their formation were intrinsic to axons or dependent on dendritic contact, or whether the assembly process was similar to that observed *in situ*.

Using atomic force microscopy (AFM), we first show that poly-D-lysine-coated (PDL) beads induce adhesion to axonal processes such that the bead is rapidly resistant to detachment within minutes of contact. Our data reveal that PDL beads can indeed induce the formation of presynaptic boutons by a mechanism that depends on F-actin reorganization and the presence of heparan sulfate proteoglycans (HSPGs), and implicate the cell surface HSPG syndecan-2 as at least one important co-receptor. Functional presynaptic differentiation is fast, on a time scale of minutes, similar to native synapse formation and can occur in the absence of postsynaptic partners, while postsynaptic assembly is dependent on the presence of differentiated presynaptic structures. These findings suggest that early recognition events that guide synaptic assembly need not be highly selective on axons, and reveal a readiness for axons to form non-cognate presynaptic connections at any site along their length. Taken together, these data strengthen previous conclusions that the presynaptic complex is primed for self-assembly once presented with appropriate triggering signals in a permissible environment (Burry, 1982; Phillips et al., 2001).

EXPERIMENTAL PROCEDURES

Neuronal Culture

All animal experimentation was approved by the institutional animal care committee and conformed to the guidelines of the Canadian Council of Animal Care. All culture media was purchased from Gibco® (Invitrogen, Burlington, Ontario, Canada). Hippocampi were dissected from embryonic day 17-18 (E17-18) Sprague-Dawley rat embryos (Charles River, Quebec, Canada) as described (Benson and Tanaka, 1998). For immunocytochemistry, cells were plated at a density of 2.0-2.5 x 10^4 cm^{-1} on PDL- (Sigma-Aldrich, Oakville, Canada) coated glass coverslips. For electron microscopy, cells were plated on PDL-treated 4-well plates (Nunc©, VWR, Mississauga, Canada) at a density of 5.0 x 10^4 cells per well. All cells were cultured in serum-free Neurobasal medium supplemented with L-glutamine and B-27. Transfection of plasmid DNA was performed using Lipofectamine (Invitrogen) according to the company protocol and transfected cells were incubated for a minimum of 48h prior to experimentation. Mouse synaptophysin-EGFP plasmid was a gift of Dr. Edward Ruthazer (McGill University), and pEGFP-C1 plasmid was purchased from Clontech (Mountain View, CA).

Lentiviral-infected hippocampal cultures were prepared using a modified Banker-style protocol (Banker and Goslin, 1998) and infected as described (Leal-Ortiz et al., 2008). Three constructs were used for these studies. These included EGFP-tagged versions of SV2, alpha-SAP97 and a mCherry-tagged version of beta-actin. In the case of SV2 and alpha-SAP97, the EGFP-fusion protein was expressed under the ubiquitin promoter from with the FUGW lentiviral vector as previously described (Schluter et al., 2006; Leal-Ortiz et al., 2008; Waites et al., 2009). With regard to the mCherry-beta-Actin construct, the CMV-enhancer/chicken beta-actin/beta-globin intron (CAG) promoter driving transgene expression in the lentiviral expression vector pLL4.4 (Kwiatkowski et al., 2007b; Kwiatkowski et al., 2009) was replaced with a ubiquitin promoter, creating pLL4.5. The EGFP expression cassette in pLL4.5 was excised and replaced with a

fusion between mCherry and human beta-actin (gift of Frank Gertler, Massachusetts Institute of Technology), creating pLL4.5 mCherry-beta-actin.

Preparation of micropatterned glass substrates

Glass substrates were patterned with polylysine using custom-made silicone elastomer stamps. To facilitate transfer of polylysine to the glass, coverslips were first surface-treated with 1% 3-glycidoxypropyl-trimethoxysilane (3-GPS, Gelest, Morrisville, PA) in toluene for 1h, transferred to a beaker containing fresh toluene and rinsed several times. Coverslips were then dried with nitrogen steam and autoclaved.

Polydimethylsiloxane (PDMS) was prepared from the Sylgard 184 Silicone elastomer kit (Dow Corning, Midland, MI) according to company protocols and poured onto a master silicone wafer etched with the following grid-like dimensions: 10μm-wide lines spaced 420μm apart with 20μm circles centered on line intersections. The PDMS was left standing on the wafer for 1h at room temperature followed by curing at 60°C for 12h, then carefully peeled off of the wafer.

For stamping coverslips, the molded PDMS stamp was first sterilized under UV light, soaked in 70% ethanol for 1 minute, then dried using nitrogen gas. The stamp was placed in a solution containing poly-D-lysine (1mg/ml in phosphate-buffered saline, pH 7.4 (PBS)) for 3 hours, dried with nitrogen gas, then applied to the treated coverslips with gentle pressure for several seconds. The coverslips were then rinsed several times with sterile water, placed into culture dishes and used for plating neurons as described above. Cultures grown on these substrates were incubated with poly-D-lysine coated beads after 7 days *in vitro* (DIV).

Preparation of polylysine-coated beads

Neurons were cultured to various stages of development (between 7 and 21DIV) before the addition of beads. For coating with poly-D-lysine (PDL) or poly-L-lysine-FITC (both

Sigma), 7μm polystyrene beads (Bangs Laboratories, Fishers, IN) were incubated with a solution of either polymer in sterile PBS (50μg/mL unless otherwise stated), overnight at 4°C with end-to-end mixing. Beads were then washed 3X in PBS by centrifugation, resuspended in Neurobasal medium, and added dropwise to the neurons at a concentration of 10^5-1.5 x 10^5 beads/coverslip. Uncoated beads were washed in PBS alone, and added to the neurons at a density 3-4 times that of PDL-coated beads to account for the increased proportion of beads that did not adhere to the cultures.

Preparation of lipid bilayer-coated beads

5μm silica beads (Bangs Laboratories) were diluted to a concentration of 9 million beads/mL in PBS, washed twice in PBS by centrifugation, then resuspended and incubated in 1mL of PBS containing 0.05mg/mL avidin overnight at 4°C. The avidin-treated beads were then washed several times and resuspended in a final volume of 500μL PBS prior to incubation with the lipids.

The following lipids purchased from Avanti Polar Lipids (Alabaster, AL) were used for the preparation of the bilayer membrane: 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), 1,2-Dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and 1,2-Distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[biotinyl (polyethyleneglycol)2000] (ammonium salt) (DSPE-PEG2000-biotin). Chloroform solutions of DOPC (1.5 mM, 75 μL), DOTAP (1.5 mM, 25 μL), and DSPE-PEG2000-biotin (0.6 mM, 5 μL) were mixed and dried in vacuum for 4h under sterile conditions. The film was then hydrated using 0.1M sucrose through rapid mixing followed by sonication in a bath sonicator for 5 minutes, which results in the formation of small unilamellar vesicles.

500μL of the vesicle solution was then mixed with 500μL of the avidin-coated silica beads, shaken gently and incubated for 10 minutes. The bead-vesicle solution was then mixed vigorously and sonicated for 1 minute followed by centrifugation (12,000rpm for

13 minutes) and the resulting pellet was then resuspended in PBS. The bilayer-coated beads were used within 1 week of preparation and added to cultures as described above.

Drug treatments

In cultures where inhibitors of HSPGs were added, heparinase (III or II), heparan sulfate (all Sigma) or heparin (Organon, Toronto, Canada) were added immediately prior to the addition of PDL-coated beads. Heparinase was diluted in Neurobasal medium and added to cells for a final concentration of 1-1.25 Sigma units/mL while heparan sulfate or heparin was diluted to concentration of either 1μg/mL or 20μg/mL. In cultures where inhibitors of actin were added, jasplakinolide (Molecular Probes, Burlington, Canada) and latrunculin A (Sigma) were diluted from a 1000X stock in DMSO (Invitrogen) to a final concentration of 5μM in Neurobasal medium and added to DIV13-15 cultures immediately prior to the addition of PDL-coated beads.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma) in phosphate buffer (PB), pH 7.4, for 25min, incubated in blocking solution (Tris-buffered saline, pH 7.4 (TBS), containing 4% normal donkey serum and 0.1% Triton-X) for 30min, followed by incubation in primary antibodies diluted in TBS containing 0.1% Triton-X and 0.5% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA), overnight at 4°C with gentle rotation. Cells were washed in TBS, incubated in fluorochrome-coupled secondary antibodies (in TBS-0.5% NDS), washed 3X in TBS, and mounted on glass slides. Primary antibodies used for our studies include rabbit polyclonal anti-Synaptophysin (Invitrogen), mouse monoclonal anti-Bassoon (Assay Designs, Ann Arbor, MI), rabbit polyclonal anti-CaV2.2 (Ab571, gift of Dr. E. Stanley, University of Toronto), mouse monoclonal anti-RIM (BD Biosciences, Mississauga, Canada), guinea pig polyclonal VGlut1 (Chemicon, Temecula, CA), rabbit polyclonal anti-Glutamate Decarboxylase 65/67 (GAD65/67)(Chemicon), mouse monoclonal anti-PSD95 (Affinity Bioreagents, Golden, CO), rabbit polyclonal anti-N-cadherin (Fannon and Colman, 1996), mouse monoclonal anti-Tau1 (Chemicon), chicken polyclonal anti-MAP2 (GeneTex, Irvine,

CA), goat polyclonal anti-syndecan-2 (Santa Cruz), mouse monoclonal anti-heparan sulfate 10E4 (Seikagaku, Japan), and mouse monoclonal anti-β-Tubulin (Developmental Studies Hybridoma Bank, see acknowledgements). All secondary antibodies (species-specific, highly cross-adsorbed IgG) were purchased from either Jackson Immunoresearch or Molecular Probes and used at a dilution of 1:200-1:500. For cultures that were probed for actin labeling, we added Alexa 488-phalloidin (Molecular Probes) to the secondary antibody solution at a concentration of 1:50.

Confocal microscopy of antibody-labeled cultures

Fixed coverslips were imaged using an Olympus (Tokyo, Japan) Fluoview FV1000 laser scanning confocal microscope with a 60x PlanApo oil immersion objective [1.4 numerical aperture (NA)] on an IX81 inverted microscope. Images of single optical sections through the neuritic plane were acquired with 1x digital zoom and 4x Kalman averaging. At least 10 separate images were taken for each condition. For double or triple immunostaining, images were acquired via sequential scanning of each individual channel along with the corresponding brightfield (differential interference contrast, or DIC image). For each coverslip, optimal parameters were adjusted manually to avoid image saturation.

Electron Microscopy (EM)

Unless otherwise stated, all EM reagents purchased from Cedarlane (Burlington, Canada). Cells were grown directly on tissue culture plastic (PDL-coated 4-well plates, Nunc) to DIV14-15, and following incubation with beads for 24h were fixed with 2% glutaraldehyde-2% PFA (Sigma) for 30min. For transmission electron microscopy, cells were then post-fixed with 1-2% OsO_4, dehydrated in graded alcohols, embedded in epon, ultrathin-sectioned (80nm thick), counterstained with uranyl acetate and lead citrate, and examined using a JEOL (Tokyo, Japan) 100CX transmission electron microscope set to 80kV voltage. For scanning electron microscopy, cells were gently removed from their wells via gentle scraping and placed directly on copper grids, dried and examined using a Hitachi S-4700 Field Emission scanning electron microscope set between 1-3kV voltage.

Combined fluorescence and Atomic Force Microscopy (AFM)

For these experiments, hippocampal neurons were cultured in 35mm glass-bottom dishes (MatTek, Ashland, MA) coated with PDL. Cells were transfected with mouse synaptophysin-EGFP at DIV6 and incubated for 2-4 days prior to experimentation. 7μm polystyrene beads were attached to an AFM cantilever tip (Veeco, model: MSCT-AUHW, Camarillo, CA) with an UV-curable adhesive (Electro-lite, Danbury, CT). The adhesive was applied to the cantilever tip using a pulled glass capillary (World Precision Instruments, Sarasota, FL) mounted on a micromanipulator stage (MX7600R, Syskiyou, Grants Pass, OR). Beads were then picked up with the glued cantilever (mounted in the AFM) from a microscope slide. For the PDL-coated bead experiments, the beaded cantilever was then incubated in 50μg/mL PDL solution overnight at 4°C.

Simultaneous adhesion and live imaging experiments were carried out on a Bioscope AFM (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA), using a 100X objective (1.45 NA) and Cascade:1k CCD Camera (Photometrics, Tuscon, AZ). Cells were mounted onto a heated stage (Warner Instruments, Hamden, CT) and kept at 37°C for the duration of the experiments (two to three hours). Here the AFM function was used to position and move the beaded cantilever with sub-micrometer precision.

Live Imaging Experiments using Lentiviral-Infected Neurons

These experiments were performed on a custom-built imaging system comprised of a spinning disc confocal head (Perkin Elmer, Fremont, CA), a Zeiss Axiovert 200M microscope, an Argon/Krypton laser (SpectraPhysics, Irvine, CA) driven by Metamorph software (Molecular Devices, Sunnyvale, CA). Neuronal coverslips were mounted in a custom-built chamber designed for closed perfusion, heated to 37°C by forced-air blower. Images were collected with a Zeiss 63x PlanNeofluar oil immersion objective (NA 1.4) and a Hamamatsu 512B CCD camera using FITC and Texas Red filter sets (Chroma, VT).

For the time lapse imaging experiments, cells were mounted onto the microscope stage and perfused with a specially prepared medium (Air medium) containing 25mM HEPES, B-27, GlutaMAX, penicillin/streptomycin, 5mM D-glucose, and 25μM beta-mercaptoethanol in a 1:1 mixture of L-15 and Hanks' balanced solutions. D-glucose and beta-mercaptoethanol are from Sigma and all other reagents are from Invitrogen.

Frames were acquired sequentially with laser intensity kept low to avoid photobleaching and laser-induced toxicity. For each movie, several frames were acquired prior to the addition of PDL beads, which were added thereafter by resuspension in Air medium and perfusion over the cells. The beads were allowed to settle and then time-lapse imaging was quickly resumed within 3min of their addition. Individual frames were acquired as stacks of 7-0.5μm sections per field at intervals of 30sec for the first hour, followed by variable intervals of 1-3min for longer movies.

Styryl FM 4-64 dye Imaging

For experiments using non-infected cultures, cells were plated on 42mm glass coverslips (Hemogenix, Colorado Springs, CO) at a density of 400,000/coverslip and cultured for 12-15 days prior to the addition of PDL-coated beads for 24h. Hank's buffered saline solution (HBSS, Invitrogen) containing Ca^{++} (1.26mM), Mg^{++} (0.9mM) and D-Glucose (5.6mM) was used in all experiments. Hyperkalemic solutions were prepared by addition of KCl for a final K^{+} concentration of 45mM. For fluorescence imaging, we used a Zeiss LSM 510 META laser scanning confocal microscope with a 63x PlanNeofluar oil immersion objective [1.4 numerical aperture] on a Zeiss Axiovert 100M inverted microscope. Laser intensity was set to 25% of the maximum to avoid photobleaching and toxicity. Imaging parameters were kept constant throughout all experimental sessions.

Coverslips were mounted onto a heated stage, rinsed 1X with warmed HBSS then depolarized in hyperkalemic HBSS containing 15μM FM 4-64 (Molecular Probes) for 90sec followed by regular HBSS containing 15μM FM 4-64 for 2 minutes. Cells were then washed several times with regular HBSS containing 1mg/mL Advasep (Cydex,

Lenexa, KS) and allowed to rest 5min in HBSS-Advasep during which the first series of images were acquired. The HBSS-Advasep was then removed and replaced with hyperkalemic HBSS for 90 seconds to facilitate destaining of hippocampal terminals. Cells were washed with plain HBSS several times and imaged once again.

For experiments using EGFP-SV2-infected neurons, cells were first incubated with beads for defined time points (1h and 24h shown here) in glial-conditioned medium at 37°C, 5% CO_2, then mounted onto the microscope stage in Air medium for selection of EGFP-SV2-positive bead sites. We used a motorized stage to sample from several different sites on a single coverslip.

EGFP-SV2-positive presynaptic boutons were labeled with the FM4-64 dye by perfusion first with Tyrode's saline solution (Leal-Ortiz et al., 2008), followed by incubation in high K+ Tyrode's solution (Tyrode's + 90 mM KCl, 31.5 mM NaCl) containing ~1μg/mL FM dye for 60 sec, followed by Tyrode's + FM dye for 30 sec. Neurons were then washed for 5 min in normal Tyrode's before imaging. Destaining was performed by perfusion with high K+ Tyrode's for 60sec followed by a 2min washout in normal Tyrode's prior to acquisition of the destained images.

Image quantification and analysis

Quantification of immunocytochemistry and colocalization was performed using NIH ImageJ software. All quantifications were calculated for at least 30 beads per condition per experiment and averaged across a minimum of 3 separate experiments per condition. All brackets beneath histograms show the total number of beads analyzed. Values in histograms are always expressed as mean ± SEM.

To quantify the recruitment of synaptic proteins under the beads, the brightfield (DIC) image was used to locate and highlight each bead contacting a neurite, and for each bead, an adjacent equal-sized area was highlighted to act as a control. Great care was taken to ensure that the adjacent site contained a similar density of neurites with respect to the

corresponding bead site to be analyzed. Beads contacting cell bodies were not included in the analysis. Fluorescence was quantified by measuring either the (i) fluorescence intensity at each bead and corresponding adjacent contact or (ii) the proportion of labeled pixels at each bead and adjacent contact following careful thresholding. Values were expressed as bead/adjacent fluorescence intensity ratios or fluorescence ratios, respectively. For the colocalization analysis (Figure 1G), we used the Intensity Correlation Analysis (ICA) plugin within the NIH ImageJ program (Li et al., 2004).

Quantification of the lentiviral-infected cultures imaged by live time lapse was performed using Metamorph software (Molecular Devices). To measure changes in fluorescence intensity, we performed line-scans of axonal/dendritic profiles with or without beads and measured their fluorescence intensities for each time point acquired. All individual values were thereafter normalized to the average baseline fluorescence intensity values for each site (prior to the addition of the beads), plotted and statistically analyzed.

All images were processed and prepared for print using Photoshop (Adobe, San Jose, CA).

Statistics

All statistics were performed and data graphed using GraphPad Prism software (San Diego, CA). For comparisons of fluorescence changes between two groups, we assessed significance using Student's t-test. For comparisons between multiple groups we used one-way ANOVA followed by Bonferroni's *post-hoc* test. To assess changes in fluorescence intensity with time (live imaging data, Figure 5), we performed Two-way ANOVA with time as the repeated measure. All data shown are mean ± SEM. In figures, statistical significance is indicated by (#/n.s.) for $p>0.05$, (*) for $0.05<p<0.01$, (**) for $0.01<p<0.001$, and (***) for $p<0.001$.

RESULTS

(I) PDL-coated beads induce membrane adhesion followed by the sub-plasmalemmal clustering of synaptic vesicle complexes under the adhesion site within axons.

Previous studies (Burry, 1980, 1982; Peng et al., 1987) revealed that cultures of cerebellar granule cells were capable of forming contact-induced clusters of vesicles within 24h on poly-L-lysine (PLL)-coated beads. To further investigate this phenomenon, low-density hippocampal cultures were grown (to 7, 15 or 21 days *in vitro* (DIV)) and thereafter incubated with 7μm polystyrene beads coated with PDL (50μg/mL)(Figure S1). PDL is an isomer of PLL, which like PLL, is typically used to promote *in vitro* adhesion of cells to a substrate. Incubation of these beads with cells for as long as 72h appeared to have no adverse affect on neuron health or on their ability to arborize their axons or dendrites as assessed by either Differential Interference Contrast (DIC) microscopy (Figure 1B, S1) or immunofluorescence with antibodies to neurofilaments (NF-H, see Figure 1B).

When PDL-coated beads were added to cultured hippocampal neurons, we were first struck by how the beads adhered to cell surface membrane domains within minutes, and were resistant to mechanical dislodgement throughout the incubation period. In contrast, uncoated beads mostly remained free in the culture medium even after several hours, and were readily washed away post-fixation. To assess the "attachment phase" more directly, we combined live imaging with atomic force microscopy (AFM). In these experiments, neurons expressing synaptophysin-EGFP [to mark neuronal processes] were exposed to PDL-coated or uncoated beads immobilized on a cantilever attached to a Bioscope-AFM. When an immobilized PDL-coated bead was gently placed against a synaptophysin-EGFP expressing axonal membrane, the bead became resistant to detachment within just a few minutes of contact (data not shown). After a 2h incubation period, moving the cantilever attached to a PDL-coated bead resulted in a corresponding displacement of the axon several microns from the site of the culture dish onto which the axon was attached, thus revealing a strong, grossly observable adhesion event (Figure 1A top, Movie S1). In

contrast, moving the cantilever bound to an uncoated bead resulted in its immediate detachment from the axon even after 30min of contact, revealing no significant adhesive interaction between uncoated beads and axons (Figure 1A bottom, Movie S2).

The ability of the beads to induce clustering of synaptic vesicles (SVs) was assessed by determining the localization pattern of the synaptic vesicle protein synaptophysin. Following a 24h bead incubation, we observed a marked enhancement of synaptophysin immunolabeling at sites of contact between neurites and PDL-coated beads in the vast majority of neurons visualized at all three stages of maturity tested (Figure 1B, Figure S1). Prominent synaptophysin-positive puncta were also found in association with PDL beads following a 72h incubation period, indicating that these bead-induced SV clusters are quite stable (Figure S1).

The morphological features of these presynaptic-bead complexes (following 24h of incubation) were next assessed by electron microscopy. By scanning electron microscopy, we observed dense and profuse neurite extensions onto PDL-coated beads (Figure 1C, top left) but not on uncoated beads (Figure 1C, bottom left). Transmission electron microscopy revealed that processes extending onto PDL-coated beads contained bouton-like swellings filled with 50nm vesicles (Figure 1C, top right), while vesicle clusters were not observed in neurites contacting uncoated beads (Figure 1C, bottom right).

We next evaluated whether SV clusters were derived from the axons of inhibitory and/or excitatory neurons. To this end, cultures were incubated with beads for 24h then immunostained with antibodies against the vesicular glutamate transporter 1 (VGlut1) or glutamic acid decarboxylase (GAD). These experiments revealed that a majority of the SV clusters were VGlut1-positive (~90%), while a smaller proportion were immunopositive for GAD (approx. 40%)(Figure 1D,E). Quantitative analysis revealed that in each case, GAD positive puncta were not VGlut1 immunoreactive, and vice versa, indicating that each arises independently from either GAD or VGlut1 positive axons, respectively (Figure 1D,F). Although there were fewer GAD-positive boutons, where

present, they displayed enhanced clustering to the same degree as VGlut1 (Figure 1G). This result suggests that PDL-coated beads robustly induce the clustering of SVs from both excitatory and inhibitory neurons.

(II) PDL-coated beads induce the formation of functional presynaptic boutons.

Although the SV clustering phenomenon had been described previously (Burry, 1980, 1982; Peng et al., 1987), it was unclear whether or not these bouton-like structures contained active zones indicative of functional *bona fide* presynaptic endings. This was first explored by immunostaining cultures of hippocampal neurons, grown for 15DIV and incubated with beads for 24h, with antibodies against a variety of SV and active zone markers (Figure 2A). In addition to synaptophysin (Fig. 2Ai), we also observed a robust enhancement of immunoreactivity for the active zone proteins bassoon (2Aii), rab3a-interacting molecule (RIM)(2Aiii), the N-type calcium channel CaV2.2 (2Aiv), N-Cadherin (2Av) and F-actin (alexa-phalloidin)(2Avi) on beads coated with PDL but not uncoated beads. There was also a small but significant enhancement of tubulin at PDL-coated but not at uncoated bead contacts, suggesting that PDL-coated beads may induce changes in microtubular organization (Figure 2Avii, and c.f. Dillon, 2005). Alternatively, the accumulation of tubulin at PDL bead sites may be accounted for by the small increase in axonal contact area at PDL-bead but not uncoated bead contacts, as determined from the expression of soluble EGFP along transfected axons (ratios: 2.38±0.48 (PDL)** vs. 1.06±0.08 (uncoated), Figure S2). Although significant, this increase cannot account for the >10-fold increases in the clustering of synaptophysin, RIM and bassoon (Figure 2Ai-iii). Taken together, these data reveal that these axonal swellings contain more than just clusters of SVs, and in fact may be functional presynaptic boutons.

To assess whether these presynaptic-like endings could recycle SVs in an activity-dependent manner, we depolarized neuron-bead cultures (DIV15 + 24h PDL-coated beads) with KCl (45mM) in the presence of the styryl dye FM 4-64 (Ryan et al., 1993; Ryan and Smith, 1995). This treatment led to the appearance of fluorescent puncta along

dendritic profiles, confirming the presence of functional presynaptic boutons at native axo-dendritic contacts. We also observed the appearance of fluorescent puncta along the perimeter of PDL-coated beads in contact with axons (Figure 2B). Exposure to a second depolarizing concentration of KCl, to cause exocytosis of dye-filled SVs, resulted in a significant decrease in the intensity of fluorescent puncta along dendrites as well as neurite-PDL bead contact sites (Figure 2B,C). Together, these data demonstrate that PDL-coated beads induce the formation of functional presynaptic boutons, closely resembling the presynaptic compartments assembled as a result of induction by natural substrates.

(III) PDL induces the formation of presynaptic endings through a mechanism involving heparan sulfate proteoglycans (HSPGs).

PDL is typically used to coat substrates to promote cell adhesion *in vitro* (Yavin and Yavin, 1974). In previous studies, it had been reported that isolated axons grown on a PDL-coated substrate form presynaptic-like structures similar in composition to the bead-presynaptic complexes reported here, but these structures were highly mobile and not thought to be triggered by the substrate itself (Krueger et al., 2003). Although our observations suggest that PDL-coated beads induce highly stable presynaptic complexes irrespective of age or culture density (Figure S1), given that both the dish and beads were coated with PDL could suggest that these two types of structures were somehow related.

To address this question, we devised the following experiment (Figure 3A). Dissociated hippocampal cells were plated on substrates already containing PDL-coated beads and allowed to differentiate in their presence for several days (DIV0 to at least DIV7). Then, we added a second population of beads, this time coated with a FITC-conjugated version of polylysine (PLL-FITC) to allow us to distinguish between the two bead populations. In control experiments, we have found PLL-FITC to be as effective as PDL at inducing presynaptic complexes when either type of bead is added to cultures after DIV7 (data not shown). However in this experimental setup, where beads were added to cultures both

before and after the developmental stage *in vitro* in which they become competent to form synapses, only the PLL-FITC beads induced the formation of presynaptic clusters, while the PDL beads added at DIV0 did not (Figure 3B). This result suggests that neurons must reach a stage of development at which they are competent to form synapses in order for polylysine to behave as a synaptogenic substrate, and prior to this, that PDL behaves primarily as a promoter of axon outgrowth and cell adhesion. Furthermore, we conclude that these presynaptic complexes induced by PDL beads are unique, both functionally and morphologically, compared to the presynaptic-like structures reported previously (Krueger et al., 2003).

PDL is a highly cationic artificial polymer. How might it induce the formation of functional presynaptic compartments? We observed that the accumulation of synaptophysin increases at bead contacts with increasing concentrations of PDL on the bead surface (Figure 3C), clearly indicating that this protein, and not the bead itself, is responsible for the observed effects. However, this clustering effect is markedly reduced in cultures incubated with beads coated with cationic lipids (Figure 3D). To confirm that the concentration of positive charge was the same, we performed a zeta potential analysis to analyze overall surface charge and found no difference between PDL- and lipid-coated beads (50μg/mL PDL beads, 46±3mV vs. lipid beads, 45±mV; data not shown). These results indicate that positive charge alone is insufficient to drive presynaptic assembly, suggesting that the configuration of charge on the PDL surface might be instead the defining characteristic that drives its inductive properties.

We then speculated that perhaps a direct interaction between some extracellular or transmembrane component and the PDL is responsible for presynaptic compartment induction. Although charge alone isn't responsible for its effects, PDL is nevertheless poised to interact with substances that are negatively charged in an electrostatic manner. Heparan sulfate proteoglycans (HSPGs) are a large and heterogeneous family of extracellular and transmembrane molecules which in part comprise the architecture of the extracellular matrix, and a growing body of literature points to a role for these molecules in synapse formation as well as other critical stages of neuronal and synaptic

development, including axon guidance and synaptic plasticity (Rauvala and Peng, 1997; Lauri et al., 1999; Bandtlow and Zimmermann, 2000; Dityatev et al., 2004; Dityatev and Schachner, 2006; Johnson et al., 2006; Van Vactor et al., 2006; Matsumoto et al., 2007). HSPGs are composed of a proteoglycan core post-translationally modified to provide anionic sites for a number of extracellular binding partners (Gallagher et al., 1986). Given that these molecules are heavily negatively charged, they have been predicted to be good substrates for interaction with molecules rich in positively charged residues such as lysines and arginines (Cardin and Weintraub, 1989). Furthermore, it has been shown in heterologous cell lines that short cationic peptides and/or motifs bind to and can drive intracellular signaling through interaction with cell-surface HSPGs (Mislick and Baldeschwieler, 1996; Chang et al., 1997; Nakase et al., 2007). We therefore hypothesized that HSPGs, which are known to be expressed in hippocampal cultures in both transmembrane and secreted form (Dow et al., 1988; Sugiura and Dow, 1994; Hsueh and Sheng, 1999), were potential endogenous targets for interaction with PDL-coated beads.

We next evaluated whether HSPGs direct the formation of presynaptic endings onto PDL-coated beads. In the first of these experiments, neurons grown for 15DIV were incubated with PDL beads for 24h in the presence of heparinase II, an enzyme that degrades HSPGs. Here, we found a dramatic decrease in the intensity of synaptophysin puncta associated with PDL beads (Figure 3E). Similarly, we observed a dose-dependent decrease in the number and intensity of synaptophysin puncta when PDL-beads were added in the presence of 1μg/mL or 20μg/mL heparan sulfate, a condition predicted to compete with the binding of endogenous HSPGs to PDL (Figure 3E, histogram). These treatments did not affect the binding of PDL beads to the axonal surface. Furthermore, we did not find any overall changes in presynaptic puncta size, number or intensity at non-bead sites, suggesting that these treatments did not have any effect on established boutons (Figure S3). Taken together, these data indicate that PDL-coated beads depend on HSPGs to facilitate the contact-mediated *de novo* assembly of presynaptic boutons.

Which HSPGs may be involved in this phenomenon? To address this question, we first asked whether HSPGs themselves could cluster directly onto the beads. Using a pan-Heparan Sulfate antibody that recognizes an epitope present in a variety of HSPGs (10E4 epitope), we found significantly enhanced HS immunofluorescence at PDL bead sites compared to uncoated bead sites (Figure S4A,C). This is consistent with the increase in synaptophysin found exclusively at PDL bead sites (Figure S4B,C). Next, we wished to assess the role of specific HSPGs. We focused our attention on syndecan-2 (syn2), a cell-surface HSPG shown previously to cluster at synapses both pre and postsynaptically (Hsueh et al., 1998; Ethell and Yamaguchi, 1999; Hsueh and Sheng, 1999; Ethell et al., 2001). We found a significant enhancement of syn2 at PDL bead sites within 1h as well as after 24h of contact (Figure 4A,C). When we dually immunostained cultures for syn2 along with synaptophysin, we found that the vast majority of bead sites displayed enhanced co-clustering of both proteins (Figure 4B,C). It should be noted that after 1h, a sizeable proportion of bead sites expressed neither syn2 nor synaptophysin, while a second smaller proportion expressed enhancement of synaptophysin only. This latter population persisted at the 24h time point, suggesting that syn2 does not become clustered in a small proportion of PDL bead sites (Figure 4B). However, after 24h, the proportion of sites that displayed no enhanced clustering was markedly reduced, appearing to be replaced by a larger proportion that was dually enhanced for both proteins. This analysis reveals a correlated accumulation of the cell surface HSPG syndecan-2 and synaptophysin at a majority of PDL bead sites, suggesting that perhaps syn2 is involved in the triggering of PDL bead-induced presynapse formation.

To better address this question, we incubated cultures with PDL beads in the presence of heparinase or 20μg/mL of heparin, which is more heavily sulfated than heparan sulfate but can similarly block interactions between HSPGs and their targets. We found that both treatments abolished the enhanced clustering of both syn2 and synaptophysin after 24h (Figure 4C,D). After 1h of PDL bead contact, we found that heparinase treatment significantly reduced, but did not entirely abolish, the clustering of syn2 and synaptophysin (Figure 4E). Since all the treatments were added at the same time as PDL beads, this may reflect a delay in the enzymatic activity of heparinase relative to the

inductive effects of the beads. However, treatment with heparin was highly effective, further reducing the PDL bead-induced clustering of both syn2 and synaptophysin after 1h down to levels not significantly different from uncoated beads (syndecan-2: 1h heparin, 1.39±0.09 vs. 24h uncoated beads, 1.16±0.07(n.s.); synaptophysin: 1h heparin, 1.35±0.07 vs. 24h uncoated, 1.14±0.11(n.s.), one-way ANOVA)(Figure 4E). Taken together, these results for the first time implicate syn2 as a specific cell-surface HSPG important in triggering associated with presynapse assembly induced by PDL beads.

(IV) Time-resolved determination of presynaptic protein recruitment and functionality at PDL bead sites.

A variety of recent studies show that synapses can form within a few hours of axo-dendritic contact both *in vitro* and *in vivo*. Therefore, our next question was: do PDL-coated beads trigger synaptic assembly in a time frame that is equivalent to that observed at native axo-dendritic contacts? To investigate this, we performed fixed time-course studies whereby neurons were incubated with PDL-coated beads for defined time periods and triple-labeled with antibodies against synaptophysin and bassoon along with alexa-phalloidin to visualize F-actin. A rapid increase in the fluorescence intensity of all three proteins was observed within the first hour of bead contact that continued to the third hour of incubation (Figure 5A,B). Beyond 3h, there was no added enhancement of fluorescence intensity of actin, while the accumulation of bassoon and synaptophysin appeared to continue, albeit modestly when compared to the 3h time point (Figure 5A,B).

Next, we used time-lapse imaging to observe the dynamics of synaptic protein recruitment to beads within the first several hours of contact. To this aim, hippocampal neurons were infected with lentivirus expressing mCherry- or EGFP-tagged synaptic proteins. In our first experiments, we focused on the axonal response of neurons by dually infecting cells with viruses expressing EGFP-tagged SV2 (EGFP-SV2) and mCherry-tagged beta-actin (mCh-actin). Here, we observed a striking reorganization of mCh-actin that occurs within the first several minutes of bead contact (Figure 5C, S6; Movie S3). The mCh-actin appears very dynamic as if it is part of a filopodial-like

process that is exploring the surface of the bead (see t=30min time point in Figure 5C). Intriguingly, we noticed that the accumulation of EGFP-SV2 is similarly rapid and appears to coincide closely with the observed reorganization of actin at bead sites (Figure 5C, bottom panel) and once recruited, appeared remarkably stable throughout the remainder of the time course (Figure 5D).

To quantify these data, we first analyzed the changes in fluorescence intensity at bead sites well as a separate, adjacent control axon for every bead site. We found a significant increase in the fluorescence intensity of both mCh-actin and EGFP-SV2 over the 60min imaging period (Figures 5E,F). In contrast, there was no significant accumulation of either mCh-actin or EGFP-SV2 at control nonbead sites (Figures 5E,F).

Next, to determine the precise temporal pattern of actin and SV2 accumulation, we performed a 2-way analysis of variance (ANOVA) with one repeated measure (time). For mCh-actin, accumulation became significant at t=28min compared to the nonbead controls (Figure 5E). This increase in mCh-actin fluorescence intensity, although remaining elevated throughout the remainder of the time lapse, was no longer significant after t=55min (Figure 5E), coinciding with the appearance of a stable but condensed focal site of mCh-actin (see Figure 5C, t=60min).

When we applied this same analysis for EGFP-SV2, we found that accumulation became significant at t=30min, just 2min later than mCh-actin (Figure 5F). However, unlike mCh-actin, this increase in intensity remained significant relative to nonbead control sites for all time points after t=30min (Figure 5F). Taken together, these data suggest that there is a close temporal relationship between actin dynamics and synaptic vesicle accumulation during presynapse assembly triggered by PDL-coated beads, whereby initial contact triggers a reorganization of the axonal actin cytoskeleton that is closely followed by the trapping of synaptic vesicles. To confirm that actin is not just temporally linked but is in fact critical for synaptic vesicle accumulation, we performed experiments whereby PDL-coated beads were added to hippocampal cultures (13-15DIV) along with disruptors of the actin cytoskeleton and incubated for 3h prior to fixation. At this

developmental stage *in vitro*, it has been shown that preexisting clusters of synaptic vesicles were unaffected by actin depolymerization (Zhang and Benson, 2001). However, in the presence of (i) Latrunculin A (LatA) or (ii) Jasplakinolide (Jas), toxins which (i) sequester actin monomers, thereby inhibiting F-actin formation or (ii) promote the stabilization of F-actin, respectively (Spector et al., 1999), we found that synaptophysin accumulation was nearly abolished at PDL bead sites (Figure 5G,H). These data reveal that actin reorganization a critical step in nascent presynapse assembly independent of neuronal age, adding to a number of studies showing that dynamic actin reorganization is an important property of axons during nascent presynapse assembly (Colicos et al., 2001; Sankaranarayanan et al., 2003a; Dillon and Goda, 2005; Cingolani and Goda, 2008).

Finally, we wished to confirm that these nascent sites could recycle transmitter. To address this question, we performed experiments using cultures singly infected with EGFP-SV2, and incubated the cultures with PDL-coated beads for 1h or 24h. This allowed us to visualize the formation of nascent presynaptic boutons at bead sites prior to stimulation, and to select for bead sites containing SV clusters. We then monitored the ability of these boutons to recycle the vital dye FM4-64. After 1h of incubation with PDL-coated beads, we observed bright EGFP-SV2-positive clusters outlining the bead perimeter at a subset of bead contacts, in both young (Figure S5A) and more mature (Figure S5E) cultures. After 24h, most bead contacts contained multiple EGFP-SV2-positive clusters (Figure S5C,G). When stimulated by a high K+ solution in the presence of the FM dye, we observed robust uptake of the FM dye at these sites (Figure S5A,C,E,G; 1X KCl panel) that was unloaded following a 2nd stimulation (Figure S5A,C,E,G, 2X KCl panel). Following quantification, we found that the unloading of the FM dye was significant at all time points tested (Figure S5B,D,F,H). These results confirm that presynaptic functionality can be achieved rapidly at nascent presynaptic endings formed at PDL-bead contacts, as has been observed between native contacts *in situ* (Ahmari et al., 2000; Friedman et al., 2000).

(V) PDL-coated beads facilitate postsynaptic differentiation that is delayed and dependent on the presence of presynaptic clusters.

It is clear both from previous work and our data that substrate-bound polylysine can induce presynaptic assembly; however, whether these beads can induce postsynaptic assembly in central neurons is unclear. To investigate the capacity of PDL beads to drive postsynaptic development, we first performed live imaging studies using cultures dually infected with the postsynaptic protein SAP97-EGFP along with mCh-actin. By visual inspection, the PDL beads adhered to dendrites with similar speed and resistance to dislodgement as was observed along axons. Furthermore, similar to our observations of PDL beads contacting axons, we also observed an accumulation of mCh-actin at sites of dendritic contact (Figure 6A,B, S6; Movie S4). In contrast, there was no net change in mCh-Actin fluorescence intensity at adjacent control sites (Figure 6C). When analyzed by 2-way ANOVA, this dendritic accumulation of mCh-actin appeared to follow a similar time course as axonal mCh-actin, as the accumulation of actin became significant at t=30.5min and remained so throughout the remainder of the time course (Figure 6C). However, we did not observe any accumulation of SAP97-EGFP-positive puncta at any time during imaging, even after several hours (Figure 6A,B,D; Movie S4). These latter results show that while beads can adhere to dendrites, and can induce actin reorganization that appears to be similar to that seen along axons, this does not lead to a postsynaptic differentiation on a time frame in which presynaptic differentiation may be observed along axons.

We hypothesized that perhaps postsynaptic protein accumulation proceeds in response to PDL beads, but is simply delayed relative to presynaptic differentiation, as has been reported previously (Friedman et al., 2000; Okabe et al., 2001). To address this question, we next performed co-immunofluorescence experiments of synaptophysin with PSD95, a marker of the postsynaptic density, following 24h of co-culture of DIV15 neurons with PDL beads. When we quantified this data we found that, like synaptophysin, PSD95 immunofluorescence is also enhanced at PDL bead sites, albeit to a significantly lower degree (Figure 6E, Table 1). Closer inspection of the data, however, revealed some interesting trends.

Where beads contacted few thin-diameter processes located distally to the cell body, indicative of axons, it appeared that only synaptophysin clustering is induced (Figure 6E, Bead 7). When beads contacted neurites closer to the cell body, presumably where there is a higher density of dendrites, PSD95 clusters were also observed (Figure 6E, Bead 1). To investigate this further, we classified the fluorescence intensity ratios into the following categories: where both synaptophysin and PSD95 were increased >2-fold, where synaptophysin alone or PSD95 alone were increased >2-fold, and where neither were increased. We found that approximately 38% of the contacts displayed dually enhanced synaptophysin and PSD95 labeling, but an even greater proportion (45.4%) of the contacts displayed enhanced synaptophysin clustering in the absence of enhanced PSD95 clustering (Table 1). In contrast, the reverse (enhanced PSD95 without enhanced synaptophysin) virtually never happened (3/207 PDL-coated beads)(Table 1). These results suggest that perhaps postsynaptic differentiation may indeed be induced at sites of bead contact, but depends on the presence of differentiated presynaptic boutons.

(VI) Interdependence of pre- and postsynaptic elements on cognate synaptic development.

Previous studies have shown that the induction of pre or postsynaptic differentiation can be achieved by the expression of subsets of synaptic adhesion molecules such as neuroligin, synCAM, or neurexin on the surface of heterologous cells in the place of native neuronal membranes (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004; Sara et al., 2005; Kim et al., 2006). These molecules are part of a growing family of synaptic recognition molecules that are thought to behave as the earliest molecular triggers of synaptic development. However, the beads used in the present study were not coated with any of these or other native molecules shown *in vitro* to drive presynaptic or postsynaptic assembly, suggesting that PDL-coated beads bypass these cognate recognition cues. To rule out the possibility that PDL bead-induced presynapse assembly relied on cues derived from nearby or contacting postsynaptic membranes, we grew hippocampal neurons on a micropatterned substrate, which facilitates the separation of

axons from dendrites by encouraging growth along defined linear pathways. In these cultures, only isolated tau1-positive axons contacting PDL-coated beads displayed robust synaptophysin clustering, while beads contacting isolated MAP2-positive dendrites did not (Figure 7A,B). This result confirms that PDL-coated beads exclusively induce SV clustering on axons and do not require the presence of dendritic membranes to do so.

The intriguing converse question to the above experiments is whether isolated postsynaptic boutons could be induced to form by PDL-coated beads, or whether differentiation of a postsynapse is dependent on presynapse assembly. Although it is generally difficult to separate axons from dendrites at stages of neuronal differentiation when postsynaptic protein clusters are forming (9DIV and beyond), we were able to observe several examples of beads contacting isolated dendrites expressing SAP97-EGFP. After 1h of incubation with PDL-coated beads, we did not observe appreciable clustering of SAP97-EGFP at sites of bead contact (Figure 7C, circles), consistent with our time lapse imaging data (Figure 6A-D). After 24h of bead contact, we did observe robust clustering of SAP97-EGFP only at sites where beads contacted several processes, presumably mixed tracts of axons and dendrites (Figure 7D, arrowheads). These data are consistent with our previous PSD95 data (Figure 6E). However, even at this 24h timepoint, there was no enhanced clustering at bead sites contacting isolated SAP97-positive processes (Figure 7D, circles). Taken together, these results clearly show that PDL-coated beads possess the capacity to bypass native cognate interactions that normally trigger presynaptic development, effectively substituting for the postsynaptic ending. In contrast, this type of interaction between bead and dendritic membrane is insufficient to drive postsynaptic assembly, and lends strength to our assertion that postsynaptic development observed at bead sites is not driven by the bead but by the presence of the presynaptic ending.

DISCUSSION

This work broadly extends some early observations revealing that axons have the remarkable capacity to form presynaptic compartments onto artificial substrates. By EM, prior studies characterized the SV clustering phenomenon in detail (Burry, 1980a; Burry, 1982b; Burry, 1983, 1985; Peng et al., 1987). Because substrates were necessarily polybasic for the clustering effect to occur, it was concluded that electrostatic-based adhesion between an axon and its target represents the first step that triggers synapse assembly (Burry, 1980). While intriguing, these studies did not sufficiently define the early assembly steps in order to place adhesion within a logical sequence of events. Furthermore, it was never clear how these artificial substrates interacted with neuronal membranes to trigger SV clustering. Finally, these studies did not determine whether the observed SV clusters represented true presynaptic boutons, nor did they define the potential of these substrates to induce postsynaptic development. In the present work, we sought to revive an old experimental approach in order to reveal novel facets of the synaptogenic process. Our most significant findings are as follows.

Adhesion as a first step

First, we confirm that adhesion rather than synaptic protein clustering is indeed the first step towards building a functional, stable presynapse. Using a combination of live imaging and atomic force microscopy, we show that PDL-coated beads are resistant to detachment well in advance of any synaptic protein accumulation. We find that SV clusters along axons are highly mobile unless and until they encounter a bead, shortly after which they stabilize and intensify with time. Furthermore, SV clusters could be recruited to bead sites placed anywhere along the length of the axon, suggesting that synapse assembly *in situ* does not necessarily take place at sites of preexisting presynaptic clusters, but rather relies on target adhesion to trigger synaptic protein recruitment.

Role of charge distribution and recruitment of endogenous factors

Our observations distinguish not just charge (Burry, 1980) but presentation of charge as critical for triggering presynapse assembly. We find that cationic polypeptides immobilized on the bead surface selectively provide a platform that is both adhesive and synaptically inductive, while positively charged lipids do not. In addition, we identify HSPGs, including the membrane spanning HSPG syndecan-2, as an important class of ECM molecules driving PDL bead-induced synapse formation. HSPGs are a family of molecules with enormous structural diversity and thus are rather intimidating to study. Yet, their ubiquitous expression, anionic structure and capacity to act as critical co-receptors for several growth factor molecules make them uniquely poised to participate in a number of cell biological processes. In the CNS, this family of molecules is becoming increasingly linked to diverse neurodevelopmental processes including neuronal proliferation, differentiation and neurite outgrowth (Bandtlow and Zimmermann, 2000; Kleene and Schachner, 2004; de Wit and Verhaagen, 2007). In the present study, we further highlight their role in synaptogenic processes by identifying them as critical mediators in PDL bead-induced synapse assembly and identify a specific cell surface molecule, syndecan2, as at least one important molecular subtype involved in these effects. Furthermore, the structure and localization of syn2 at the cell surface suggests a mechanism whereby the bead binds directly to axonal membranes and their receptors, thereby activating signaling processes that lead to presynapse assembly (Figure 8A). Yet, at the moment we cannot rule out the possibility that other cell surface or secreted HSPG subtypes are also involved, possibly by adsorbing onto the bead surface and further recruiting other necessary soluble components (Figure 8A). It will be of great interest in the future to identify other molecular mediators that can interact in a heterophilic manner with PDL beads. Nevertheless, these findings underscore an important role for the ECM as an adhesive support and a scaffolding structure that can promote synaptogenesis.

Presynaptic complexes are true boutons, not mere synaptic vesicle clusters

The ensuing presynaptic specializations formed between neuronal processes and PDL-coated beads are more than simple SV-filled varicosities (Burry, 1980b, 1982a; Peng et al., 1987), but functional excitatory or inhibitory presynapses. These bead-presynaptic complexes are highly stable, unlike the mobile presynaptic-like clusters observed along isolated axons (Krueger et al., 2003). They contain presynaptic proteins in their correct dispositions, depend on F-actin reorganization for their assembly, and are capable of the uptake and release of the styryl dye FM4-64 as quickly as within 1h following PDL-bead contact. When considered in the context of other studies on presynaptic development (Ahmari et al., 2000; Friedman et al., 2000; Jontes et al., 2000; Shapira et al., 2003; Tsuriel et al., 2006), these observations strongly suggest that the assembly process triggered by PDL-coated beads is highly similar to that which occurs *in situ*.

Postsynaptic development only driven by the nascent presynapse

In contrast to the readiness by which the presynaptic ending forms to the bead, the bead does not act as a postsynaptic inducer (Figure 8B). Where postsynaptic differentiation was observed, it was nearly always in relation to already formed presynaptic structures on the bead, suggesting that the nascent presynapse is critical in directing postsynaptic differentiation in this system (Figure 8B). The mechanism by which this takes place is unclear but could involve the encouragement of selective interactions between cognate pre- and postsynaptic molecules beneath the bead, a fitting explanation given that in other studies of postsynapse formation *in vitro*, the presynaptic membrane could only be replaced by endogenous synaptogenic inducers (Graf et al., 2004; Kim et al., 2006; Ko et al., 2006). Although synaptogenic inducers derived from postsynaptic membranes can also drive presynaptic development (Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003; Kayser et al., 2006; Dalva et al., 2007), our system shows that presynapse formation can proceed in the absence of the postsynaptic membrane on which they are expressed.

Permissive vs. inductive: What is necessary to trigger the formation of a synaptic ending?

Following his groundbreaking observations that transected optic nerve axons can regrow to their target destinations in the tectum, Sperry hypothesized that the establishment of neuronal circuitry is critically dependent on the expression of selective cytochemical markers at the appropriate location and concentration (Sperry, 1963). In the context of synapse formation, a growing consensus in the field is that cell adhesion molecules (CAMs), several classes of which are enriched at central synapses (Gerrow et al., 2006; Dalva et al., 2007; Shapiro et al., 2007), act as the molecular tags which correctly identify synaptic partners to each other, and provide the adhesive substrate to lock in synaptic membranes. Other molecules also demonstrated to be synapse-inducing fall into a variety of categories including soluble growth factors (Hall et al., 2000; Withers et al., 2000; Umemori et al., 2004; Ledda et al., 2007), extracellular matrix factors (Noakes et al., 1995; Gautam et al., 1996; Fox et al., 2007; Bogdanik et al., 2008; Sato et al., 2008), and astrocyte-derived molecules (Ullian et al., 2001; Christopherson et al., 2005).

The molecular diversity and selective interactions of these molecules satisfies the specificity that is required in accordance with Sperry's hypothesis. However, one must address the concept of synaptic molecules being permissive versus those that are truly inductive, a controversy that arises when one examines the published data focused on certain adhesion molecules. Despite clear evidence that they can induce synaptogenesis *in vitro* (Scheiffele et al., 2000; Biederer et al., 2002; Dean et al., 2003; Graf et al., 2004; Kayser et al., 2006), and that altering their expression levels alters the number of synapses *in vitro* (Ethell et al., 2001; Chih et al., 2005; Sara et al., 2005), their genetic deletion *in vivo* does not dissolve synaptogenesis (Henkemeyer et al., 2003a; Missler et al., 2003; Fugita et al., 2006; van der Weyden et al., 2006; Varoqueaux et al., 2006; Dudanova et al., 2007). This would suggest that *in vivo*, synaptic CAMs most critically function at later stages of maturation rather than synaptic induction.

Another issue relates to whether or not synaptic inducers act at within defined time frames during nascent synaptogenesis. This has been raised in a recent study at the NMJ,

in which it was shown that agrin, FGF, laminin-β2, and distinct collagen IV chains, which could all be classified as synaptic inducers, were expressed at discrete times during development and regulated distinct aspects of synapse formation *in situ* (Fox et al., 2007). Thus, a major challenge in the field will be to classify chemotactic synaptogenic molecules in accordance with the complexity present within the nascent synaptic environment.

Finally, given the extraordinary production of synapses during the late gestational and early postnatal periods of mammalian brain development, many of which are eliminated at later stages, it has been hypothesized that initial synaptic connections lack precision and refinement of preexisting connections that are initially formed promiscuously lead to the establishment of mature neuronal circuits (Changeux and Danchin, 1976; Katz and Shatz, 1996; Webb et al., 2001; Jontes and Phillips, 2006; Zhang, 2006). These *in vivo* observations have not been complemented by current cellular models and yet if true, suggest that a high degree of specificity is not required to trigger synapses to form in the earliest stages.

The surprising ease with which the presynaptic compartment self-assembles in our system therefore underscores an underestimated capacity for neurons to form rather indiscriminate synaptic connections. Our observations are unlikely to be an artifact of culture, but support a large body of literature demonstrating that axons possess the capacity to drive synaptogenesis following contact with a variety of unnatural targets (Schacher et al., 1985; Sur et al., 1988; Haydon and Zoran, 1989; Zwimpfer et al., 1992), some of which were shown to be functional in behavioral assays (Jacobson and Baker, 1968). While the identification of cell-surface and possibly secreted HSPGs does suggest that the bead requires certain endogenous, and thus specific, factors to drive presynapse assembly, we nevertheless conclude that point-to-point specificity between natural targets is not necessarily required. Instead, such specificity could arise during later stages of synaptogenesis, such as following strengthening by activity- and experience-dependent mechanisms (Changeux and Danchin, 1976; Jontes and Phillips, 2006). A developmental strategy whereby more generalized requirements are needed to form

synapses could ensure that sufficient numbers of synapses are formed during development, when many more synapses are formed than retained en route to the formation of mature neuronal circuits (Katz and Shatz, 1996).

Conclusions

In the present study, we have shown that a simple system of cultured neurons and PDL-coated beads can be ideally suited to probe the earliest events by which nascent synapses form. This system, that can define the temporal appearance of synaptic molecules with precise spatial control will likely be useful in many types of studies of synaptic development.

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FIGURE LEGENDS

Figure 1. PDL-coated beads induce the formation of adherent synaptic vesicle complexes on axons.

(A) Bioscope-AFM images of axon-bead contacts (white circles). Top panel, PDL-coated bead in contact with a synaptophysin-EGFP-transfected axon for 2h. Note how the axon follows the movement of the PDL bead via the cantilever to which the bead is attached, indicating a significant adhesion. Bottom panel, an uncoated bead manipulated in the same way results in the immediate detachment of bead from the axon, revealing that no significant adhesion had taken place. Bar, 10μm.

(B) DIV15 rat hippocampal neurons incubated with PDL-coated beads for 24h (DIC, far left) labeled with antibodies to heavy chain neurofilament (green) and synaptophysin (red). Lower panel, zoom of the box shown in the larger DIC image. Bar, 20μm.

(C) Scanning (SEM, left panel) and transmission (TEM, right panel) electron microscopic images of neurons co-cultured for 24h with PDL-coated (top) or uncoated (bottom) beads. By SEM, we observed that neurites extend dense and numerous processes onto PDL-coated beads (top left), but fail to extend processes onto uncoated beads (bottom left). By TEM, we find that these dense processes contain bouton-like swellings and accumulate synaptic vesicles when contacting PDL-beads (top right), whereas neurites contacting uncoated beads do not form these varicosities (bottom right). Bars, 1μm (SEM), 250nm (TEM).

(D) DIV15 hippocampal neurons incubated with PDL-coated beads for 24h labeled with antibodies to VGlut1 (green) and GAD (red). Dashed box in full DIC image (top left) corresponds to location of close up panel (bottom). Bar, 20μm.

(E) Proportion of bead contacts displaying enhancement (fluorescence intensity ratio >2) of VGlut1 or GAD.

(F) Colocalization of VGlut1 and GAD puncta at bead sites. Compared to the distributions of synaptophysin and bassoon (black bar), which are highly covariant, we observe that the VGlut1 and GAD staining distributions are segregated from one another even when on the same bead (blue bar), as would be expected if these puncta are derived from different axons.

(G) Fluorescence intensity ratios of VGlut1 and GAD. No significant difference in fluorescence intensity ratio was observed between VGlut1 and GAD, suggesting that PDL-coated beads possess the capacity to induce both inhibitory and excitatory presynaptic endings equally robustly. For this and all other histograms, numbers in brackets denote the total number of beads quantified ($n \geq 3$ experiments). Dashed line, ratio value=1 (which would be expected if no change in fluorescence intensity was observed).

Figure 2. PDL-coated beads induce the formation of functional presynaptic boutons.

(A) DIV15 neurons incubated for 24h with either PDL-coated (left image panel) or uncoated (right image panel) beads were fixed and immunostained for a variety of presynaptic and cytoskeletal markers (i-vii). For each fluorescence image, the corresponding DIC image is represented on the left to note the location of the bead. Bars, 10μm. Arrowheads denote the bead sites. Histograms, fluorescence ratios comparing PDL-coated vs. uncoated beads (*$p<0.05$, ***$p<0.001$).

(B) Representative image panel of (DIV15) neurons co-cultured with beads for 24h (phase contrast image, top) prior to depolarization-induced loading (1X KCl, middle) and unloading (2X KCl, bottom) of the synaptic dye FM 4-64. Arrowheads denote the bead sites. Bar, 20μm.

(C) Histogram, measurements of fluorescence intensity following dye loading (1X KCl) and unloading (2X KCl) at bead contacts (20-30 beads per experiment, n=5 experiments, ***$p<0.001$).

Figure 3. PDL bead-induced presynaptic boutons are different from isolated axonal clusters, and form in a dose-dependent manner by a mechanism involving heparan sulfate proteoglycans (HSPGs).

(A,B) Addition of beads at different developmental stages distinguishes inductive capacity of PDL on the substrate versus the bead.

(A) Experimental design. At the time of plating, dissociated hippocampal cells were added to a PDL-coated substrate containing PDL-coated beads. After 7DIV, PLL-FITC coated beads were added and left for 24h, fixed and imaged as usual.

(B) DIC and fluorescence images of synaptophysin (red) and PLL-FITC beads (green). Only the PLL-FITC coated bead (green circle) induces synaptophysin clusters while the PDL coated bead added at the time of plating (white circle) does not.

(C) Representative images of DIV7 axons contacting beads coated with increasing concentrations of PDL for 24h. Histogram, measurements of fluorescence at neurite-bead contacts with increasing PDL concentrations (**, $p<0.01$). Bar, 2μm. Dashed circles indicate the location of the beads.

(D) Beads coated with a synthetic lipid bilayer containing DOTAP, a cationic lipid, were incubated with neuronal cultures (DIV12-14) for 24h. Left, representative images of cultures incubated with DOTAP beads (DIC, top left) and immunolabeled for synaptophysin (green, bottom left). Bar, 10μm. Far right, DOTAP beads with rhodamine (red, bottom right) incorporated into the lipid bilayer confirm that the bilayer remains intact when incubated with neurons for 24h. Bar, 5μm.

(E) Representative images of neurons (DIV15) incubated for 24h with PDL or uncoated beads with or without heparinase or heparan sulfate. Histograms, fluorescence ratio measurements in response to the different treatments (**, $p<0.01$, ***, $p<0.001$, n.s., between 20μg/mL heparan sulfate and uncoated beads). Scale and circles are the same as

in (C).

Figure 4. Syndecan-2 (syn2) is a cell-surface HSPG that mediates PDL bead-induced presynapse assembly.

Hippocampal cultures were grown to DIV11-13 prior to the addition of beads for these experiments.

(A) Histogram, fluorescence intensity ratio measurements of syndecan-2 accumulation at PDL bead sites. Syn2 accumulation at PDL bead sites after both 1h and 24h is significantly higher compared to 24h of co-culture with uncoated beads (***, $p<0.001$).

(B) Diagram showing the proportion of bead sites displaying dually or singly enhanced syn2 and/or synaptophysin after 1h or 24h of incubation. For this analysis, the fluorescence intensity ratios for both syn2 and synaptophysin were quantified and binned according to whether a single bead site had a >2-fold enhancement of syn2, synaptophysin or both. A large proportion of bead sites are dually enhanced after just 1h (0.57), and this proportion is further increased after 24h (0.77).

(C) Representative images of cultures incubated with PDL-coated beads and dually stained for syn2 (green) and synaptophysin (red). The cultures were incubated for either 1h (top panel) or 24h (lower 3 panels) in the presence or absence of the HSPG disruptors heparinase (III) and heparin (20µg/mL). Circles in fluorescence images denote the bead site shown the corresponding brightfield (DIC) panel. Bar, 5µm.

(D,E) Histograms, quantification and analysis of syn2 and synaptophysin co-clustering at PDL bead sites after 24h (D) or 1h (E) of incubation. (***, $p<0.001$ compared to the No treat (PDL) condition; n.s., not significant compared to the No treat (Unc) bead condition.)

Figure 5. Coordinated recruitment of multiple presynaptic proteins to PDL bead sites proceeds by a mechanism dependent on F-actin reorganization.

(A,B) Time course study of the recruitment of actin, synaptophysin, and bassoon to PDL

bead contacts. Representative images and analysis are derived from three independent experiments (DIV11-13 neurons), with a minimum of 50 beads analyzed per time point per experiment. Circle, location of the bead.

(A) Single neurite-PDL bead contacts labeled with antibodies to synaptophysin (red) and bassoon (blue) as well as alexa-phalloidin to stain F-actin (green). Bar, 2μm.

(B) Measurements of fluorescence intensity at neurite-bead contacts following incubation with PDL-coated beads (*$p<0.05$, **$p<0.01$, # $p>0.05$ vs. 3h).

(C) Individual frames collected during live time lapse imaging of a DIV8 hippocampal culture dually infected with SV2-EGFP (green) and mCherry-beta-actin (red). Bar, 2μm.

(D) Kymograph of the complete set of time lapse frames from (C), showing the relationship of actin and SV2 accumulation at the bead site. We observed a rapid and highly dynamic accumulation of both actin and SV2 that appears to stabilize in the latter half of the time course.

(E) Quantification and analysis of mCherry-beta-actin fluorescence intensity at PDL bead and adjacent control sites (n=5). Top, comparison of fluorescence intensity values taken before (t=-2min) and after (t=60min) the addition of beads at bead (left) and adjacent (right) sites (*, $p<0.05$, n.s., $p>0.05$, paired t-test). Bottom, time course of fluorescence intensity at bead and adjacent control sites (*, $p<0.05$, n.s., $p>0.05$, 2-way ANOVA).

(F) Quantification and analysis of SV2-EGFP fluorescence intensity at bead and adjacent control sites (n=7). Top, comparison of fluorescence intensity values taken before (t=-2min) and after (t=60min) the addition of beads at bead (left) and adjacent (right) sites (*$p<0.05$; n.s., $p>0.05$). Bottom, time course of fluorescence intensity at bead and adjacent control sites (**$p<0.01$, 2-way ANOVA).

(G,H) DIV13-15 hippocampal cultures were incubated with PDL-coated beads for 3h,

either alone (no treatment) or in the presence of jasplakinolide (Jas) or latrunculin A (LatA)(5μM), which were added immediately prior to the addition of the beads. (G) Representative image panel of cultures following fixation and staining for actin and synaptophysin. Bar, 5μM. Note the decrease in F-actin fluorescence intensity for both jasplakinolide and latrunculin A, the former due to competition of the phalloidin agent for the same binding site as jasplakinolide, the latter due to a true loss of F-actin due to the sequestration of actin monomers.

(H) Quantification of fluorescence intensity ratios of synaptophysin (C) in the presence or absence of these treatments. ***, $p<0.001$ compared to PDL beads alone (No treatment), one-way ANOVA.

Figure 6. PDL-coated beads can facilitate postsynaptic differentiation on beads where presynaptic clusters are also observed.

(A) Individual frames collected during live time lapse imaging of dendrites from DIV13 hippocampal neurons dually infected with SAP97-EGFP (green) and mCh-actin (red). Circle, location of the bead. Bar, 2μm.

(B) Kymograph of the complete set of time lapse frames from (A), showing relationship of mCh-actin and SAP97 accumulation at the bead site. We observe that actin accumulates at the bead site while SAP97 fluorescence does not appear to change.

(C) Quantification and analysis of mCh-actin fluorescence intensity at bead and adjacent control sites (n=9). Top, comparison of fluorescence intensity values taken before (t=-2min) and after (t=150min) the addition of beads at bead (left) and adjacent (right) sites (***$p<0.001$, n.s., $p>0.05$). Bottom, time course of fluorescence intensity at bead and adjacent control sites (**$p<0.01$, 2-way ANOVA).

(D) Quantification and analysis of SAP97-EGFP fluorescence intensity at bead and adjacent control sites (n=9). Top, comparison of fluorescence intensity values taken before (t=-2min) and after (t=150min) addition of beads at bead (left) and adjacent (right)

sites ($p>0.05$). Bottom, time course of fluorescence intensity at bead and adjacent control sites. No significant changes in fluorescence intensity were observed at any time point.

(E) Representative image panel of a DIV15 neuron co-cultured with beads for 24h (DIC) and immunostained for synaptophysin (red) and PSD95 (green). In the upper left DIC image, the beads are numbered to demarcate their proximity relative to the cell body (arrowhead). Bottom panels show two neurite-PDL-bead contacts, one of which exhibits numerous synaptophysin as well as PSD95 clusters (Bead 1) and the other showing synaptophysin clustering alone (Bead 7). Bar, 20μm.

Table 1. Quantification and analysis of PSD95-synaptophysin dual labeling experiments.

The fluorescence ratios for both PSD95 and synaptophysin were quantified and binned according to whether a single bead site had a >2-fold enhancement of PSD95, synaptophysin or both. The pooled fluorescence ratios are also shown for each category.

Figure 7. Dependency of presynaptic development on postsynaptic assembly, postsynapse development on presynaptic assembly.

(A,B) Representative images of DIV7 neurons cultured on a micropatterned substrate and triple stained for Tau1 (green), MAP2 (blue) and synaptophysin (red) following incubation with PDL-coated beads for 24h. Notice the separation of axons from dendrites and the selective enhancement of synaptophysin exclusively on axons (arrowheads) and not on dendrites (arrows). Left-hand images are the merged fluorescence and DIC images to denote the location of the beads. Bars, 20μm.

(C,D) Representative confocal images of DIV9, SAP97-EGFP-infected cultures (green) incubated with PDL-coated beads. Circles and arrowheads denote the location of beads shown in corresponding DIC (right) and/or merge (left) images. Bars, 10μm.

(C) DIV9 cultures + 1h PDL beads. Top panel, singly infected SAP97-EGFP culture. Lower panel, dually infected SAP97-EGFP/mCh-actin (red) culture immunostained for MAP2 (blue).

(D) DIV9 cultures + 24h PDL beads. Top panel, singly infected SAP97-EGFP (green) culture immunostained for MAP2 (blue). Bottom panel, dually infected SAP97-EGFP/mCh-actin (red) culture immunostained for MAP2 (blue).

Figure 8.

(A) Model to describe the function of the ECM in PDL bead-induced presynapse formation. The ECM is rich in a variety of heavily charged and complex molecules, including HSPGs, that forms a scaffold for the heterophilic binding of soluble and transmembrane proteins. The binding of a PDL bead to an axon (*left*) could therefore encourage (i) the heterophilic binding of transmembrane HSPGs, including syn2 and potentially other cell-surface receptors, (ii) the presentation of soluble synaptogenic ligands to the axon, or both (*middle*). This results in the triggering of signaling mechanisms resulting in the assembly of a presynapse (*right*).

(B) Model for distinguishing the responsiveness of axons vs. dendrites to form synaptic-like contacts to PDL-beads. *Top*, upon contact with a PDL-coated bead, an axon isolated from any native targets will rapidly form a functional presynaptic ending. In contrast, a bead contacting an isolated dendrite (*middle*) will adhere, but not trigger the formation of a postsynaptic density even after 24h. *Bottom*, a PDL bead making simultaneous contact with an axon and a dendrite can encourage the formation of a native synapse that, given the inability of the PSD to form on isolated dendrites, is likely driven by the presynaptic bouton induced to form by the bead.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. PDL beads form stable synaptic vesicle clusters independent of neuronal age.

Representative image panels of hippocampal neurons cultured for 7 (top), 15 (middle) and 21 (bottom) days prior to the addition of PDL-coated beads. Cells were incubated with beads for (A) 24h or (B) 72h, fixed and stained for synaptophysin (red). White box in fluorescence images demarcate the close-up region shown beneath. Bars, 20μm.

Figure S2. Small changes in axonal contact area are accompanied by large changes in synaptic vesicle clustering at PDL bead but not uncoated bead sites. (A-C), representative image panels of uncoated (A) and PDL-coated (B,C) beads contacting EGFP-positive axons (green) and stained for synaptophysin (red). Note the distinct increases in both axonal volume and synaptophysin clustering selectively at PDL-bead sites. Scale bars, 10μm. (D) Quantification of GFP fluorescence at PDL vs. uncoated bead sites. (E) Quantification and analysis of GFP vs. synaptophysin immunofluorescence at PDL bead sites (**, $p<0.01$; ***, $p<0.001$, unpaired t-test).

Figure S3. Treatment with heparinase or heparan sulfate does not affect the number, size or intensity of established presynaptic puncta. (A) Method of quantification. A number of regions of interest (ROI, 44μm diameter) were selected at random from the DIC image (representative image, left) that excluded PDL bead sites. These sites on the corresponding synaptophysin fluorescence image (right) were thereafter analyzed. (B) Table showing all parameters analyzed (average ROI, number and size of puncta). No values are significantly different from the 24h PDL bead, no treatment condition.

Figure S4. Heparan sulfates accumulate onto PDL-coated but not uncoated beads. (A,B) Representative image frames of showing the accumulation of HS (A) or synaptophysin (B) onto PDL-coated (a-e) or uncoated (f-j) beads. Numbers on the lower right-hand side (in μm) denote the location in the z-stack above the neuritic plane (0.0μm

shown in a). (C) Quantification and analysis of the fluorescence intensity ratios at PDL coated vs. uncoated bead sites (*, $p<0.05$, Student's t-test).

Figure S5. Presynaptic boutons are rapidly functional following bead contact.

Analysis of presynaptic functionality following depolarization-induced loading (1X KCl) and unloading (2X KCl) of the synaptic dye FM 4-64. (A,C,E,G) Representative images of depolarization-induced uptake and unloading of the FM 4-64 dye in SV2-EGFP-infected cultures. At bead sites displaying clusters of SV2-EGFP (green), we observed both uptake and unloading of the FM 4-64 dye (red). White circle outlines the perimeter of the bead.

(A) DIV7+1h PDL beads.

(C) DIV7+24h beads.

(E) DIV12+1h PDL beads.

(G) DIV12+24h PDL beads.

(B,D,F,H) Histograms, measurements of fluorescence intensity following dye loading (1X KCl) and unloading (2X KCl) at bead contacts. Numbers in brackets represent the total number of puncta analyzed from at least 5 different sites per culture from 2-3 cultures per condition (except (H), in which 5 sites were analyzed from 1 culture but see Figure 2C)(***, $p<0.001$).

(B) DIV7+1h PDL beads.

(D) DIV7+24h PDL beads.

(F) DIV12+1h PDL beads.

(H) DIV12+24h PDL beads.

Figure S6. Individual frames derived from supplemental movies M3 and M4.

Image panel derived from individual frames within supplemental movies 3 and 4. DIC images show the imaged field before (far left) and after (center left) the addition of PDL-coated beads. The fluorescence images (center right and far right) show the location of the beads (circles) prior to their addition to the culture. Bar, 10μM.

(A) Movie S3, mCh-actin/SV2-EGFP.

(B) Movie S4, mCh-actin/SAP97-EGFP.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. PDL-coated beads firmly adhere to axons: Evidence by Atomic Force Microscopy. PDL-coated beads were applied to synaptophysin-EGFP (green) expressing neurons via a cantilever attached to an atomic force microscope. Following 2h of incubation, lateral movement of the cantilever resulted in a corresponding displacement of the axon that remained attached to the bead.

Movie S2. Uncoated beads do not adhere to axons: Evidence by Atomic Force Microscopy. Uncoated beads, when applied to synaptophysin-EGFP (green) expressing neurons in the same way as in Movie 1, easily detached from axons when pulled away from contact via the cantilever. In this movie, the uncoated bead remained in contact with the axon for 30min, a time point at which PDL-coated beads have already firmly attached to neurons (previous observations).

Movie S3. Dual live imaging reveals a rapid reorganization and accumulation of mCh-actin (red) and SV2-EGFP (green) on axons at sites of PDL bead contact. A representative example of an axon (from a DIV8 culture) contacting a PDL-coated bead for 60min. Note how the bead site (center of field, see Figure S6A) is devoid of SV2-EGFP positive clusters prior to adhesion of the bead. We observed that actin quickly reassembles itself at the site of bead contact in a matter of minutes and that SV2-EGFP-positive synaptic vesicle clusters are recruited near-simultaneously. Even after 60min of bead contact, these clusters of actin and SV2 are remarkably stable.

Movie S4. Dual live imaging of mCh-actin and SAP97-EGFP. A representative example of SAP97-EGFP (green) and mCh-actin (red) dendrites from a DIV13 culture contacting a bead. Here we observe a steady accumulation of actin (center of field, see Figure S6B) but no corresponding recruitment of SAP97-EGFP positive clusters, even after 150min of bead contact.

FIGURES

Figure 1

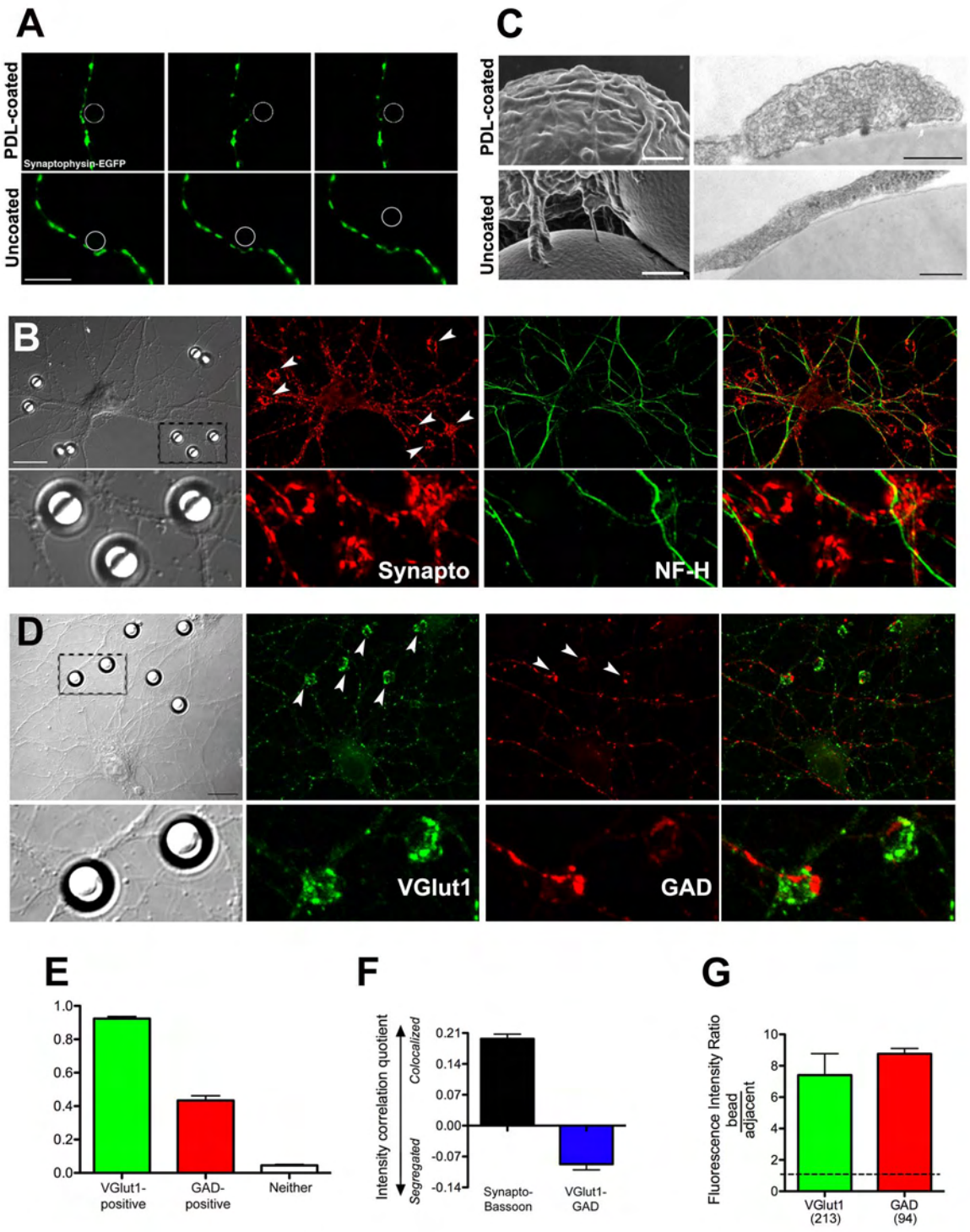

A
PDL-coated
Synaptophysin-EGFP
Uncoated
C
PDL-coated
Uncoated
B
Synapto
NF-H
D
VGlut1
GAD
E
1.0
0.8
0.6
0.4
0.2
0.0
VGlut1-positive
GAD-positive
Neither
F
Intensity correlation quotient
Colocalized
Segregated
0.21
0.14
0.07
0.00
-0.07
-0.14
Synapto-Bassoon
VGlut1-GAD
G
Fluorescence Intensity Ratio
bead
adjacent
10
8
6
4
2
0
VGlut1 (213)
GAD (94)

Figure 2

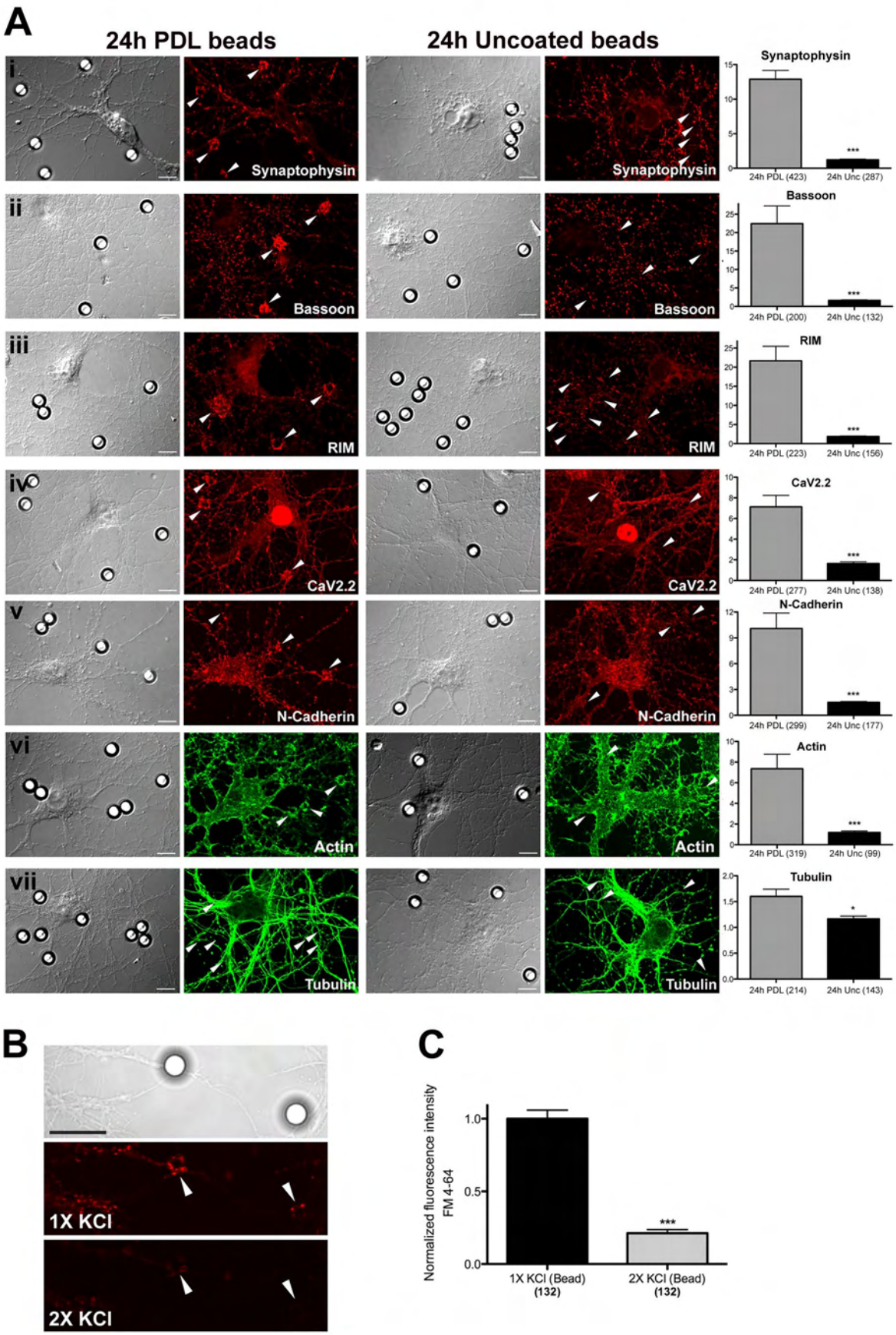

A
24h PDL beads
24h Uncoated beads
i
Synaptophysin
Synaptophysin
Synaptophysin
24h PDL (423)
24h Unc (287)
ii
Bassoon
Bassoon
Bassoon
24h PDL (200)
24h Unc (132)
iii
RIM
RIM
RIM
24h PDL (223)
24h Unc (156)
iv
CaV2.2
CaV2.2
CaV2.2
24h PDL (277)
24h Unc (138)
v
N-Cadherin
N-Cadherin
N-Cadherin
24h PDL (299)
24h Unc (177)
vi
Actin
Actin
Actin
24h PDL (319)
24h Unc (99)
vii
Tubulin
Tubulin
Tubulin
24h PDL (214)
24h Unc (143)
B
1X KCl
2X KCl
C
Normalized fluorescence intensity
FM 4-64
1X KCl (Bead)
(132)
2X KCl (Bead)
(132)

Figure 3

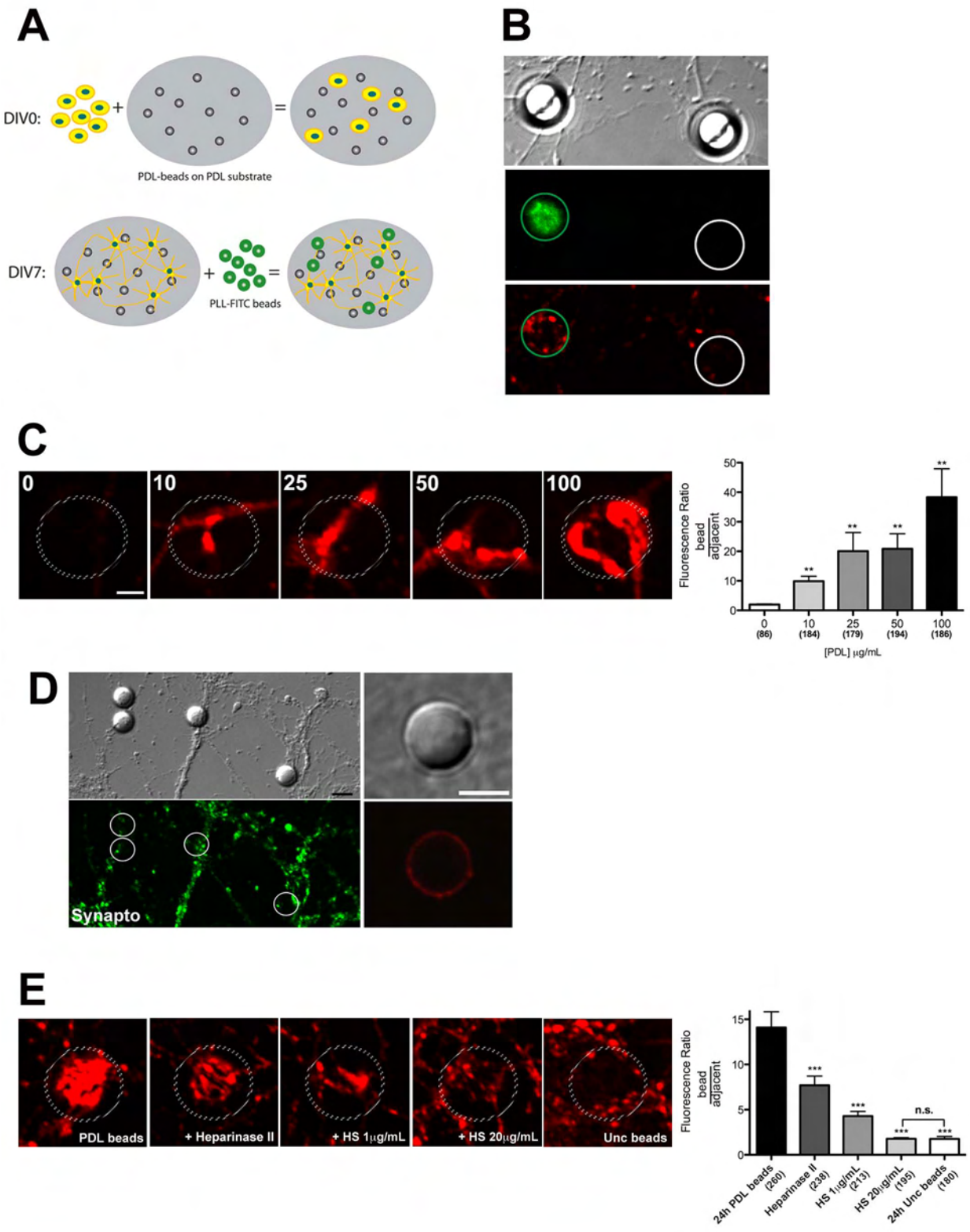

A
DIV0:
PDL-beads on PDL substrate
DIV7:
PLL-FITC beads
B
C
0
10
25
50
100
Fluorescence Ratio
bead
adjacent
[PDL] µg/mL
D
Synapto
E
PDL beads
+ Heparinase II
+ HS 1µg/mL
+ HS 20µg/mL
Unc beads
n.s.
24h PDL beads (260)
Heparinase II (238)
HS 1µg/mL (213)
HS 20µg/mL (195)
24h Unc beads (180)

Figure 4

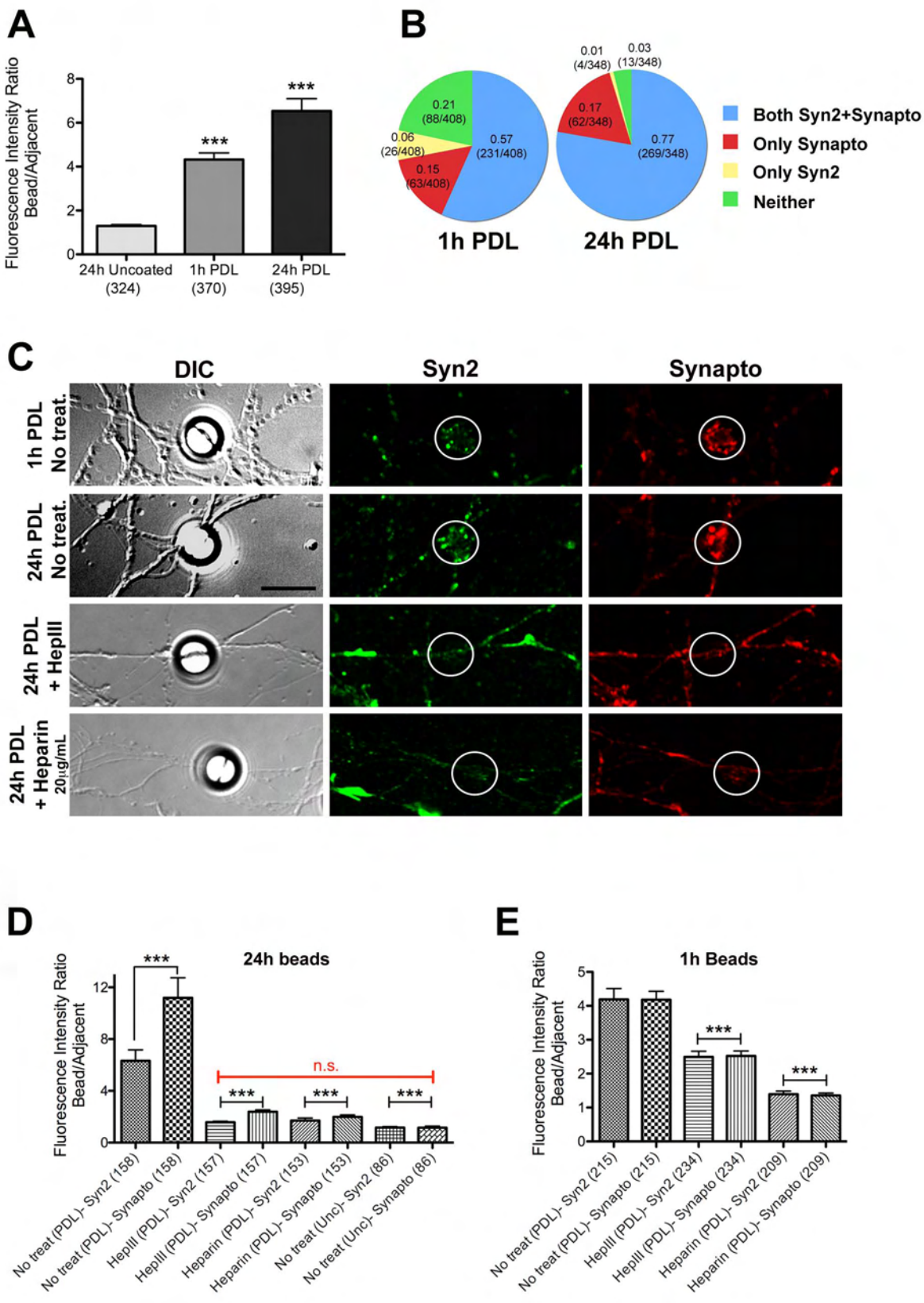

A
Fluorescence Intensity Ratio Bead/Adjacent
***
***
24h Uncoated (324)
1h PDL (370)
24h PDL (395)
B
0.21 (88/408)
0.06 (26/408)
0.57 (231/408)
0.15 (63/408)
0.01 (4/348)
0.03 (13/348)
0.17 (62/348)
0.77 (269/348)
1h PDL
24h PDL
Both Syn2+Synapto
Only Synapto
Only Syn2
Neither
C
DIC
Syn2
Synapto
1h PDL No treat.
24h PDL No treat.
24h PDL + HepIII
24h PDL + Heparin 20µg/mL
D
24h beads
Fluorescence Intensity Ratio Bead/Adjacent
***
n.s.
***
***
***
No treat (PDL)- Syn2 (158)
No treat (PDL)- Synapto (158)
HepIII (PDL)- Syn2 (157)
HepIII (PDL)- Synapto (157)
Heparin (PDL)- Syn2 (153)
Heparin (PDL)- Synapto (153)
No treat (Unc)- Syn2 (86)
No treat (Unc)- Synapto (86)
E
1h Beads
Fluorescence Intensity Ratio Bead/Adjacent
***
***
No treat (PDL)- Syn2 (215)
No treat (PDL)- Synapto (215)
HepIII (PDL)- Syn2 (234)
HepIII (PDL)- Synapto (234)
Heparin (PDL)- Syn2 (209)
Heparin (PDL)- Synapto (209)

Figure 5

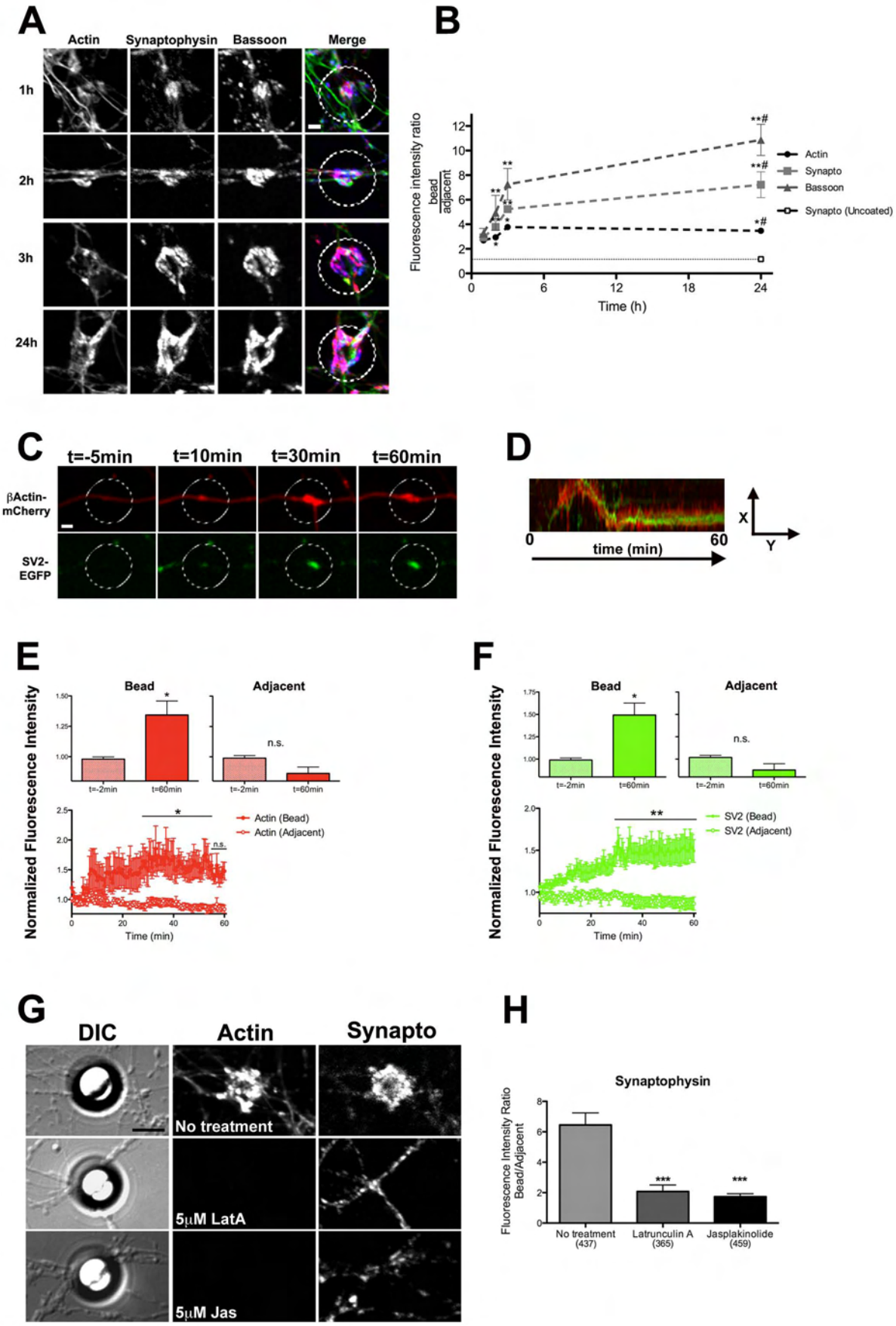
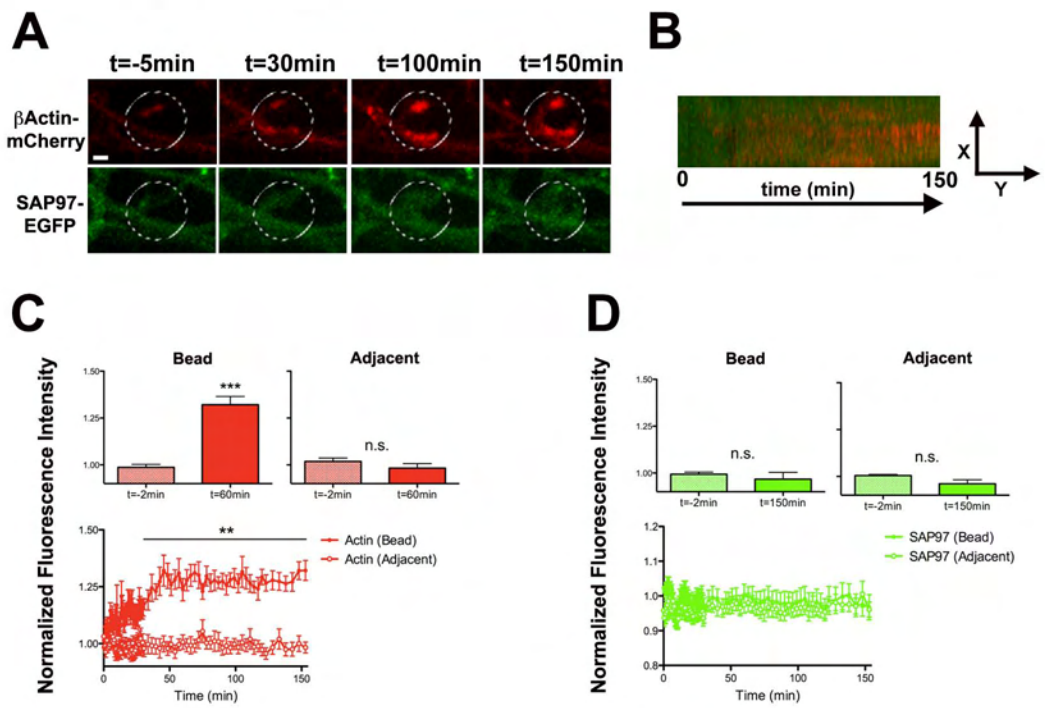

A
Actin
Synaptophysin
Bassoon
Merge
1h
2h
3h
24h
B
Fluorescence intensity ratio bead adjacent
Time (h)
Actin
Synapto
Bassoon
Synapto (Uncoated)
C
t=-5min
t=10min
t=30min
t=60min
βActin-mCherry
SV2-EGFP
D
time (min)
X
Y
E
Bead
Adjacent
Normalized Fluorescence Intensity
n.s.
Actin (Bead)
Actin (Adjacent)
Time (min)
F
Bead
Adjacent
Normalized Fluorescence Intensity
n.s.
SV2 (Bead)
SV2 (Adjacent)
Time (min)
G
DIC
Actin
Synapto
No treatment
5μM LatA
5μM Jas
H
Synaptophysin
Fluorescence Intensity Ratio Bead/Adjacent
No treatment (437)
Latrunculin A (365)
Jasplakinolide (459)

Figure 6



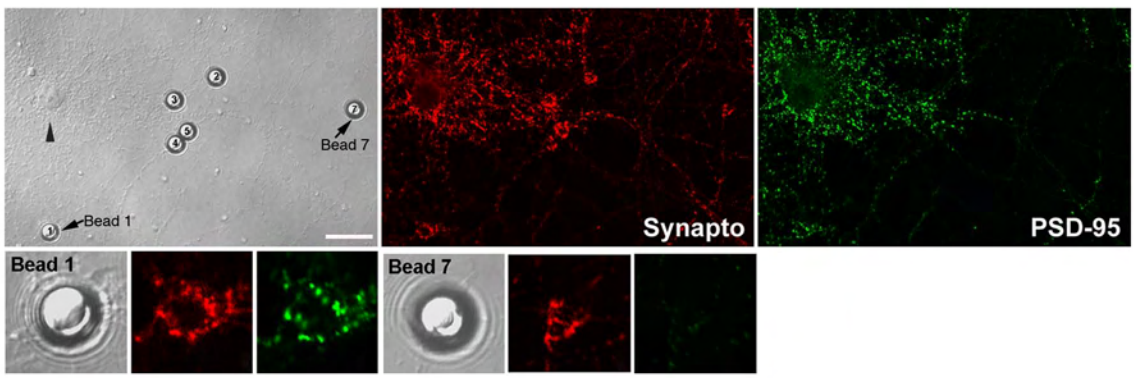


Table 1

	Total		Both enhanced		Just Synaptophysin		Just PSD95		Neither
Number of Beads	207		79		94		3		31
Proportion	100%		38.2%		45.4%		1.4%		15%
Fluorescence intensity ratio (mean±SEM)	Synapto	PSD95	Synapto	PSD95	Synapto	PSD95	Synapto	PSD95	
	14.7±1.9	3.4±0.5	17.1±2.7	7.5±1.2	17.5±3.5	0.8±0.1	1.6±0.1	2.9±0.4	

Figure 7

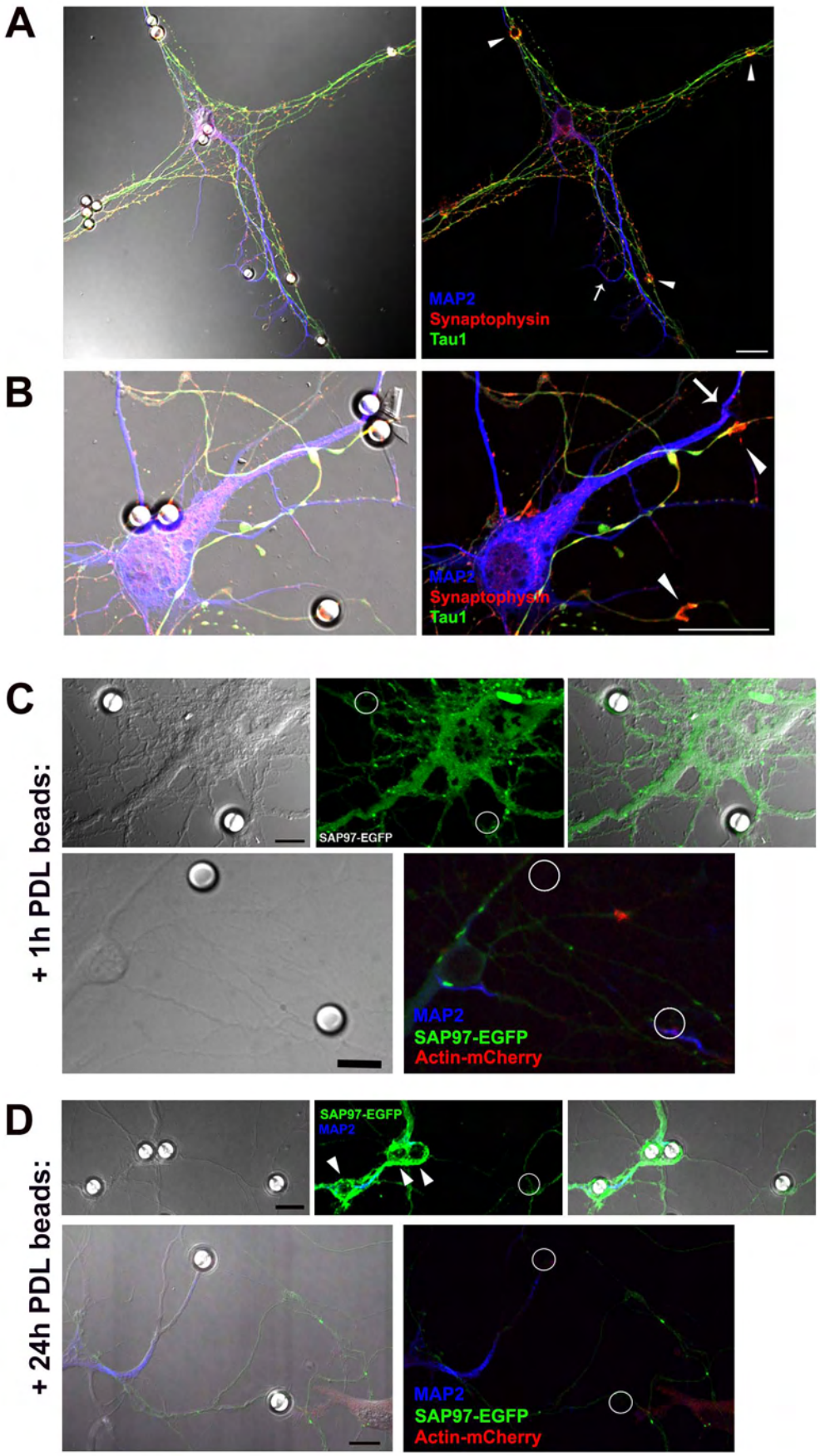

A
MAP2
Synaptophysin
Tau1
B
MAP2
Synaptophysin
Tau1
C
+ 1h PDL beads:
SAP97-EGFP
MAP2
SAP97-EGFP
Actin-mCherry
D
+ 24h PDL beads:
SAP97-EGFP
MAP2
MAP2
SAP97-EGFP
Actin-mCherry

Figure 8

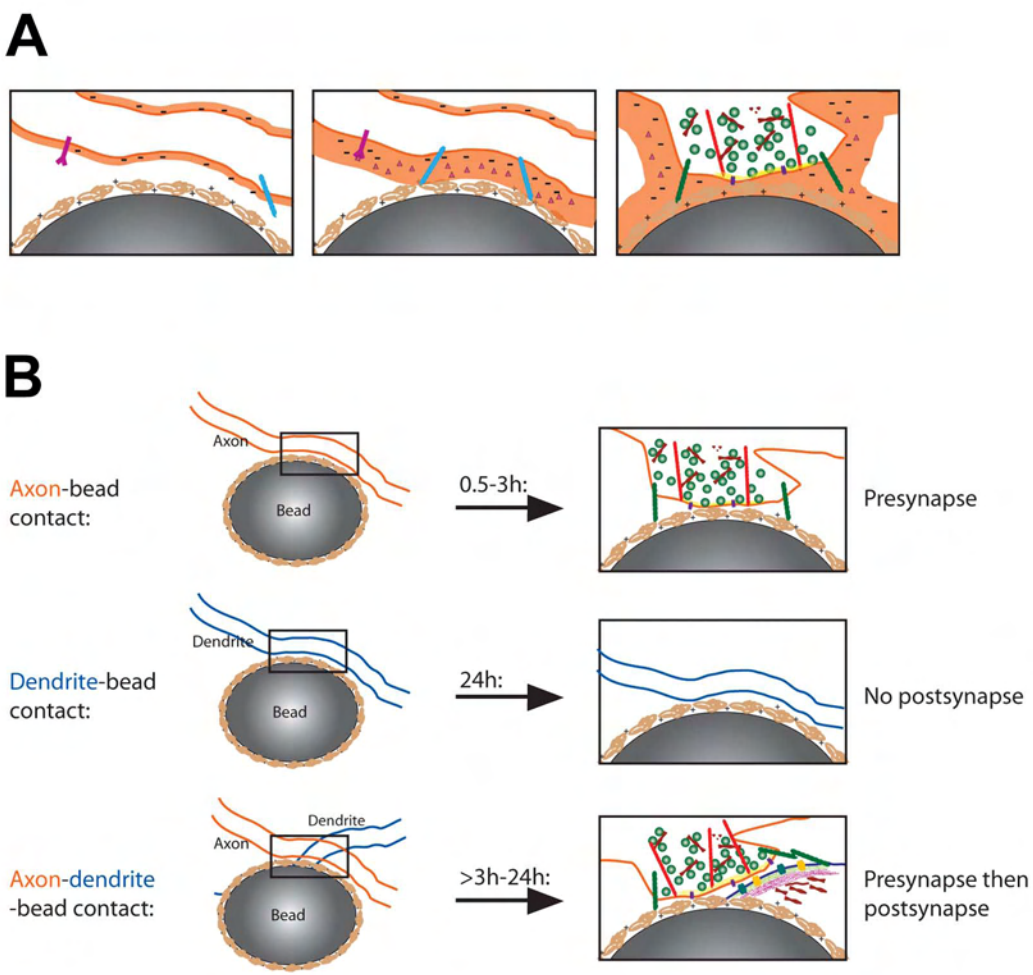

A
B
Axon-bead contact:
Axon
Bead
0.5-3h:
Presynapse
Dendrite-bead contact:
Dendrite
Bead
24h:
No postsynapse
Axon-dendrite -bead contact:
Dendrite
Axon
Bead
>3h-24h:
Presynapse then postsynapse

Figure S1

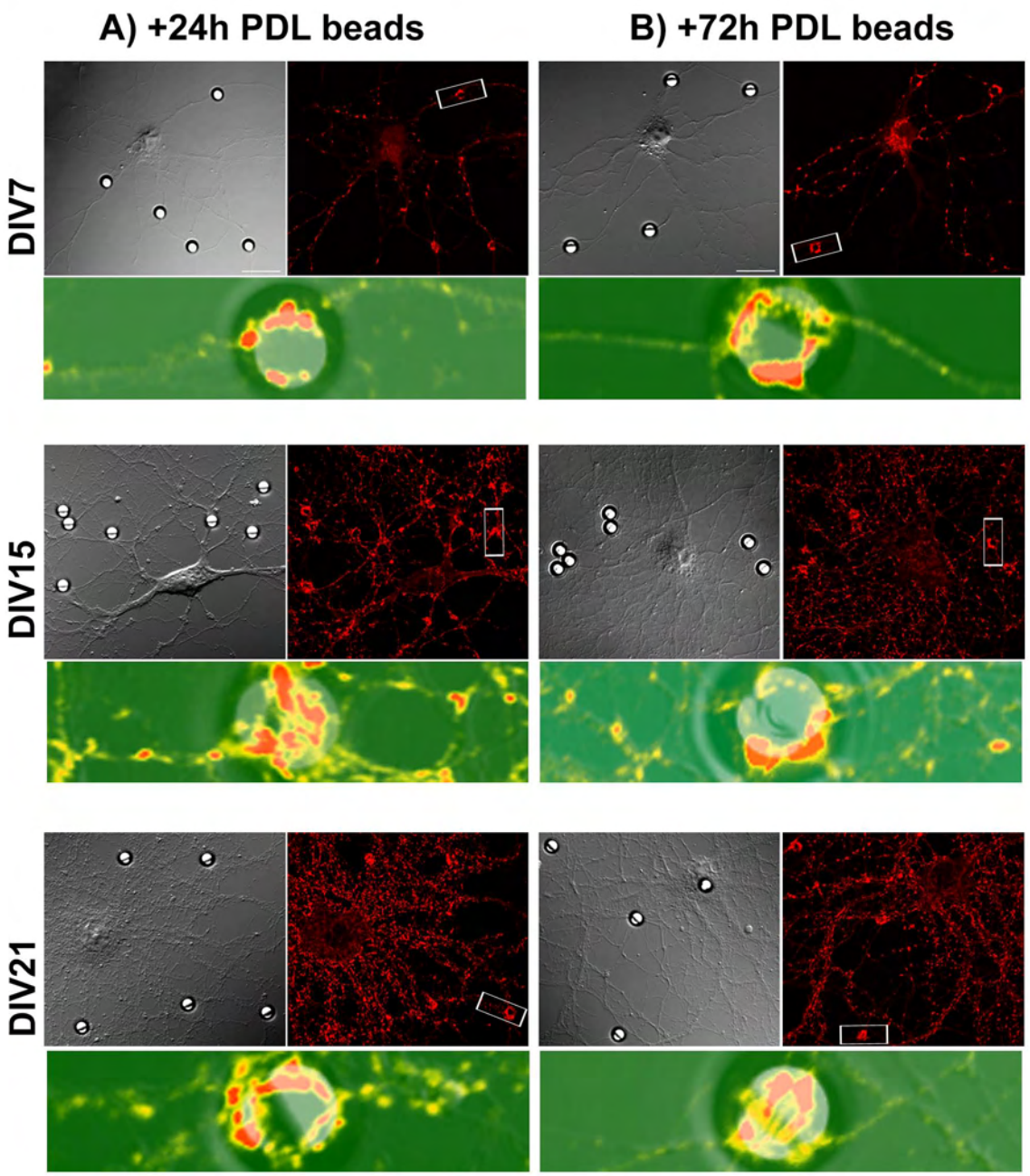


Figure S2

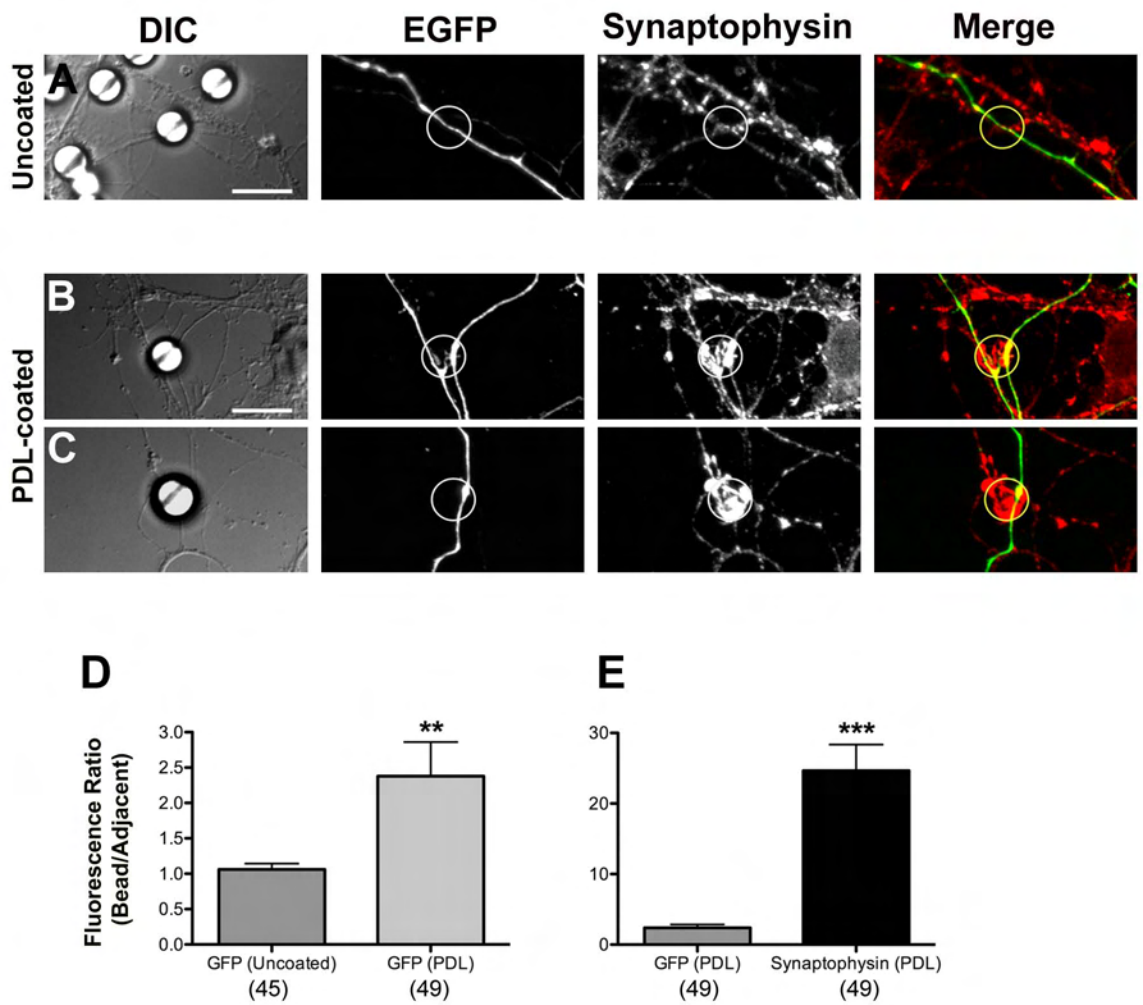
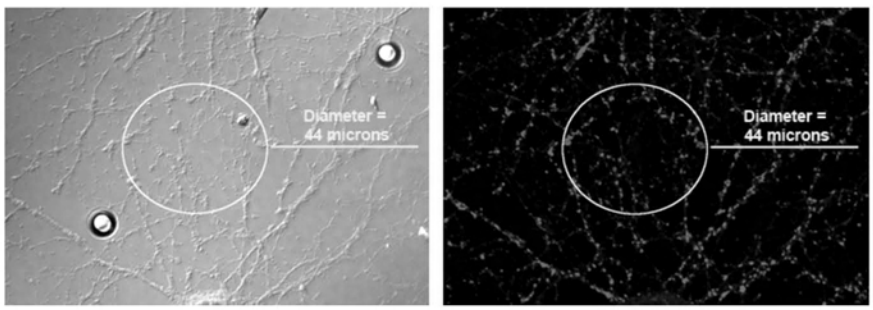

DIC
EGFP
Synaptophysin
Merge
Uncoated
PDL-coated
A
B
C
D
Fluorescence Ratio (Bead/Adjacent)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
GFP (Uncoated)
(45)
GFP (PDL)
(49)
E
30
20
10
0
***
GFP (PDL)
(49)
Synaptophysin (PDL)
(49)

Figure S3

A



B

		Average per ROI:						
	ROI length (μm)	Fluorescence intensity		# of Synaptophysin -positive puncta		Average punctal length (nm)		Number of ROIs per experiment (number of experiments)
		Mean	SEM	Mean	SEM	Mean	SEM	
PDL beads alone	44	26.7	1.6	188	8.8	323	26.7	48(4)
PDL beads + Heparinase II	44	28.1	2.7	156.4	10	461.5	73.3	36(3)
PDL beads + 1μg/mL Heparan Sulfate	44	29.3	1.6	189	11.7	380.9	37.9	36(3)
PDL beads + 20μg/mL Heparan Sulfate	44	27.2	2	152	8.9	406.6	32.7	36(3)

Figure S4

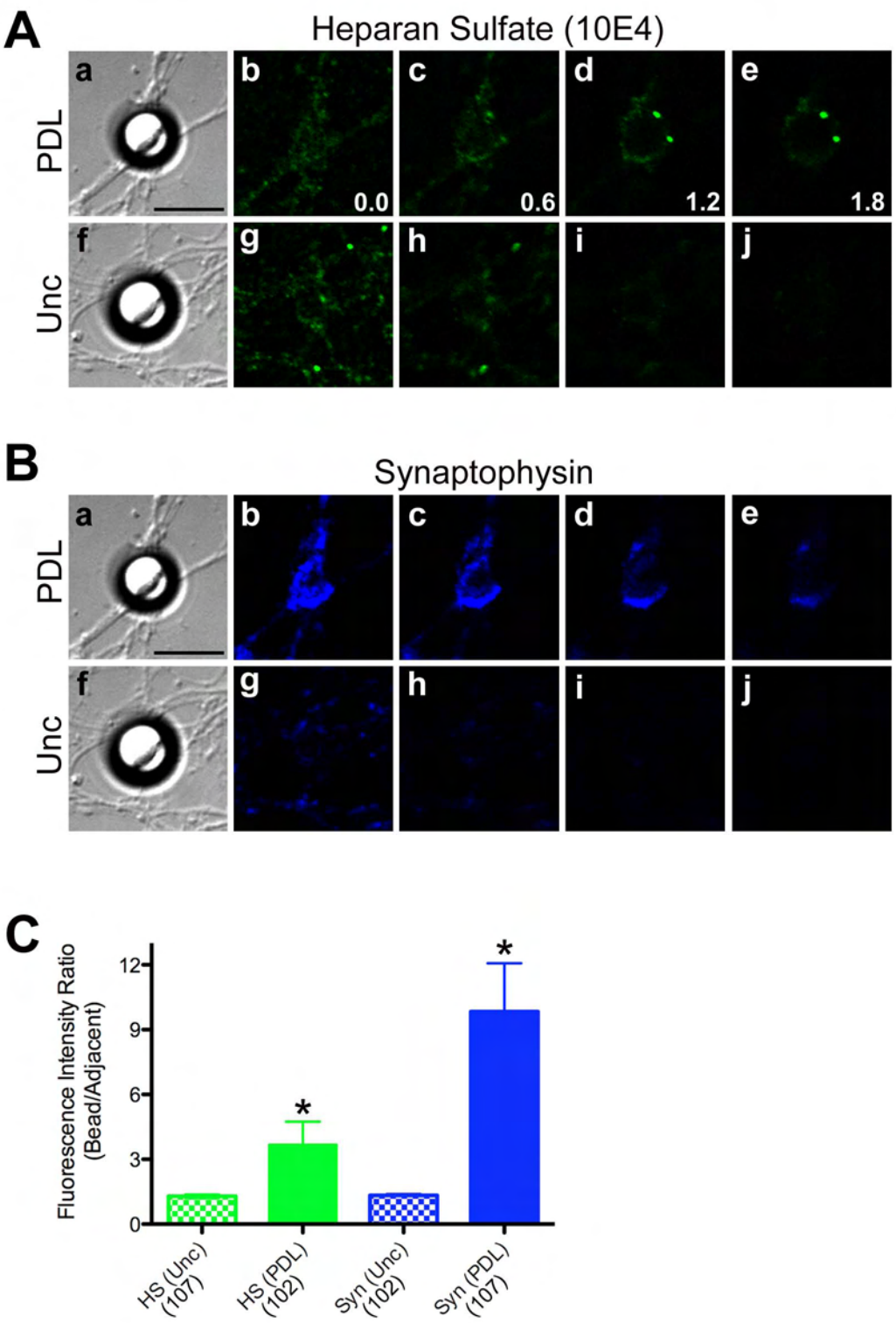

A
Heparan Sulfate (10E4)
PDL
Unc
a
b
c
d
e
f
g
h
i
j
0.0
0.6
1.2
1.8
B
Synaptophysin
PDL
Unc
a
b
c
d
e
f
g
h
i
j
C
Fluorescence Intensity Ratio (Bead/Adjacent)
12
9
6
3
0
*
*
HS (Unc) (107)
HS (PDL) (102)
Syn (Unc) (102)
Syn (PDL) (107)

Figure S5

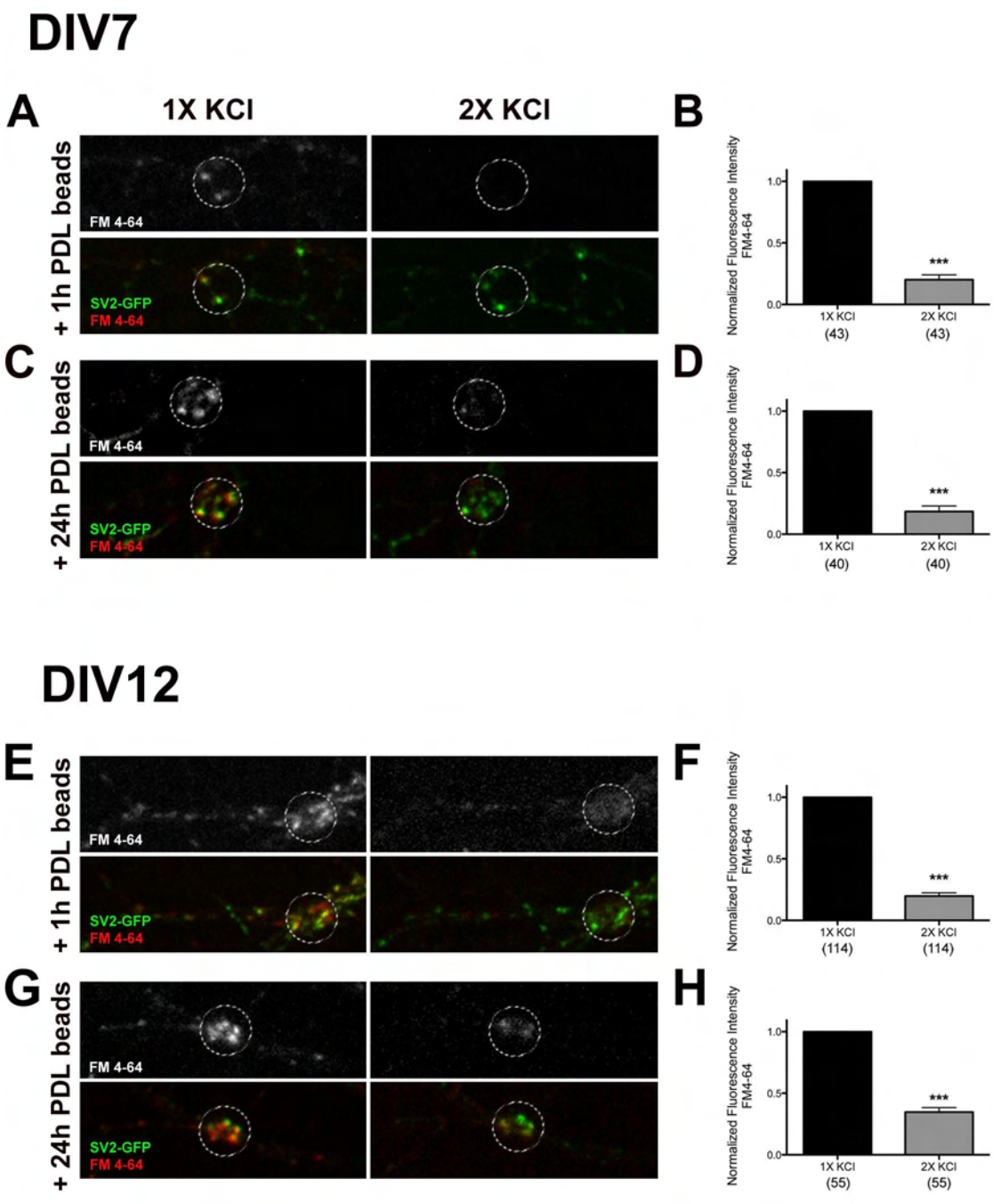
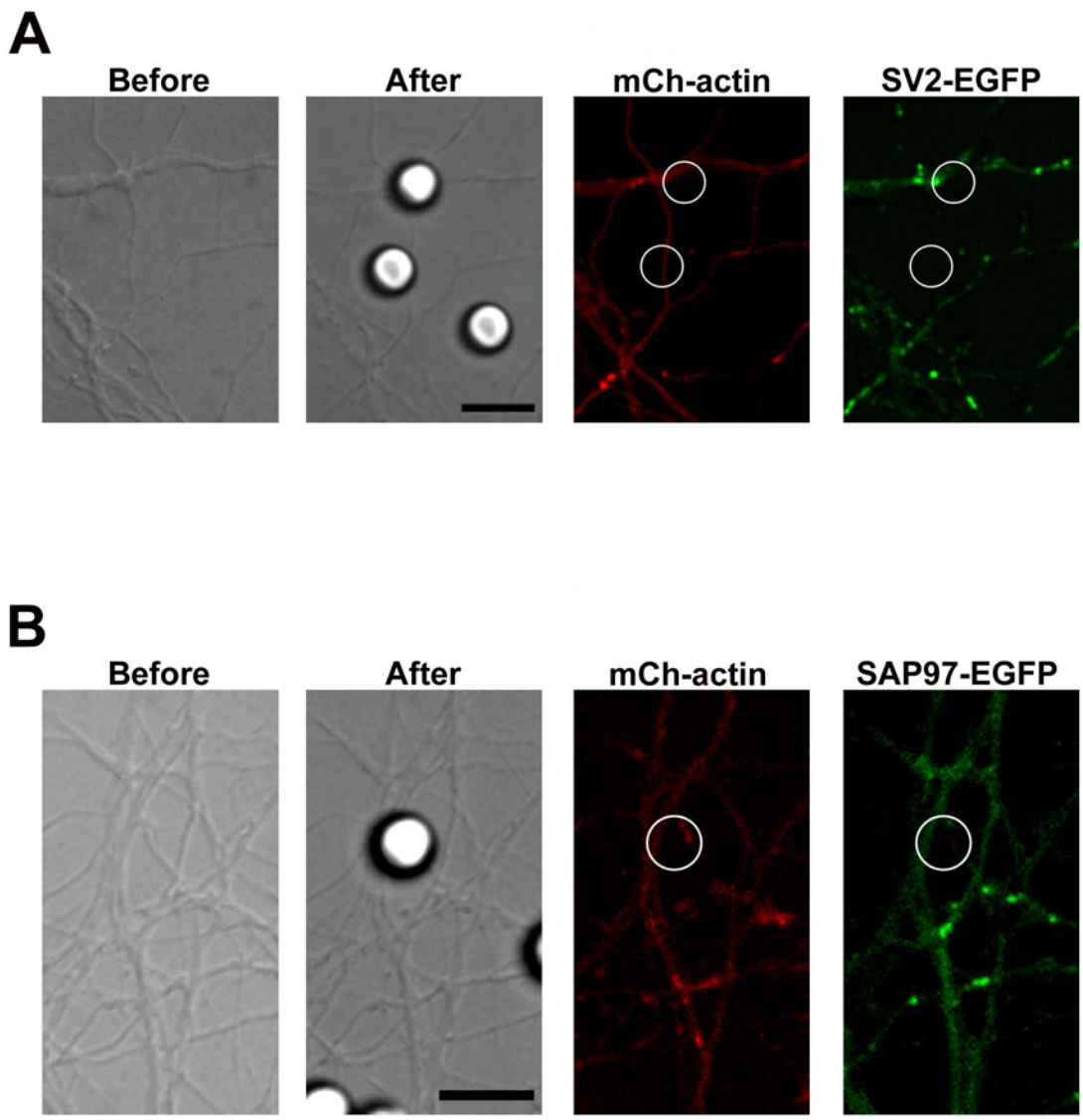

DIV7
A
1X KCl
2X KCl
+ 1h PDL beads
FM 4-64
SV2-GFP
FM 4-64
B
Normalized Fluorescence Intensity FM4-64
1.0
0.5
0.0
***
1X KCl
(43)
2X KCl
(43)
C
+ 24h PDL beads
FM 4-64
SV2-GFP
FM 4-64
D
Normalized Fluorescence Intensity FM4-64
1.0
0.5
0.0
***
1X KCl
(40)
2X KCl
(40)
DIV12
E
+ 1h PDL beads
FM 4-64
SV2-GFP
FM 4-64
F
Normalized Fluorescence Intensity FM4-64
1.0
0.5
0.0
***
1X KCl
(114)
2X KCl
(114)
G
+ 24h PDL beads
FM 4-64
SV2-GFP
FM 4-64
H
Normalized Fluorescence Intensity FM4-64
1.0
0.5
0.0
***
1X KCl
(55)
2X KCl
(55)

Figure S6


A
Before
After
mCh-actin
SV2-EGFP
B
Before
After
mCh-actin
SAP97-EGFP

RESULTS PART (B): SUPPLEMENTAL THESIS RESULTS

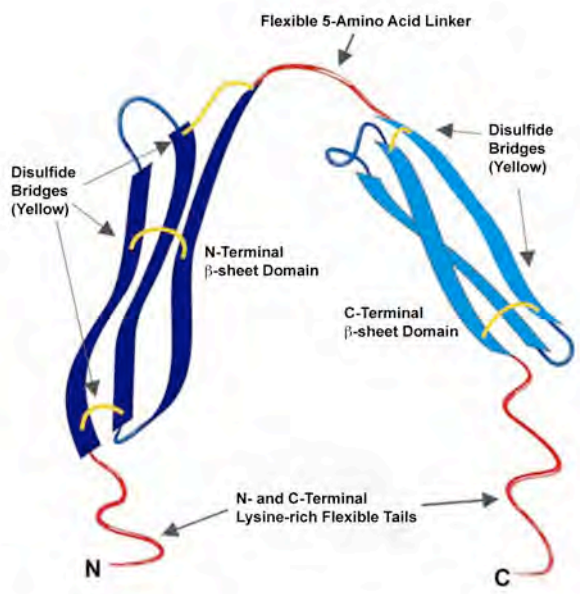
PREFACE

This section outlines some additional experiments that were not contained within the submitted manuscript "Rapid assembly of functional presynaptic boutons triggered by adhesive contacts." There are three sets of experiments that will be described with an accompanying figure, background rationale and description of the results. These sets of experiments will be divided into three separate parts (Part I-III). For the detailed materials and methods, please see Appendix I: supplemental thesis methods.

PART I: Pleiotrophin/HB-GAM, a lysine-rich growth factor that promotes presynapse formation.

Background

Pleiotrophin, also known as heparin-binding growth-associated molecule (HB-GAM), is a growth factor first isolated in a heparin affinity-chromatography screen for new ECM molecules that enhanced neurite outgrowth (Rauvala, 1989; Rauvala and Peng, 1997). Molecular cloning revealed pleiotrophin/HB-GAM (herein referred to as PTN) to be an 18kDa protein rich in lysine and arginine residues particularly at the N- and C-termini (Li et al., 1990; Merenmies and Rauvala, 1990)(Figure 1.1). Since then, PTN has been shown to be a potent enhancer of neurite outgrowth and its expression to be highly developmentally regulated, showing a predominant expression along fiber tracts in perinatal and early postnatal animals and little expression in adults [reviewed in (Rauvala and Peng, 1997)].



Characteristic	HB-GAM
Primary sequence	136 amino acids
% cationic residues (lysine + arginine)	24 amino acids
% cysteine residues	7 amino acids
Intra-chain disulfides	5 amino acids
Apparent molecular mass	18kDa
Heparin-binding	Strong, reversed by 1M NaCl

*Information based on studies of Merenmies & Rauvala, 1990; Li et al., 1990; reviewed in Rauvala and Peng, 1997.

Figure 1.1. Structure of pleiotrophin/HB-GAM. Left, schematic representation of HB-GAM tertiary structure. Adapted from (Raulo, 2006). Note the lysine-rich N- and C-terminal tails. Right, table outlining the features of the HB-GAM (PTN) primary sequence. Information taken from sources outlined below the table.

The predominant receptor for PTN appears to be N-syndecan, a transmembrane HSPG. This has been shown by (1) HB-GAM affinity columns, which isolated a 200kDa protein revealed to be N-syndecan (Raulo et al., 1994), (2) inhibition of HB-GAM-based neurite outgrowth in the presence of N-syndecan function-blocking antibodies or soluble N-syndecan-derived protein fragments (Kinnunen et al., 1996; Kinnunen et al., 1998) and (3) a highly correlated expression pattern during development (Nolo et al., 1995; Rauvala and Peng, 1997).

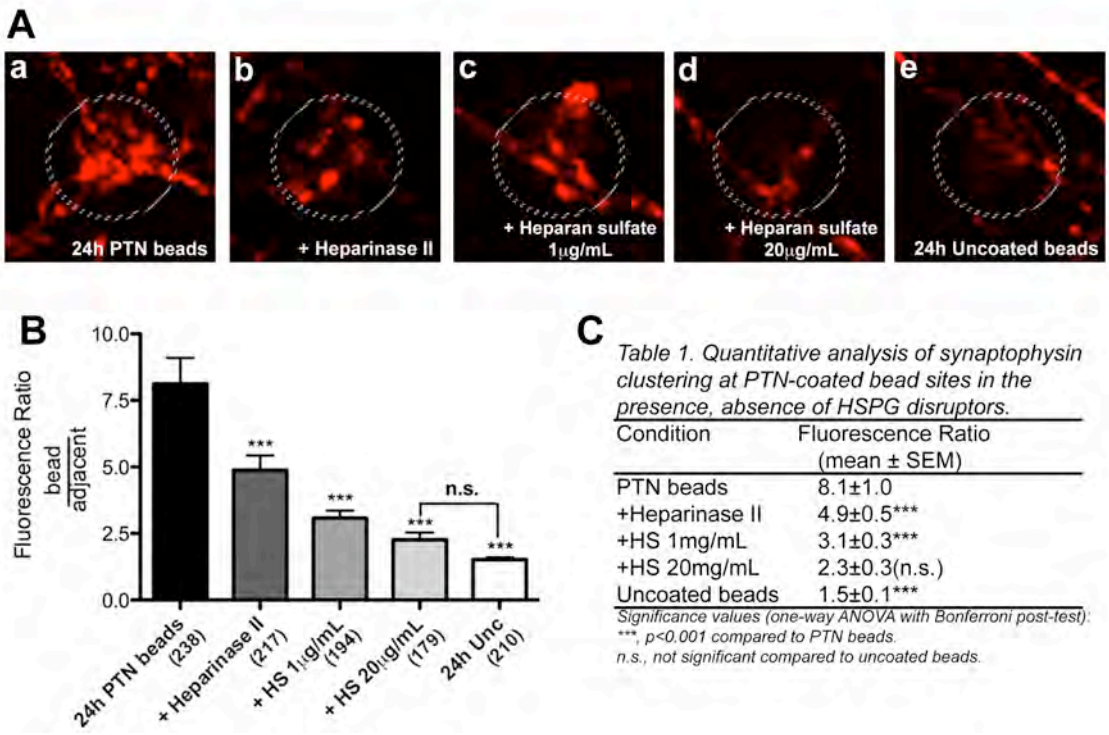
We were intrigued by the cationic-rich amino acid sequence of PTN. In the early seminal studies, it was suggested that PTN, given the clusters of high positive charge on both of its termini, could promote neurite outgrowth in a similar manner as poly-lysine: by electrostatic adhesion (Rauvala, 1989; Merenmies and Rauvala, 1990). Furthermore, it has been reported that PTN can induce presynaptic differentiation (Dai and Peng, 1996b; Rauvala and Peng, 1997), although it had never been confirmed that the inductive effect

involved HSPGs. If true, this molecule represented an avenue through which we could understand how PDL triggers synaptogenesis; specifically, if the cationic properties of PDL promote its affinity for HSPGs, then we should expect a similar phenomenon to take place for PTN. We therefore wished to confirm that PTN could indeed induce presynaptic differentiation by a mechanism involving HSPGs.

Results

PTN-coated beads induce synaptophysin clustering at sites of bead contact

In these experiments, hippocampal cultures were grown to 15DIV and thereafter incubated with beads coated with PTN for 24h. These experiments were quantified from 3 independent experiments and the data is shown in both histogram (B) and table (C) form. In these cultures, we observed a dramatic increase in the intensity of synaptophysin puncta at PTN bead-neurite contacts (Figure 1.2Aa) compared to uncoated bead contacts (Figure 1.2Ae). Following quantification, we found this increase at PTN bead sites to be highly significant compared to uncoated bead sites ($p<0.001$, One-way ANOVA with Bonferroni's post-test, Figure 1.2B,C). When heparinase II, an enzyme that degrades HSPGs, was added to cultures along with PTN-coated beads, there was a significant attenuation of the synaptophysin immunofluorescence (Figure 1.2Ab,B,C). Similarly, the presence of soluble heparan sulfate led to a dose-dependent decrease in synaptophysin fluorescence. The addition of 1μg/mL of soluble HS, like heparinase, also significantly attenuated the enhancement (Figure 1.2Ac,B,C), while the addition of 20μg/mL abolished synaptophysin fluorescence at PTN-coated bead contacts to levels observed beneath uncoated beads (Figure 1.2Ad, B,C). Taken together, these data suggest that PTN, like PDL, can facilitate the clustering of synaptic vesicles, as previously suggested (Dai and Peng, 1996b; Rauvala and Peng, 1997) and does so by a mechanism involving HSPGs.



C

Table 1. Quantitative analysis of synaptophysin clustering at PTN-coated bead sites in the presence, absence of HSPG disruptors.

Condition	Fluorescence Ratio (mean ± SEM)
PTN beads	8.1±1.0
+Heparinase II	4.9±0.5***
+HS 1mg/mL	3.1±0.3***
+HS 20mg/mL	2.3±0.3(n.s.)
Uncoated beads	1.5±0.1***

Significance values (one-way ANOVA with Bonferroni post-test):
****, p<0.001 compared to PTN beads.*
n.s., not significant compared to uncoated beads.

Figure 1.2. PTN induces the clustering of synaptophysin by a mechanism involving heparan sulfate proteoglycans (HSPGs). Imaging and analysis of cultures incubated with beads for 24h and immunolabeled with synaptophysin (red). (A) Representative images of DIV15 neurons incubated for 24h with PTN-coated beads alone (a) or in the presence of heparinase II (b), or soluble HS [1μg/mL (c) or 20μg/mL (d)]. (e), Cultures incubated with uncoated beads). Circle, location of the bead. (B) Histogram, fluorescence ratio measurements in response to the different treatments. Brackets, total number of beads analyzed from three independent experiments. (C) Table outlining quantification and analysis of the different conditions.

PART II: The distribution of glial cells in hippocampal cultures and relationship with PDL-bead induced presynapse formation.

Background

Protocols for the culture of hippocampal neurons can differ greatly. The most complex protocols, in which hippocampal cells are inverted over a layer of glia (predominantly astrocytes) in the presence of inhibitors of cell proliferation, tend to produce the healthiest and longest-lasting cultures since the cells benefit from paracrine trophic support from glia as well as from contact with neurons (Kaech and Banker, 2006). Our hippocampal dissection and culture protocol instead relies on the presence of serum-free supplements to provide trophic support to the hippocampal cultures as they differentiate (see supplemental thesis methods). This widely used protocol for the culture of hippocampal neurons is admittedly much easier to carry out and also produces very robust cultures. However, we made no direct attempts to control the proliferation of glia except to culture hippocampal cells from rat embryos at a developmental stage (E17/18) when they still do not contain many glia (in this region). We were therefore curious to examine the possibility that non-neuronal cells were present in our cultures, and if so, to determine if this affects PDL bead-induced presynapse formation.

To carry out these experiments, we used cultures (DIV14-15) incubated with PDL-coated beads for 24h and stained them with various combinations of the following four antibodies: anti-NeuN, which specifically stains the nuclei of neurons, anti-RIP, a cytoplasmic marker for both immature and differentiated oligodendrocytes, anti-GFAP, an astrocyte-specific cytoplasmic marker, and anti-synaptophysin. These experiments were carried out using a total of three independent cultures.

Results

Astrocytes are present in hippocampal cultures and have a variable distribution

Figure 2A shows three representative images of neuron-bead cultures stained for the distribution of neurons (NeuN, green) and astrocytes (GFAP, red). By visual inspection, we observed three different types of astrocyte distribution. In certain regions of the coverslip, we could observe large populations of astrocytes with few neurons around (Figure 2Aa), although this was very rare. Much more common were observations of neurons in the total absence of astrocytes (Figure 2Ab) or the presence of astrocytic processes extending and touching neurons but not necessarily in contact with their neuronal processes (Figure 2Ac). Taken together we conclude that astrocytes were certainly present in the cultures but had a variable distribution, and appeared to leave the majority of axons and dendrites untouched.

Oligodendrocytes are sparsely distributed in the cultures and do not impede PDL-bead induced presynapse formation

We next performed immunostains on cultures to assess the distribution of oligodendrocytes and their relationship to PDL-bead induced presynapse formation. To do this, cells were dually immunostained with antibodies to RIP, an oligodendrocyte-specific marker, and synaptophysin. Figure 2B shows two representative RIP-synaptophysin image panels. The above image panel shows cultures incubated with PDL-coated polystyrene beads (Figure 2Ba-d), while the lower image panel shows cultures incubated PDL-coated 5μm silica beads (Figure 2Be-h); these beads are much harder to distinguish given their flat appearance and similar refractive index relative to the substrate and are therefore demarcated by the black arrowheads (Figure 2Be).

We found that the distribution of oligodendrocytes was highly sparse, with very few cells present along an entire coverslip. Most of the oligodendrocytes appeared to be immature, elaborating few processes and exhibiting a punctate distribution of the cytoplasmic marker RIP (Figure 2Bg), and we found a single differentiated cell in all three experiments examined (Figure 2Bc). In the above image panel (Figure 2Ba-d), a PDL-

coated polystyrene bead contacts the highly differentiated oligodendrocyte directly (Figure 2Ba, white arrowhead). There appears to be no synaptophysin clustering at this oligodendrocyte-PDL bead contact (Figure 2Bb, right circle). In contrast, there is another bead (black arrowhead) touching an axon that does have synaptophysin clustering (Figure 2Bb, left circle).

The lower panel shows what appears to be a much less differentiated oligodendrocyte, which was far more commonly observed (Figure 2Be-h). There are 2 beads directly contacting both an axon and an oligodendrocyte process, and there are clearly synaptophysin clusters along the axon (Figure 2Bf, white circles). Taken together, these results suggest that oligodendrocytes, where present, have no detrimental effect on PDL bead-induced presynapse formation along axons, but do not respond to the beads themselves.

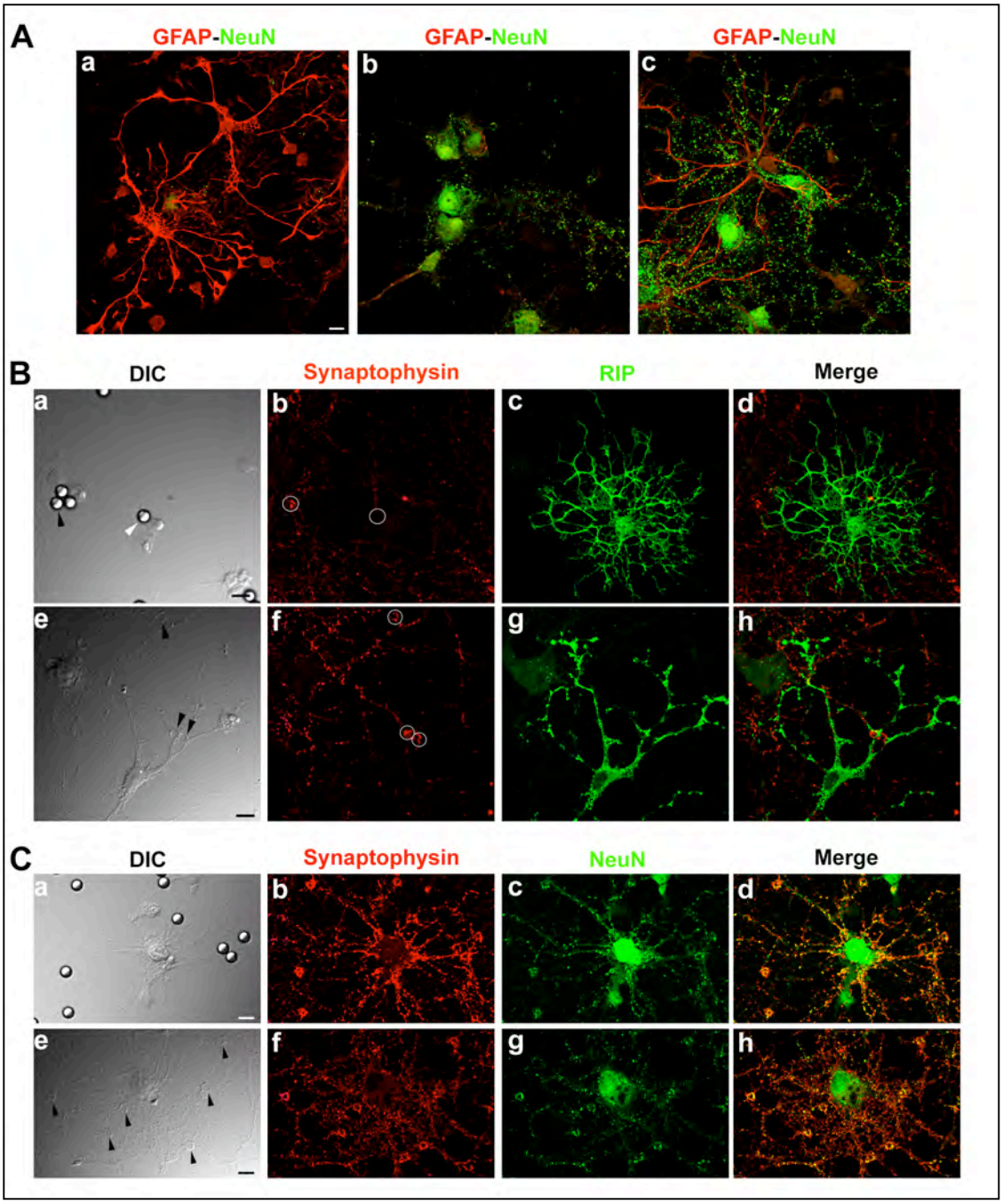
PDL-coated beads induce synapse formation selectively along neuronal processes

Figure 2C shows two representative image panel of cultures immunostained for synaptophysin (red) and the neuron specific marker NeuN (green). Like Figure 2B, the above (Figure 2Ca-d) and lower (Figure 2Ce-h) image panels show cultures incubated with PDL-coated polystyrene or silica beads, respectively (bead sites demarcated by black arrowheads).

In both sets of images we observed robust synaptophysin clustering at sites of PDL-bead contact. Furthermore, we found that the processes contacting PDL-beads arise from cells containing NeuN-positive nuclei (Figure 2Cb-d and 2Cf-h). We did observe a curious distribution of NeuN, whereby neuronal processes contained NeuN-positive puncta that appeared enhanced at PDL bead sites. It is unclear whether this distribution is an artifact of the staining protocol, although considering that we observed this phenomenon using two different secondary antibodies this seems unlikely. Alternatively this distribution could be an issue with the primary antibody itself. However, considering that the sole purpose of using NeuN was to identify neuronal cells, the antibody did serve its purpose

and we remain comfortable in concluding that PDL-coated beads induce presynapse formation selectively on neurons.

Figure 2. The distribution of astroglial cells in hippocampal-PDL bead cultures and relation to bead-induced presynapse formation (following page). (A) Representative (separate) images of GFAP (red) and NeuN (green)-stained hippocampal cultures. (B) Representative image panels of synaptophysin (red) and RIP (stained hippocampal cultures). (a-d) Image panel centering on a differentiated RIP-stained oligodendrocyte with two PDL-beads in the field (white and black arrowheads, Ba). The bead contacting the oligodendrocyte (white arrowhead in Ba, right circle in Bb) does not contain synaptophysin clusters while the bead contacting presumably axons (black arrowhead in Ba, left circle in Bb) has synaptophysin-positive clusters. (e-h) Image panel showing an immature oligodendrocyte as well as a differentiated neuron along with several PDL-coated silica beads (black arrowheads in Be). Beads contacting both axons and the oligodendrocyte contain numerous synaptophysin-positive clusters (lower two circles in Bf). (C) Representative image panels of synaptophysin (red) and NeuN (green)-stained cultures incubated with PDL-coated polystyrene (a-d) or silica (e-h) beads. Black arrowheads in (Ce) denote the bead sites. Robust synaptophysin clusters are seen along processes that extend from primary neurons. Scale bars, 10μm (all).


A
GFAP-NeuN
GFAP-NeuN
GFAP-NeuN
a
b
c
B
DIC
Synaptophysin
RIP
Merge
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b
c
d
e
f
g
h
C
DIC
Synaptophysin
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PART III: Negatively charged substances do not trigger the induction of postsynaptic specializations.

Background

In Burry's seminal studies, he showed that beads coated with positively-charged compounds exclusively induced presynapse formation, while negatively-charged compounds including poly-glutamic acid did not (Burry, 1980a). From these findings, he concluded that complementary electrostatic adhesion between axons and dendrites is the first step towards synapse formation, whereby clusters of positive charges along axonal membranes would attract negatively charged postsynaptic membranes and/or surface molecules *in situ* (Burry, 1980). We find that PDL-coated beads, while highly adhesive, do not directly trigger postsynaptic development along dendrites (*see Results Section (A): Manuscript*) suggesting that unlike the presynaptic ending, the postsynaptic compartment cannot be induced to form in response to electrostatic adhesion alone. However, if the 'complementary charges' hypothesis is correct, then perhaps attempting to trigger postsynaptic development using a presynaptic trigger is the wrong approach. Is it therefore possible that a concentrated presentation of negatively charged compounds could trigger postsynaptic development along dendrites?

In these experiments, we performed our neuron-bead co-culture assay on DIV14-15 cells. We coated beads with either PDL (as a positive control) or poly-L-glutamic acid (PLG) at three different concentrations: 50μg/mL (same as PDL), 100μg/mL, or 1mg/mL. These beads were incubated with the cultures for 24h, fixed and dually stained for both synaptophysin and PSD95.

Results

PDL-coated beads induce clusters of both synaptophysin and PSD95

As shown previously, we find that PDL-coated beads contacting a plexus of both axons and dendrites can induce the clustering of both PSD95 and synaptophysin after 24h of contact (Figure 3A). These clusters can be observed both in the neuritic plane (0.0μm, Figure 3Ab,c) as well as above the focal plane of the neuron (1.2μm, Figure 3Ad,e). The presynaptic clustering effect was always more robust, whereby synaptophysin clusters were observed at nearly every neurite-bead contact imaged while PSD95 clusters were only observed at a subset (although Figure 3Ad,e shows three highlighted beads).

Poly-L-glutamic acid-coated beads have reduced adhesivity and do not induce any PSD95 clustering

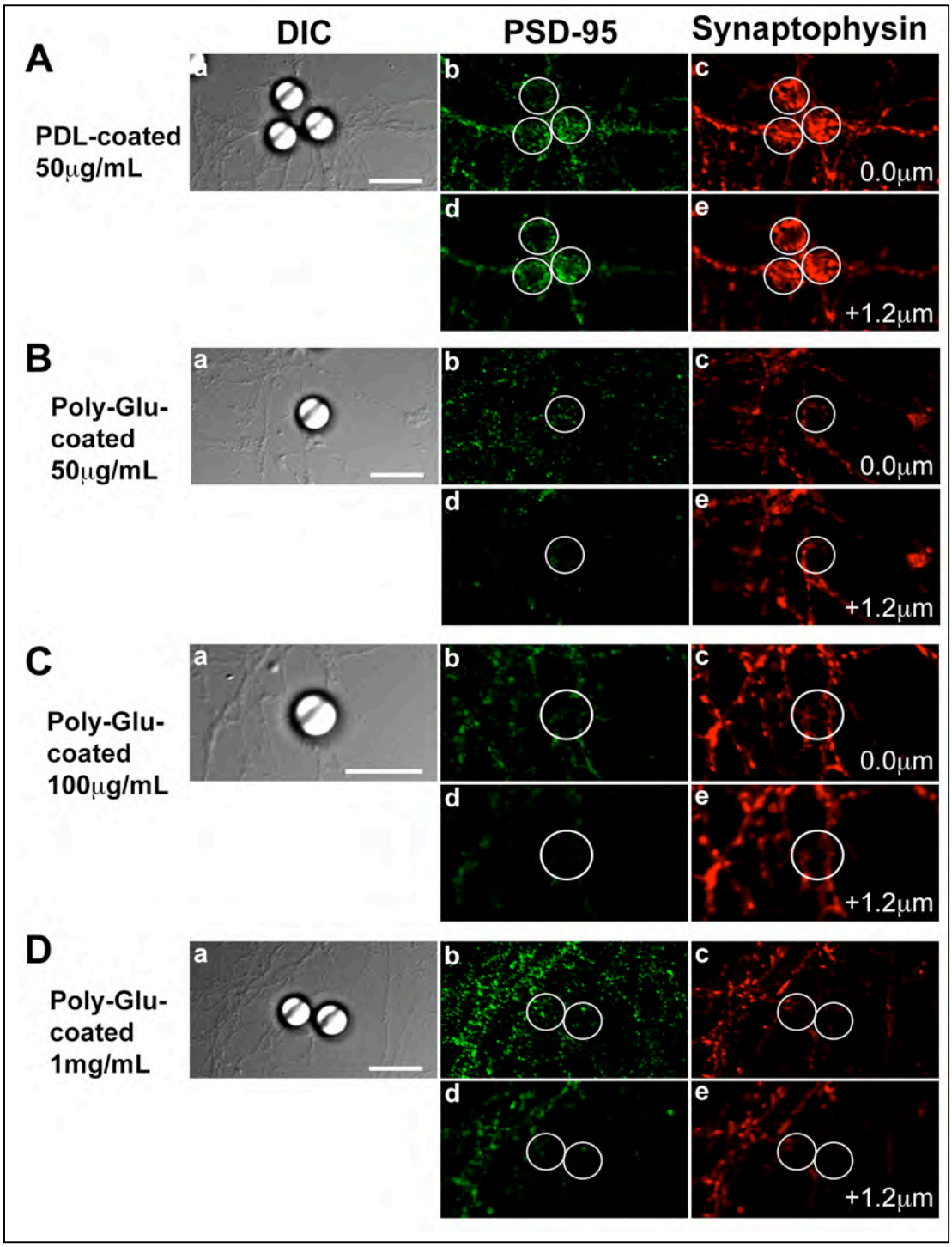
In these experiments we used PLG-coated beads for the first time. The presence of the beads did not appear to negatively affect the health of the cultures, as they remained highly arborized throughout the duration of the bead co-culture. By visual inspection, we found that PLG beads were not nearly as adherent as PDL-coated beads, taking longer to settle on the dish and having a much higher proportion wash away after 24h without ever having attached. By confocal imaging, we found that PLG-coated beads induced neither synaptophysin-positive nor PSD95-positive clusters at sites of contact at any concentration tested (Figure 3B-D). These results clearly indicate that anionic compounds do not trigger postsynaptic development.

There are many possible explanations for these results. It is not surprising that negatively charged beads do not stick to neuronal membranes if axonal and dendritic membranes are similarly negatively charged and thereby would not be electrostatically attracted to PLG-coated beads. By extension, it makes sense that PDL beads would be attracted to all neuronal membranes regardless of whether they are contacting axons and dendrites. Given that PDL-coated beads selectively induce presynaptic development along axons, and not postsynaptic development along dendrites, suggests that mechanisms other than complementary charge-charge interactions are involved in presynaptic induction.

Furthermore, these results suggest that charge-charge interactions are similarly insufficient to predict whether postsynaptic development can occur in response to artificial substrates.

Therefore, perhaps PDL-coated beads trigger presynaptic development by a mechanism involving complementary electrostatic charge-based *adhesion*- whereby PDL-beads induce heterophilic adhesion between bead and axon, and adhesion induces presynapse formation. This is a sufficient requirement for axons to trigger the synaptogenic process, but is insufficient for dendrites, which instead require more specific signals to initiate postsynaptic development. Furthermore, these studies suggest that complementary electrostatic interactions between axon and dendrite *in situ*, as suggested by Burry (Burry, 1980) is not enough— interactions between axon and dendrite must not just be complementary, but adhesive.

Figure 3. Negatively charged PLG-coated beads do not induce postsynaptic development (following page). For each panel, the top three images (DIC+fluorescence, images a-c) show the focused neuritic plane while the two lower images (d,e) are of the same field 1.2μm above the focal plane. Scale bars, 10μm. (A) PDL beads. Note the enhancement of both synaptophysin and PSD95 clusters around the beads. (B-D) PLG-coated beads. We used three different concentrations ranging from 50μg/mL (same as PDL)(B) to 20X that concentration (1mg/mL)(D). We found that none were able to induce the clustering of either synaptophysin or PSD95 at sites of bead contact.


DIC
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Synaptophysin
A
PDL-coated
50μg/mL
a
b
c
0.0μm
d
e
+1.2μm
B
Poly-Glu-
coated
50μg/mL
a
b
c
0.0μm
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C
Poly-Glu-
coated
100μg/mL
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e
+1.2μm
D
Poly-Glu-
coated
1mg/mL
a
b
c
d
e
+1.2μm

DISCUSSION

PREFACE

This final section will discuss relevant considerations of PDL bead-induced synapse formation that have not been mentioned in previous sections (I and II), and will suggest possibilities for how PDL-coated beads interact with the extracellular milieu to promote synaptogenesis (III). It will end with a discussion of the utility of the system to probe questions related to nascent synapse development (IV), as well as potential therapeutic applications (V).

(I) PDL beads and the postsynapse

In this thesis, we found that PDL-coated beads could trigger two types of synaptic development: either the exclusive formation of presynaptic endings, or the formation of both pre and postsynaptic endings at bead sites that was presynaptically driven. Where direct comparisons can be made, we found that the time course of presynaptic development is entirely consistent with that observed in previous studies, lending strength to our assertion that PDL-beads produce presynaptic endings that form and function much like those *in situ*. Furthermore, our findings that the nascent presynapse is a critical player driving postsynapse formation is also consistent with previous work (Friedman et al., 2000; Okabe et al., 2001). However, the results from our time course studies of postsynaptic development diverge from previous studies and may reflect some important differences in the requirements for, and the process of, nascent postsynapse assembly.

In our model system, we observed the rapid assembly of functional presynaptic boutons within 1hr of adding PDL-beads to mature cultures. In contrast, postsynaptic assembly (where it was observed) occurred over a much longer period of time (several hours) and was protracted even relative to other published studies (Friedman et al., 2000; Okabe et al., 2001)*(see Results Part (A): Manuscript, Figure 6)*. What is responsible for this

restrictiveness in general, as well as the enhanced delay in our system? We propose two possibilities.

Possibility I: Differential kinetics of presynaptic vs. postsynaptic accumulation?

We know from previous studies that presynaptic endings can form rapidly in both young (<DIV5)(Sabo et al., 2006) as well as more mature cultures (>DIV10)(Ahmari et al., 2000; Friedman et al., 2000; Colicos et al., 2001), findings which are supported by this thesis. We also know that in immature cultures (7DIV or less), extra-synaptic clusters of postsynaptic proteins such as neuroligins, GKAP and N-methyl D-aspartate (NMDA) receptors are often associated with the subsequent co-clustering of presynaptic proteins (Washbourne et al., 2002; Gerrow et al., 2006). However, these findings have not been replicated in more mature cultures such as those used in our study.

One parsimonious explanation is that the signals that direct pre and postsynapse assembly occur simultaneously during initial axodendritic contact, but the kinetics of assembly is somewhat dependent on the available pool size of synaptic proteins. In this regard, excess pools of postsynaptic proteins in immature neurons could readily accumulate at nascent axodendritic contacts once synapse assembly has been triggered, either before or in close temporal sequence with presynaptic proteins. Alternatively, it is plausible that the ectopic clustering of these excess pools can directly trigger presynaptic assembly in young neurons. However, with neuronal development and synaptogenesis, perhaps this excess pool of postsynaptic proteins becomes reduced. As a result, the assembly of postsynaptic endings would rely more heavily on *de novo* protein synthesis to supply the pool of necessary proteins, resulting in a temporal delay.

If correct, it is intriguing that the situation would be so different presynaptically, given the many studies revealing the lability of protein exchange and accumulation along axons. It is known that immature axons can form presynaptic boutons to mature postsynaptic sites (Fletcher et al., 1994; Barker et al., 2008), that isolated axons can recycle transmitter (Kraszewski et al., 1995; Dai and Peng, 1996a; Krueger et al., 2003),

and that previously formed mature boutons can exchange their contents (Darcy et al., 2006; Tsuriel et al., 2006; Tsuriel et al., 2009). Studies in this thesis further support the concept that the presynaptic ending is a highly dynamic construct that can be readily assembled from, most likely, an excess or easily freed pool of presynaptic proteins (given the speed with which they can be assembled). Collectively, these studies suggest that a rich pool of mobile presynaptic proteins may be more than just a property of axons, but may be required for normal synaptic function throughout development.

How can this explain the enhanced delay in postsynaptic development that we observe at PDL-bead sites? Considering that instead of a native pre-post pair being induced to form simultaneously, the presynapse is selectively triggered to form first in this system. If the postsynaptic "trigger" is therefore further delayed relative to pre, it is plausible that even more time would be required for postsynaptic protein synthesis and/or accumulation.

Possibility II: Differential requirements for triggering?

Although not explored in detail, another explanation for this delay is that perhaps many of the presynaptic molecules that normally direct postsynaptic differentiation are captured by the surface properties of the PDL-beads. Therefore, they would not be present in sufficient numbers within these hemi-presynaptic boutons to trigger a dendritic response that would lead to postsynaptic assembly. This would imply that unlike the presynapse, postsynaptic differentiation requires factors derived from its cognate synaptic membrane and/or the endogenous extracellular milieu. This conclusion is consistent with studies to date, whereby postsynapse assembly has been shown to be induced only by the heterologous expression or exposure to naturally occurring synaptogenic molecules (Graf et al., 2004; Kim et al., 2006; Ko et al., 2006). Assessing which of these possibilities are correct will depend on experiments that better focus on the kinetics of assembly at PDL-bead sites versus native axodendritic contacts, in conjunction with studies that compare the assembly process at PDL-bead sites versus beads or cells presenting native synaptogenic factors.

(II) Is presynaptic morphology regulated by its target?

A striking feature of these bead-induced presynaptic boutons is their size relative to native axodendritic synapses, appearing to form enlarged puncta that grow and spread around the perimeter of the bead (*see Results Part (A): Manuscript, Figure 1, S1*). The beads used in this study were 7μm in diameter- much larger than any dendritic target within the CNS that an axon could encounter. What might this say about the role of the postsynaptic target in determining presynaptic morphology? When considering the ultrastructure of synapses whose targets lie outside of the nervous system, including the NMJ as well as between synapses of afferent vagal axon terminals and myenteric ganglia, it is clear that presynaptic endings are also much bigger than those observed between CNS neurons [see (Gabella, 1972; Ceccarelli et al., 1973; Heuser and Reese, 1973; Hayakawa et al., 2006); reviewed in (Sanes and Lichtman, 1999)], in accordance with the size of the muscle or ganglion cell surface, respectively.

Although presently circumstantial, these data point to an intriguing possibility that the presynaptic bouton is somehow physically regulated by its target, whereby in this system, the combined size and uniform coating of the PDL-bead represents a surface onto which a presynaptic ending is allowed to develop unrestrained. Furthermore, it is likely that our observations are encouraged by *in vitro* conditions that lack restrictive physical and chemical factors present *in vivo*. Therefore, perhaps the *in situ* environment in which synapses are formed represents an optimal balance between permissive and restrictive factors to ensure that not only number but synapse size is correct for the optimal establishment of neuronal circuitry. Manipulating our *in vitro* conditions to address these possibilities is an important avenue of future work.

A strategy to investigate this further would be to modify the physical characteristics of PDL-coated substrates to see if this affects bouton size, number, or distribution. For example, beads of every size range from 100nm to several hundred microns are available commercially, each of which have different curvatures and thus appear qualitatively different to the axon even if coated with the same substance. These beads could easily be combined in a culture dish to see if differences in bead size or curvature affect SV cluster

density, area or volume. Another approach would be to engineer flat substrates to display polylysine in various concentrations and configurations provided that cultures may be exposed to them after a certain stage of development is reached (P.T. Yam, Colman Lab, ongoing studies). As a readout assay, one could easily combine immunofluorescence cytochemistry with 3-D reconstruction of the newly formed boutons and volumetric analysis. Taken together, these studies could yield intriguing insight into the physical role of the target in assembly and morphology of the presynaptic ending.

(III) Further insight into mechanism(s) of PDL bead-induced presynapse formation.

In this thesis, we have performed experiments aimed at understanding how PDL beads trigger presynapse assembly on axons. We have found that (i) the beads are highly adhesive along axonal membranes prior to synaptogenesis, (ii) interfering with endogenous heparan sulfates and/or HSPGs inhibits the effects of PDL-coated beads, (iii) heparan sulfates themselves adsorb onto the bead surface when assayed after 24h and (iv) factors from the postsynaptic ending are not required for PDL bead-induced presynapse assembly. From this collection of findings, we may make certain conclusions but several possibilities remain. Below, I will discuss the different possibilities based on our results, and suggest ways to refine our understanding of how PDL-beads exert their effects.

In the manuscript, we hypothesize that PDL-coated beads adhere directly to surface membranes and suggest that HSPGs, either adsorbed onto the bead surface from the surrounding medium or bound to the bead along with neuronal membranes, are critical endogenous mediators *(see Results Part (A): Manuscript, Figure 3E).* Given that both neuronal membranes as well as HSPGs are negatively charged would imply that the core molecular interaction between PDL beads and their endogenous targets is based on complementary electrostatic attraction. In turn, these molecular interactions facilitate adhesion, and together this triggers presynaptic assembly on axons.

The finding that PDL beads adsorb HSPGs onto their surface at sites well above contact with axons (*see Results Part (A): Manuscript, Figure S4*) would support the possibility that secreted HSPGs have an important role. In contrast, the rapid adhesivity of the beads to the neuronal surface, perturbation of bead adhesion by exogenous addition of HS as well as the finding that syndecan2 clusters at bead sites (*see Results Part (A): Manuscript, Figure 4*) would support the other possibility: that transmembrane forms of HSPGs facilitate the binding of axons to the bead surface. Another piece of information may be gleaned from the heparinase II experiments (*see Results Part (A): Manuscript, Figure 3E)* whereby the addition of beads in the presence of heparinase II significantly attenuated but did not abolish PDL bead-induced synaptophysin clustering. In this latter case, either we added too little heparinase to inactivate all forms of HSPGs, or instead have uncovered another possibility- that there are other endogenous mediators, membrane-bound or otherwise, involved in this phenomenon.

A method to determine which HSPGs and/or other endogenous factors facilitate bead-induced presynapse formation would be to carry out a candidate screening approach. Specifically, one could address this question by using RNAi to knock down the expression of known synapse inducers and determining if this affects PDL bead-induced presynapse formation. If transmembrane HSPGs are important, an obvious first choice would be to knock down syndecans (-2 or -3). Otherwise, one could try neurexins, SynCAM, or any of the other transmembrane molecules previously shown to induce presynapse formation.

Perhaps the most direct way to assess this would be to use a biochemical approach, by directly isolating bead-presynaptic complexes and compiling a full list of proteins attached to the beads by mass spectrometry. This approach would yield information that could feed a number of projects, but above all could shed considerable insight into exactly what is on the bead and axonal surfaces that could behave as synaptogenic triggers.

Probably the trickiest technical aspect to this last idea is to devise a method to isolate pure preparations of beads and presynaptic endings that do not contain fragments of the soma or dendrites. However, I suggest an easy approach that has been tried in a pilot experiment with Dr. G. Gopalakrishnan (Program in NeuroEngineering, Montreal Neurological Institute). This approach is based on studies in heterologous cells, whereby fragments of the plasma membrane were isolated from the cell body simply by sandwiching the cells between the coverslip on which they were cultured and a second coverslip that is placed on top, then removed following a brief application of pressure (Perez et al., 2006a; Perez et al., 2006b). We reasoned that this approach could be applied to neuron-bead cultures to isolate bead-presynaptic complexes. A schematic for our initial approach follows, whereby to see if this could work, we labeled neuronal membranes with the fluorescent vital dye DiI (Figure 1A).

We found that if the second coverslip is removed carefully that indeed, the beads can be removed from their parent coverslip along with patches of DiI-labeled membranes, presumably derived from presynaptic plasma membranes (Figure 1B-D). The yield (i.e., isolated bead-presynaptic complexes) was quite low when derived from a single coverslip, although pooling the isolated complexes from several coverslips at once could yield enough material for mass spectroscopic analysis. While extremely preliminary, such an approach could prove very useful as a way to isolate pure preparations of bead-presynaptic complexes in general, and to isolate synaptogenic factors at PDL-bead contacts if this is the central question.

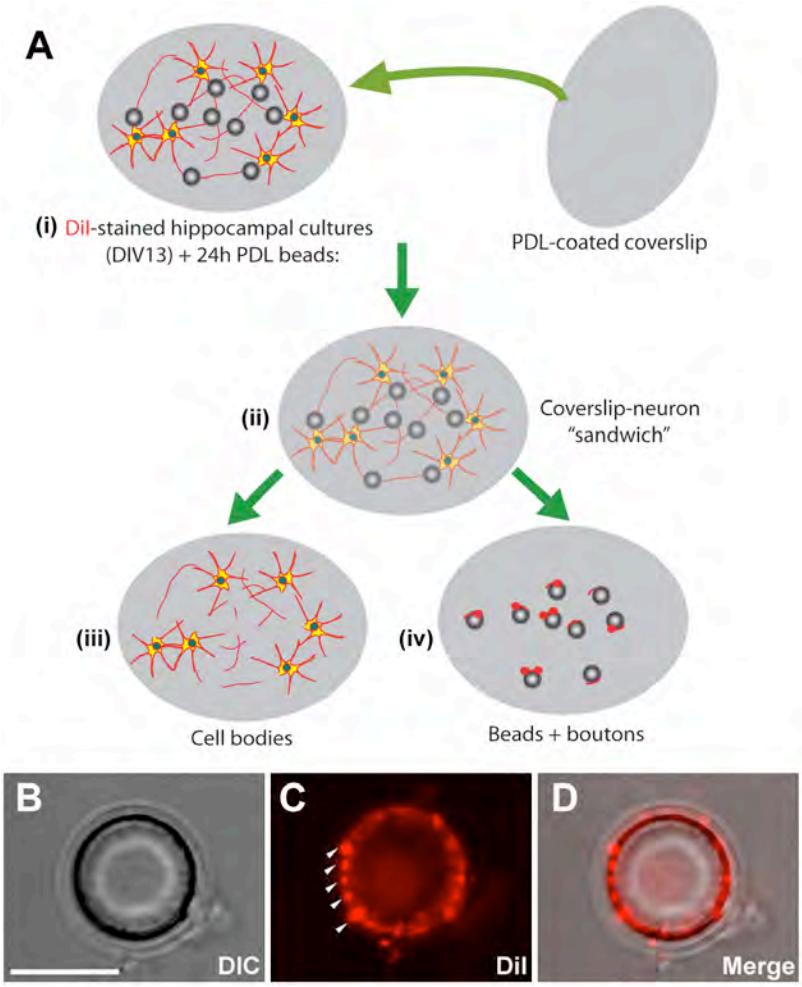


Figure 1. A method to isolate PDL bead-presynaptic complexes. (A), model for the approach. Hippocampal cultures would be grown and incubated with PDL beads as per the usual protocol. In this example, cultures were stained with DiI to label neuronal membranes (i). A PDL-coated coverslip is laid on top of the cultures (ii) and removed with as little lateral movement as possible, resulting in the separation of the cultures from the beads (iii) along with patches of presynaptic plasma membranes attached to the beads (iv). (B-D) Image panel of a bead isolated in this way. Note the DiI labeled clusters along the bead surface in (C)(white arrowheads). Scale bar, 5μm.

(IV) Implications of this model system for the field of synaptogenesis

Defining the nascent synapse

A major challenge in the field of synapse biology has been the definition of synapse "age". Synapses have been studied genetically, biochemically, electrophysiologically, microscopically, behaviourally- and yet, we still don't understand the precise molecular differences between a nascent synapse, a stabilized synapse, a mature synapse, and an aging synapse. These are fundamental questions in neurobiology, some of which we have attempted to address in this thesis.

First, we have created a system that can truly define the nascent presynapse, with the unique advantage of facilitating robust *de novo* assembly of synaptic proteins with precise experimental control. This is achieved by the very fact that the moment an axon touches a bead is a true "time zero" after which we can reliably expect presynaptic proteins to accumulate. In contrast, other paradigms that combine live imaging of cultures expressing fluorescently labeled proteins in the absence of any synaptic triggers rely on the chance but no real assurance that a new synapse will appear during the imaging session. While *in vitro* systems that rely on the addition of heterologous fibroblasts or beads expressing synaptogenic inducers offer similar advantages, PDL-beads are still much simpler to prepare, resistant to degradation and suitable for applications that other systems producing hemi-synapses are not, most notably the ability to induce native pre-post pairs (when axons and dendrites are both in contact with each other and the bead).

Early molecules of the forming presynapse- which come first?

We have shown that following bead contact and adhesion, actin reorganization and synaptic vesicle accumulation are very early events, as is the accumulation of proteins contained within PTVs such as bassoon. Can we make even more precise determinations of which proteins accumulate first? By widening our pool of candidate molecules, one could certainly continue using a combined lentiviral-live imaging-based approach (as shown in Figure 5C-J) and study the temporal accumulation of other presynaptic proteins

at PDL-bead sites. Molecules such as RIM, N-Cadherin and ERC2 have been attempted in pilot studies (A.L.L. with Drs. A.V. Kwiatkowski and C. Maas, Stanford University) and we observed that the accumulation of RIM and ERC2 followed a similar time course as SV2 while N-Cadherin, like SAP-97, did not accumulate at bead sites even after several hours (data not shown). Therefore, the use of a robust and reproducible model system to test a battery of candidate molecules could yield important information into the temporal dynamics of nascent presynapse assembly.

One could also tackle these studies using biochemical approaches such as the one suggested above (Figure 1), whereby beads could be incubated with cultures for increasing periods of time but rather than fixing and staining them (*as shown in Results Part (A): Manuscript, Figure 5A-B*), bead-presynaptic complexes could be isolated and subject to mass spectrometry. In this way, one could observe the entire complement of proteins that accumulate at bead sites as a function of time. This approach could be combined with electron microscopy, to follow the morphological changes that occur at isolated bead-presynaptic complexes. Tubulovesiclar and dense-cored vesicles have been noted at putative nascent sites in previous studies (Kraszewski et al., 1995; Ahmari and Smith, 2002). Our system would be a unique way to confirm that these different types of vesicles are indeed markers of nascent synapses.

This latter approach does depend on a high degree of physical interconnectedness between presynaptic proteins, both with themselves and eventually to the presynaptic membrane attached to the bead. This may be a significant caveat if studying the earliest stages of assembly, when it is perhaps optimistic to expect that the sophisticated scaffolding and transmitter release apparatus is sufficiently in place to be removed intact. And yet, differences in the integrity of the isolated complexes as a function of time would still be a highly informative outcome.

Nascent synaptogenesis and vesicle fusion

Many laboratories have reported that isolated SV clusters can recycle transmitter in an activity-dependent manner (Kraszewski et al., 1995; Dai and Peng, 1996a; Coco et al., 1998; Verderio et al., 1999; Zakharenko et al., 1999; Krueger et al., 2003; Matteoli et al., 2004). However, some studies have shown these SV clusters have unique properties that distinguish them from SV cycling at mature synapses, namely a differential sensitivity to Ca++ (Coco et al., 1998), an increased sensitivity to brefeldin A, a GTP-binding protein inhibitor that affects intracellular trafficking (Zakharenko et al., 1999), and a reduced sensitivity to tetanus toxin mediated by a TTX-resistant isoform of VAMP2 (Verderio et al., 1999; Matteoli et al., 2004). Although isolated presynaptic clusters have been shown to exhibit mature release characteristics (Krueger et al., 2003), this same study showed that the presence of the postsynaptic element further affected the structure and function of the presynaptic bouton by increasing the size of the recycling SV pool and influencing the coupling of calcium influx with neurotransmitter release. Taken together, these studies reveal several mechanisms for transmitter release, and suggest that presynaptic release properties are influenced by the postsynaptic element. However, it remains unclear whether these "immature" forms of SV cycling are characteristic of just mobile readily-releasing presynaptic sites, or hallmark features of a nascent presynapse. Furthermore, it is unclear how and when the postsynaptic element influences the release properties of the presynaptic ending.

We show that transmitter can be recycled within 1h of bead contact along axons (*see Results Part (A): Manuscript, Figure S5*). However, we have never directly tested whether bead-presynaptic boutons induced to form by PDL beads exhibit some of these immature release characteristics observed at isolated native boutons at some point in their early life. If we can assume that PDL beads induce the formation of *bona fide* presynaptic endings, then perhaps we can use our system to test whether these properties of isolated boutons are in fact features present in all nascent presynaptic boutons, and to determine at what 'age' these boutons transition into presynaptic endings that exhibit mature release characteristics. Direct comparisons can easily be made in this system between bead-axon and bead-axon-dendrite contacts and thus one could directly assess

whether presynaptic boutons more rapidly acquire mature release characteristics if they are contacting a putative postsynaptic element.

Nascent synaptogenesis as a function of neuronal maturation

Studies of synapse formation *in vitro* have utilized cultures that can be considered highly immature (DIV3) to fully mature (DIV10-21) and have observed a range of mechanisms of assembly, some driven by the postsynaptic element and many by the presynaptic element (Friedman et al., 2000; Bresler et al., 2001; Okabe et al., 2001; Gerrow et al., 2006; McAllister, 2007). It is interesting that the assembly of postsynaptic endings tends to be more rapid in younger cultures (Washbourne et al., 2002; Gerrow et al., 2006). However, the direct question of whether the characteristics of assembly change as a function of neuronal differentiation has yet to be addressed. This question is inherently challenging in the absence of focal synaptic inducers, since it is very difficult to (i) separate the process of synaptogenesis from other forms of neuronal differentiation in young neurons, most notably neurite outgrowth and protein synthesis and (ii) to observe newly forming synapses in mature cultures when many have already been formed.

The present system is once again ideal for such studies. Using PDL-beads as a way to stimulate presynapse formation, we have shown that dynamic actin reorganization is required for all newly forming boutons independent of neuronal age *(see Results Part (A): Manuscript, Figure 5G-H)*. This result is in contrast to previous studies suggesting that the role of actin in presynaptic assembly is developmentally regulated (Zhang and Benson, 2001). Our result is not contradictory, but corrects an experimental confound in the previous study given that they were studying not one, but two overlapping phenomena that could not be resolved with respect to each other: that of neuronal differentiation and synaptic differentiation. By observing differences in uniformly-treated cultures, they could only see the effect of actin disrupting agents on presynapse number in young cells, presumably when they are just forming their first wave of synapses. In mature cells, LatA was added presumably after many boutons presumably had already been formed. The latter finding establishes that fully formed presynaptic endings retain

their structural characteristics in the absence of actin, but could not directly address the requirement of actin reorganization for *de novo* assembly. The precise experimental control offered by PDL beads thus allowed us to focus our attention on boutons that could clearly be defined as "new", allowing us to directly address the role of actin in presynapse assembly by pharmacological as well as live imaging techniques.

In our study, we show that PDL-coated beads can induce synaptophysin clustering along axons as early as 7DIV and that cultures as mature as 21DIV can also be induced to accumulate synaptophysin clusters *(see Results Part (A): Manuscript, Figure S1)*. In spite of the similar outcome, these cells are exposed to beads at different stages of *in vitro* development and it is thus plausible that they derive their presynaptic material using different mechanisms. For example, we know that in cultures that are at least 7DIV, protein synthesis is not required for normal exchange of presynaptic material between boutons (Tsuriel et al., 2006) nor for SV accumulation at PDL-coated bead sites (Burry, 1985). Is the same true of younger cultures, which presumably have much less presynaptic material?

It is also known that established boutons can readily exchange their material (Darcy et al., 2006; Tsuriel et al., 2006), and can in some instances break off to form independent functional clusters (Krueger et al., 2003). Does this imply that new boutons forming in mature cultures exclusively derive their contents from preexisting boutons? To address these questions, one could combine time-lapse imaging of cultures expressing SV2-EGFP or other fluorescently labeled presynaptic proteins in the presence of various inhibitors of protein synthesis and transport. In this way, one could determine the source of presynaptic material at PDL-bead sites as a function of neuronal development.

Inductive capacity of the axon revealed by PDL beads

Finally, it is worth noting that our studies imply that axons are fully competent to form presynaptic endings anywhere along their length (Jontes and Phillips, 2006). This concept was recently challenged in a study by Sabo and colleagues, where they observed

that presynaptic boutons establish preferentially at sites where STVs stabilize prior to target contact (Sabo et al., 2006). From these studies, they conclude that synapses can only form at restricted sites along axons where presynaptic proteins stabilize, which they define as "pause sites" (Sabo et al., 2006). In our studies, we dropped beads at random and were struck by how rapidly SV2-GFP labeled clusters could accumulate. This never seemed to be dependent on location, as PDL-beads contacting axonal sites distal as well as proximal to the soma, isolated from contact with dendrites and those contacting dendrites, all resulted in robust presynaptic assembly. We cannot find any reason to assume that the beads selected for sites along the axons predestined to assemble synapses, and thus conclude that the axon is remarkably flexible and fully able to respond to synaptogenic triggers irrespective of the location along the axon where the "trigger" is placed.

Taken together, the above proposed experiments underscore the utility of this system to address fundamental questions in neurobiology, the most relevant being the ability to define many aspects of the nascent presynapse.

(V) PDL beads: Implications for disease and therapeutic strategies

The discussion above focused exclusively on the use of PDL beads to resolve fundamental questions in synapse biology. However, can these beads be exploited therapeutically? If axons *in situ* can respond to PDL beads as they do *in vitro*, then perhaps these beads can be used to encourage synaptogenesis following any number of diseases in which synapses are affected, including neuronal damage by injury or stroke.

In fact, we already know that PDL beads implanted into healthy brains induce the formation of presynaptic SV clusters (Burry, 1983). This is an encouraging finding that offers substantive hope that perhaps these beads can be used to a therapeutic endpoint. To first address this possibility *in vitro*, damaged axons (by laser ablation or other forms of introduced physical trauma) would have to be encouraged to regrow on a permissive substrate and then exposed to PDL-coated beads, which hopefully would trigger the

reformation of lost synapses and potentially the reconstruction of damaged circuits. If this can be achieved *in vitro*, then addressing the *in vivo* state could involve injection of PDL coated beads into a lesion site to determine whether they can encourage synapses to form or neurite outgrowth or both, considering that substrate bound poly-lysine is known to encourage both. While highly speculative at this point, this approach if successful does hold promise as a strategy to facilitate regeneration in the nervous system.

CONCLUSION

Poly-lysine, an adhesive cationic polymer, encourages synaptogenesis by triggering the assembly of presynaptic endings. We find that the entire postsynaptic membrane can be replaced by a PDL-coated bead, suggesting that axons are fully primed for self-assembly once presented with the appropriate inductive cue. The positively charged PDL-bead surface possesses a remarkable capacity for binding to axonal membranes and likely drives the clustering of endogenous factors from the extracellular milieu and/or the axonal membrane. Given that the ECM is ubiquitous, highly adhesive, and a critical scaffold for the heterophilic clustering of many morphogenic factors points to the possibility that the ECM contributes trophic and adhesive support to trigger presynapse formation. In contrast, we find that an artificial adhesive substrate is insufficient to directly trigger postsynapse assembly, which can only be observed at bead sites already containing presynaptic-like endings. We conclude that the postsynaptic membrane is comparatively restrictive in its target selection, relying on native cues from the presynaptic membrane or extracellular environment to trigger assembly.

In this thesis, we suggest from these findings that (i) the earliest stages of presynapse assembly do not require high specificity but simply require that a target be adhesive as a first step and (ii) the nascent presynapse, in turn, is a critical player driving postsynapse assembly. These findings support studies showing that postsynapse assembly is protracted relative to pre, as well as the many studies showing that presynaptic endings can easily form at ectopic sites, or in the absence of dendritic contact altogether. Furthermore, they are consistent with hypotheses of synaptogenesis based on the concept of selective stabilization, whereby synapses are formed exuberantly before they are pruned back after their formation (Changeux and Danchin, 1976; Katz and Shatz, 1996; Jontes and Phillips, 2006). This process is seen from rodents to humans (Lund et al., 1977; Huttenlocher, 1979; Rakic et al., 1986; Webb et al., 2001; Levitt, 2003) and is plausibly based on a mechanism that allows synapses to form unrestrained during development. Taken together, the work described in this thesis attempts to address some

fundamental questions in neurobiology, and will hopefully prove highly useful in addressing many others in the future.

APPENDIX I: SUPPLEMENTAL THESIS METHODS

PREFACE

This section describes in detail the most critical protocols used throughout the thesis, in particular, protocols for the dissection and culture of neurons, bead preparation, processing of cells for immunocytochemistry and transmission electron microscopy, as well as the parameters for image acquisition and quantification. In addition, these protocols include the methods for the additional experiments *(see Results section (B))* not contained within the manuscript "Rapid assembly of functional presynaptic boutons triggered by adhesive contacts." All other protocols may be found in the materials and methods section of the manuscript (*see Results section (A): Manuscript)*.

BASIC PROTOCOL I: Dissection and culture of hippocampal neurons

Cultures derived from the hippocampus are the in vitro preparation of choice for many types of studies, particularly those focused on the establishment of neuronal polarity as well as studies of synaptic development. These cultures follow a well-characterized pattern of development, beginning with the extension of a series of primary undifferentiated neurites, the single spontaneous extension of one neurite beyond all others, which becomes the axon, growth and arborization of axons and dendrites, and eventually the synthesis of synaptic proteins and formation of synaptic connections (Banker and Cowan, 1977; Dotti et al., 1988; Kaech and Banker, 2006). There are many protocols for the culture of these neurons and most are derivatives of the classic protocol of Banker, in which cells are cultured on coverslips that are inverted over an astrocyte feeder layer (Banker and Goslin, 1998; Kaech and Banker, 2006). This method was used for the live imaging experiments of lentiviral-infected proteins (*see Results section (A): Manuscript, Figure 5 and materials and methods*). However, for the majority of the experiments contained within this thesis, neurons were dissected and cultured in a defined artificial medium as follows.

Materials

Cell culture incubator, set to 37°C and humidified at 5% CO2

Culture and dissection media (*all from Invitrogen*):

Advanced DMEM (1X) *(#12491-015)*

B-27 Serum-Free supplement (50X) *(#17504-044)*

Horse serum *(#16050-122)*

Neurobasal medium (unsupplemented, 1X, no L-glutamine) *(#21103-049)*

Penicillin-Streptomycin-Glutamine (100X) *(#10378-016)*

0.25% Trypsin-EDTA *(#25200-056)*

Culture tools:

Coverslips, 18mm diameter, autoclaved *(e.g. Fisher, #12-545-100)*

Falcon tubes (15mL), plastic, sterile *(e.g. VWR, #CA21008-929)*

Hemocytometer (e.g. Fisher Scientific, # 02-671-54)

Petri dishes, 60mm plastic tissue culture-grade, sterile

Petri dishes, 10cm plastic, sterile

Plastic transfer pipettes, sterile *(e.g. VWR, #CA414004-002)*

Plates, 4-well, plastic tissue culture-grade, sterile

Pasteur pipets, 9" (glass), autoclaved *(e.g. VWR, #14673-043)*

Tabletop centrifuge

Trypan blue solution (e.g. Invitrogen, #15250-061)

Vacuum aspirator with tubing to accommodate glass pipets

Dissection tools:

Dissecting microscope, at least 5x magnification with transmitted light base

Forceps, fine (2), Dumont # 5 (Fine Science Tools, #11252-23)

Forceps, 1x2 teeth, small (Fine Science Tools, #11023-12)

Forceps, 1x2 teeth, large (Fine Science Tools, #11021-15)

Scissors, Iris (Fine Science Tools, #14060-10)

Scissors, Surgical (Fine Science Tools, #14002-16)

Poly-D-lysine, lyophilized *(Sigma, #P1149)*

Rat, Sprague-Dawley, pregnant at E17/18 (Charles River, Quebec)

Sterile ddH_2O (Millipore-filtered)

Please note that that all steps must be taken with utmost care and using sterile procedures (unless otherwise stated).

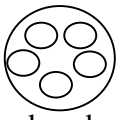
Protocol

(i) Prepare the medium

1. Prepare the supplemented Neurobasal medium (to be used for the culture of neurons at the time of plating and thereafter): Per 500mL bottle of unsupplemented Neurobasal medium add 10mL (one bottle) of B-27 and Penicillin-Streptomycin-Glutamine to a 1X final concentration. Mix and store at 4°C for up to 2 months.
2. Prepare the DMEM-horse serum medium (to be used during the dissection). Add 10mL of horse serum to 90mL of Advanced DMEM, mix and prepare 5mL aliquots. Store at 4°C for up to 3 months.

(ii) Prepare the substrates

3. Arrange 15 coverslips per 10cm Petri dish so that all lie flat and none are touching each other.
4. Cover completely with a solution of 50μg/mL PDL diluted in sterile ddH_2O. (If using 4-well plates, add enough PDL solution to completely fill bottom of each well to be used.)
5. Leave overnight in laminar hood or refrigerator and wash 3X with sterile water on the morning of dissection. (*Note: cover with foil if leaving in hood with UV light turned on. Also, one can do this step the morning of dissection if necessary, simply place PDL-coated surfaces in the cell culture incubator for 2-3h and then rinse.*)
6. Transfer the coverslips to a 60mm dish, 5 slips per dish and arrange flat:



7. Add 5mL per dish of supplemented Neurobasal medium, place inside the cell culture incubator until the cells are ready to be plated. *(Note: This helps the medium to achieve the correct pH and temperature prior to the addition of cells, thereby improving cell survival.)*

(iii) Prepare dishes and tools needed for the dissection

8. Place dissection tools inside a plastic or glass beaker containing 70% ethanol. Be sure to line bottom of the beaker with 1-2 lint-free tissues *(e.g. Kimwipes)* to protect the tips of the dissection tools from dulling or bending.
9. Prepare a 10cm Petri dish containing unsupplemented Neurobasal medium as well as 3-10cm Petri dishes containing sterile water in which to wash and place the embryos.
10. Prepare several 10cm (2-3) and 60mm (6-8) dishes containing unsupplemented Neurobasal medium and place beside dissection hood (for collecting the brains, hippocampi).
11. Spray dissection hood with 70% EtOH and turn on blower just prior to beginning dissection.

(iv) Remove the embryos

This step may be done at the lab bench.

12. Line lab bench with a clean blue diaper and have ready the 10cm Petri dishes filled with water, unsupplemented Neurobasal medium and utensils in 70% ethanol, along with a spray bottle containing 70% ethanol.
13. Euthanize the pregnant rat by CO_2 asphyxiation.
14. Transfer rat to the blue diaper at bench and place belly side up. Spray the belly thoroughly with 70% ethanol.
15. Using large toothed forceps, lift superficial layer of skin/fur and cut with surgical scissors to open belly. Create pocket underneath skin by using scissors to loosen skin from underlying connective tissue/muscle. Be sure not to puncture underlying tissue (to avoid contamination).
16. Clean tools briefly in 70% EtOH, then cut muscle/tissue to expose the internal organs. Avoid touching the fur.
17. With large toothed forceps, find the embryos and remove using surgical scissors, keeping the embryonic sac intact as much as possible. Cut away as much excess tissue as possible as the embryos are removed.
18. Pass embryos through Petri dishes containing water, removing as much blood as possible. In between passages, spray embryos with 70% ethanol.

19. Transfer embryos to dissection hood in the last Petri dish containing Neurobasal.

(v) Dissect the brains and isolate the hippocampi

20. Have everything in place at the dissection hood: Tools (fine forceps, iris scissors in beaker containing 70% ethanol), 10cm and 60mm Petri dishes containing Neurobasal, 70% ethanol spray bottle.
21. Using the small toothed forceps and surgical scissors, remove embryos from amniotic sac and decapitate.
22. Place heads inside fresh 10cm Petri dish containing Neurobasal.
23. Dissect brains: Holding brain using small toothed forceps, dorsal side up, cut skin and skull in a rostral direction beginning at the base of the head.
24. Fold away skull using forceps and gently scoop out brain from the ventral surface.
25. Place collected brains inside a 10cm Petri dish containing Neurobasal.
26. Transfer all brains at least once to a new 10cm Petri dish (to remove excess blood).
27. Transfer brains to 60mm Petri dishes, 3 brains per dish.
28. Using fine forceps, divide brain in half by separating the 2 hemispheres.
29. Remove the meninges.
30. Gently separate cortex from midbrain and turn over to reveal ventral side. The hippocampus lies within the medial ventral surface of the cortex, and may be identified by its white colour and curved shape.
31. Remove hippocampus using forceps/scissors and place inside fresh 60mm Petri dish containing Neurobasal.
32. Continue with remaining hemisphere, brains.
33. Continue as described above until you have collected enough tissue.

(Note: All subsequent steps should be performed in the cell culture hood.)

34. Transfer hippocampi to sterile 15mL Falcon tube.
35. Gently remove medium using sterile plastic Pasteur pipet and add 5mL of 0.25% Trypsin-EDTA solution.
36. Incubate for 21min in a 37°C water bath.
37. Remove trypsin with sterile plastic pipet and add 5-10mL of Advanced DMEM-10% horse serum. Incubate 5-10min in a 37°C water bath.

38. Transfer tissue to new 15mL Falcon tube containing 2-3mL unsupplemented Neurobasal medium.
39. Wash tissue 2X with 1mL unsupplemented Neurobasal medium.
40. Eliminate medium following 2nd wash, add 2-3mL fresh unsupplemented Neurobasal medium, and triturate gently using sterile plastic pipet until cells are dispersed and medium appears homogeneous.
41. Optional step: Bring volume up to 5mL using HBSS, mix cells and let settle 5-10min (to remove remaining large debris). Transfer supernatant to a fresh 15mL Falcon tube.
42. Centrifuge for 3min at 1000g.
43. Aspirate medium and resuspend cells in supplemented Neurobasal medium.

(vi) Count and plate the cells

Count the cells:

44. In an eppendorf tube, combine 50μL each of the cell suspension and Trypan blue solution.
45. Add to 10μL to the hemocytometer and place glass coverslip carefully on top to avoid bubbles.
46. Count 1-16 square area (found at each of the 4 corners of the counting chamber).
47. Multiply number of cells by $2x10^4$; this is the number of cells/mL.
48. Plate cells according to the desired density. For culture of cells not to be transfected, plate at minimal density (approximately 50,000-75,000 cells per 18mm coverslip). For cells to be transfected, plate at a higher density (75,000-100,000 cells per 18mm coverslip). For cells to be cultured in a 4-well plate for EM, plate at a density of 50,000 cells/well (and final volume of 1mL/well).
49. Place dishes in culture incubator and leave for at least 3 days prior to changing the medium. (*Note: For cells plated on coverslips, the coverslips may begin to float overnight. Be sure to check the cultures within a few hours of plating (or the next morning at the latest) to put back into place using sterile forceps*.)

50. Change the medium by replacing 1/3 of the volume per dish/well with fresh, warmed supplemented Neurobasal medium, every 2-3 days, until cells have reached the desired stage of development for experimentation.

SUPPORTING PROTOCOL I: Transfection of cells with pEGFP-C1 plasmid

These transfections were performed to assess the relative changes in axonal volume at sites of PDL-bead contact, by filling the axon with GFP expressed from a soluble EGFP construct. The results are described in Results section (B): Part IV.

Materials

pEGFP-C1 plasmid DNA *(Clontech, #6084-1),* concentration of 1μg/mL

Lipofectamine^{TM} 2000 plasmid transfection reagent *(Invitrogen, #11668-019)*

Neurobasal medium (unsupplemented, 1X, no L-glutamine) *(Invitrogen, #21103-049)*

Neurobasal medium supplemented with B-27, L-glutamine and penicillin-streptomycin (See Basic Protocol I)

Cultured hippocampal neurons, 7-8 days in vitro (DIV), cultured on 18mm coverslips at a density of 75,000-100,000 cells/coverslip

Plates, 12-well tissue culture-grade, sterile

Protocol

(i) Prepare the DNA-Lipofectamine complexes

1. In a sterile eppendorf tube, combine 1.6μg/well of DNA with 100μL/well of unsupplemented Neurobasal medium.
2. In a second sterile eppendorf tube, combine 4μL/well of Lipofectamine 2000 reagent with 100μL/well of unsupplemented Neurobasal medium. (*Note: Steps 1 and 2 contain the correct volume and quantity of reagents for transfection in 12-well plates.)*
3. Add the DNA solution to the Lipofectamine solution dropwise, mix gently and leave untouched at room temperature for 25min.

(ii) Transfer the coverslips and transfect the cells

4. To a new 12-well plate add 500μL/well of fresh supplemented Neurobasal medium. Place the plate in the incubator *(Note: This allows the medium to reach proper temperature and pH prior to addition of the cells).*
5. When step 3 is complete, transfer the coverslips to be transfected into the 12-well plates containing the warmed supplemented Neurobasal medium. Add 500μL/well of the cell-conditioned medium. *(Note: Return the remaining cell-conditioned medium to the incubator. Do not discard. This will be needed at the end).*
6. Add 200μL/well of the DNA-Lipofectamine mixture dropwise.
7. Mix by very gently pipetting up and down and/or rotating the plate.
8. Return plate to incubator and leave for 5-6h.
9. Remove all medium and replace with the saved neuron-conditioned medium.
10. Return the 12-well plate to the incubator and leave the cells for at least 48h for optimal DNA expression.

BASIC PROTOCOL II: Preparation of coated beads.

This protocol describes the methods used for coating beads with poly-D-lysine (PDL), poly-L-lysine-FITC, poly-L-glutamic acid or the endogenous growth factor pleiotrophin (HB-GAM). The glutamic acid and both lysine polymers were coated by passive adsorption onto the bead surface while pleiotrophin/HB-GAM was linked to beads by covalent attachment. Beads of different composition and size were used for certain experiments in the thesis and these will be noted where appropriate.

Note: Use sterile procedures at all times.

Materials

Beads (all from Bangs Laboratories, stock stored at 4°C):

Polystyrene beads, 7.2μm diameter, 10% solids *(#PS06N/5856)*

Polystyrene beads, 6.5μm amino-functional, 10% solids *(#PA06N/5509)*

Silica beads, 4.9μm diameter, 9.9% solids *(#SS05N/4364)*

Solutions:

1) **PBS (1X):**

To 450mL of ddH_2O add: 4g NaCl
0.1g KCl
0.7099g Na_2HPO_4
0.12g KH_2PO_4

Adjust the pH to 7.2 with HCl/NaOH.

Adjust volume to 500mL with ddH_2O.

Filter-sterilize and store at room temperature or 4°C.

2) **0.2M Ethanolamine in PBS, filter-sterilized**

3) **10mg/mL bovine serum albumin (BSA), Fraction V in PBS, filter-sterilized**

4) **Storage buffer:** 10mg/mL BSA-PBS solution + 0.1% NaN_3 and 5% glycerol, filter-sterilized

5) **8% glutaraldehyde in PBS**

Proteins:

Pleiotrophin/HB-GAM, human recombinant, lyophilized *(Cell Sciences, #CRP600B)*

Poly-L-glutamic acid, molecular weight 50,000-100,000 *(Sigma, #P4886)*

Poly-D-lysine, molecular weight 150,000-300,000 *(Sigma, #P1149)*

Poly-L-lysine, FITC-labeled, molecular weight 15,000-30,000 *(Sigma, #P3543)*

Protocol

(i) Preparation of poly-D-lysine coated beads

Note: Unless otherwise stated, these beads were used for all experiments in this thesis.

1. In a 15mL Falcon tube, resuspend 10million 7μm polystyrene beads (stock = $4.612x10^8$ beads/mL) in 3mL PBS. Be sure to mix the bead stock very well as the beads tend to settle when not being used.
2. Wash 3x in sterile PBS by centrifugation, spinning at 3500xg for 3-4min each time. The beads are highly disperse and tend not to pellet well, so be sure to add no more than 3mL PBS for each wash and to be careful when aspirating PBS.
3. After final wash, resuspend beads in 12-14mL of a poly-D-lysine solution (50μg/mL in PBS unless otherwise stated). Place at 4°C on a rotating shaker for overnight end-to-end mixing.
4. The next day, wash beads 3x in sterile PBS by centrifugation.
5. Resuspend in either PBS (if storing coated beads at 4°C) or in supplemented Neurobasal medium (if using all of them immediately) to a concentration of 10^6 beads/mL.
6. Beads coated in this way can be stored for up to one week at 4°C.
7. For uncoated bead controls, follow all the same steps and simply replace the PDL or other protein solution in step 3 with a similar volume of PBS. These can be stored for longer periods of time (up to one month).

(ii) Preparation of pleiotrophin/HB-GAM coated beads

8. These beads were used exclusively for the experiments testing the presynaptic clustering effect of pleiotrophin in response to inhibitors of HSPGs (*See Results (B): Part I*).

9. In a sterile eppendorf tube, resuspend 8million 6.5μm amino-functional polystyrene beads (stock = $6.6x10^8$ beads/mL) in 1mL PBS. Be sure to mix bead stock very well as they tend to settle when not being used.
10. Wash beads in PBS 3x by centrifugation (13,000xg, 2min each wash).
11. Resuspend beads in 8% glutaraldehyde-PBS solution (to activate NH2-functional groups) and incubate at room temperature for 4h with end-to-end mixing.
12. Wash beads in PBS 3x by centrifugation (13,000xg, 2min each wash).
13. Resuspend beads in an eppendorf tube containing 10μg pleiotrophin/HB-GAM diluted in 500μL PBS.
14. Incubate overnight at 4°C on a rotating shaker with end-to-end mixing.
15. The next day, incubate beads in 0.2M ethanolamine in PBS for 30min, followed by 10mg/mL BSA in PBS for 40min.
16. Wash beads 2x in PBS by centrifugation (13,000xg, 2min each wash).
17. Resuspend beads in storage buffer (if storing coated beads at 4°C) or in supplemented Neurobasal medium (if using all of them immediately) to a concentration of 10^6 beads/mL.
18. Beads coated in this way can be stored for up to two weeks at 4°C.
19. For uncoated bead controls, follow all the same steps and replace the pleiotrophin/HB-GAM solution in step 5 with a similar volume of PBS. These can be stored for longer periods of time (up to one month).

(iii) Other beads, substances used in thesis experiments

For experiments using either the poly-L-lysine, FITC-conjugated (*see Results (A): Manuscript, Figure 3 and materials and methods)* or the poly-L-glutamic acid polymers *(see Results (B): Part III)*, 7μm polystyrene beads were used and the protocol is identical to that outlined for poly-D-lysine (see protocol above: (i) Preparation of poly-D-lysine coated beads)). Several concentrations of poly-L-glutamic acid were used ranging from 50μg/mL to 1mg/mL, while experiments with poly-L-lysine-FITC used beads coated with a 50μg/mL solution. For experiments where silica beads were used *(see Results (B): Part II)*, these were coated using a protocol that is identical to that outlined for poly-D-lysine.

SUPPORTING PROTOCOL II: Addition of beads to hippocampal cultures

Rather than being a protocol per se, this section will briefly list certain tips for the addition of beads to cultures.

Whether being used fresh or after storage at 4°C, be sure to thoroughly mix the bead suspension several times not just to disperse but also to dissolve clumps. These clumps do not dissolve after their addition to the cultures, making quantification and interpretation difficult.

Remove the culture wells/dishes containing slips from the incubator only after the beads are dispersed. Add the beads dropwise, then use a sterile plastic pipet or a 1mL pipet to mix the culture medium thoroughly with the beads while being careful not to disturb the cells. This is the best way to ensure an even distribution of beads throughout the coverslips. Return the dishes to the incubator immediately.

Check dishes 30min later to see if the beads have adhered and to see if you have added enough. Depending on the desired length of incubation, you can add more (24h or longer), but if the experiments involve short periods of incubation this is not recommended.

Number of beads to add: For all coated beads, add approximately 100,000 beads per 18mm coverslip or well. More can be added, but take care to avoid too many as this can affect the health of the cells. One can determine whether too many are added by observing the cultures briefly under a microscope and doing a random check of how many beads adhere to a single neuron. More than 15 beads/neuron is not recommended.

For uncoated beads, this rule does not apply and one must in fact add many times more beads to the cultures, as uncoated beads do not adhere and very few will remain even after prolonged incubation periods. For this condition, add several million uncoated

beads per 60mm dish or 1million beads per well in a 4-well plate. This will ensure that a single coverslip will have enough beads to perform quantification (approximately 10-100).

BASIC PROTOCOL III: Fixation and preparation of bead-neuron cultures for transmission electron microscopy.

The following protocol describes the methods used for preparing neuron-bead cultures for transmission electron microscopy. Note that the steps for counter-fixation with lead citrate and uranyl acetate along with the ultrathin sectioning and mounting onto copper grids are omitted; these tasks were expertly performed by M. Lavallée (Montreal Neurological Institute).

Materials

Anhydrous ethanol

Cells, hippocampal cultures grown in 4-well plates

Drying oven, set to 60°C

Epon reagents (can be purchased from Electron Microscopy Sciences or as kit from SPI-Chem, *www.2spi.com*):

- Epon 812 unpolymerized resin
- DDSA
- NMA
- DMP-30 polymerizing reagent

Magnetic stir bars and stir plate

Modeling clay (can be purchased at any art supply store)

Plastic beakers and pipets

Plastic embedding capsules (Electron Microscopy Sciences, #70000)

Propylene oxide (e.g., Sigma #471968)

Solutions:

1) **0.2M Phosphate buffer (PB), pH 7.4**

To 500mL ddH_2O add: 11.6g Na_2HPO_4

2.5g NaH_2PO_4-H_2O

Check pH with litmus paper (do not adjust pH with HCl).

2) **0.1M PB, pH 7.4**

Combine equal volumes 0.2M PB and ddH_2O.

3) **4% paraformaldehyde (PFA) in 0.1M PB, pH 7.4**

Heat 30mL ddH_20 in a small (50 or 100mL) glass beaker until just beginning to boil (will lose some volume in the process). Add 2.0g PFA powder and a stir bar. While stirring, add 1-3 drops of NaOH solution until PFA solution becomes clear. Filter using Whatmann paper (wet paper first with ddH_2O) and collect filtrate in a flask on ice. Add 25mL 0.2M PB (total final volume should be approx. 48-50mL).

4) **2% Glutaraldehyde-2% PFA in PB** (make immediately prior to use)

160mL 25% glutaraldehyde

840mL 0.1M PB

1000mL 4% PFA in 0.1M PB

Per 4-well plate, need approx. 2mL (500mL/well).

5) **1% Osmium tetroxide (OsO_4) in 0.1M PB** (make immediately prior to use)

1:1:2 of 4% OsO_4: 0.2M PB: 0.1M PB.

Per 4-well plate need approx. 2mL (500mL/well).

6) **Dilutions of anhydrous ethanol (in ddH_2O):**

In separate 50mL Falcon tubes *(e.g. VWR, #CA89048-930)*, prepare the following ethanol dilutions:

50%

70%

80%

90%

95%

100% (no dilution).

Protocol

(i) Prepare the Epon

All steps should be performed in a chemical fumehood. Use plastic beakers as these are easier to clean and/or less expensive to discard than glass beakers.

1. Combine 15.5mL Epon 812 and 25mL of DDSA in a plastic beaker. Label “solution A”.
2. Combine 12.5mL Epon 812 and 11.1mL NMA in a separate plastic beaker. Label “solution B”.
3. Mix solutions A and B gently for at least 30min.
4. In a separate plastic beaker mix 30mL of solution A, 20mL of solution B, and 0.75mL of DMP30. This is the mixture that will constitute the final epon (100% epon) with which to embed the fixed samples. Mix gently for about 1 hour.
5. Note: Upon addition of DMP30 solution should immediately turn a deep orange. Over the 1h period of mixing, the epon will turn from the deep orange to a golden yellow colour (indicating that the epon has polymerized). This solution can be stored in fridge for 2-4 days, well-wrapped. Plastic beakers and stir bars, if they are to be reused, should be immediately rinsed in a hot water-bleach solution to remove traces of epon reagents.
6. Prepare 1:1 and 1:3 solutions of propylene oxide:100% epon. (Note: these solutions are to be used during the process of dehydrating the samples prior to embedding. Below are quantities sufficient to embed 2-4-well plates.)
7. 1:1 solution: 3mL propylene oxide and 3mL 100% epon.
8. 1:3 solution: 1.5mL propylene oxide and 4.5mL 100% epon.
9. Leave solutions mixing on a stir plate (in the fumehood) until ready to use.

(ii) Fix and dehydrate the neuron-bead cultures (in 4-well plates)

10. All steps should be performed in a chemical fumehood.
11. Remove 4-well plates from the incubator and rinse cells gently with 0.1M PB 2X.
12. Fix cells in 2% glutaraldehyde/2% PFA in 0.1M PB, pH 7.4, for 30min.
13. Rinse cells 2X with 0.1M PB.
14. Post-fix cells in 1% OsO_4 in 0.1M PB for 30min.
15. Rinse cells 2X with 0.1M PB.
16. Dehydrate cells in a graded ethanol series as follows, aspirating wells with little time delay:
17. 2x 50% EtOH

18. 2x 70% EtOH
19. 1x 80% EtOH
20. 1x 90% EtOH
21. 1x 95% EtOH
22. 2x 100% EtOH
23. Apply 1:1 Epon, enough to cover bottom of wells. Incubate 1min, remove.
24. Apply 1:3 Epon, enough to cover bottom of wells. Incubate 3min, remove.
25. Apply 100% epon, enough to cover bottom of wells.
26. Wrap plates in parafilm and place at 4°C overnight.

(iii) Prepare the Epon molds (next day)

27. Cut the ends and caps off of the plastic embedding capsules (molds) using a razor blade, one capsule per well.
28. Remove 4-well plates from 4°C and drain off the excess epon from the wells.
29. Place mold on top of the cells (cap-side down).
30. Holding the mold in place with one hand, surround the mold with a complete layer of modeling clay. *(Note: This prevents the epon from leaking out of the mold.)*
31. Be sure that the plastic mold is secure on top and has formed a sufficient seal at the bottom (Note: Can check this by looking at the bottom of the plate, a well-sealed mold usually has the residual epon pooling slightly around the edges.)
32. Fill each plastic mold to the top with 100% epon.
33. Place the lid of the 4-well plate on top and then place lead weights on top of the lid.
34. Place plates in a drying oven set to 60°C for 8-10hrs.
35. (Note: Do not leave for too much longer than this otherwise you will not be able to remove the hardened blocks easily and will break the underlying plastic into your samples.)
36. Remove plates from the drying oven and quickly remove as much excess modeling clay as possible.
37. Snap the blocks out from the 4-well plates by loosening forcefully from the base. Do this <u>while the blocks are still hot</u>. This ensures that the plastic from the plate will not break off onto the epon block.

38. Place blocks back into the 60°C drying oven for 1-2 days or until they have hardened sufficiently for cutting.

BASIC PROTOCOL IV: Fixation and preparation of bead-neuron cultures for immunocytochemistry.

The following protocol was used throughout the thesis for fixation of coverslips for antibody labeling.

Materials

Antibodies, primary (see Appendix II for a list of primary antibodies used, source and dilution)

Antibodies, secondary (see Appendix II for a list of secondary antibodies used, source and dilution)

Cells, hippocampal cultures grown on 18mm coverslips

Flat rotating shaker

GelTol mounting medium *(Thermo Scientific)*

Microscope slides, glass, 75x25mm, 1mm thick *(e.g. Fisher, # 12-544-7)*

Nail polish, clear (can be purchased at any drugstore)

Normal donkey serum (e.g. Jackson laboratories, # 017-000-121)

Solutions (can be stored at 4°C for several weeks and up to 3 months):

1) **0.2M Phosphate buffer (PB), pH 7.4**

To 500mL ddH_2O add: 11.6g Na_2HPO_4

2.5g NaH_2PO_4-H_2O

Check pH with litmus paper (do not adjust pH with HCl).

2) **0.1M PB, pH 7.4**

Combine equal volumes of 0.2M PB and ddH_2O.

3) **4% paraformaldehyde (PFA) in 0.1M PB, pH 7.4**

Heat 30mL ddH_20 in a small (50 or 100mL) glass beaker until just beginning to boil (will lose some volume in the process). Add 2.0g PFA powder and a stir bar. While stirring, add 1-3 drops of NaOH solution until PFA solution becomes clear. Filter using Whatmann paper (wet paper first with ddH_2O) and collect filtrate in a flask on ice. Add 25mL 0.2M PB (total final volume should be approx. 48-50mL).

4) **0.1M Tris-buffered saline (TBS)**

To 450mL ddH_2O add: 6.057g Tris base

4.5g NaCl

Adjust pH to 7.4 using HCl. Make up volume to 500mL with ddH_2O.

Note: For long-term storage of fixed coverslips, filter-sterilize the PB and TBS solutions and fix cells under sterile conditions.

5) **Blocking buffer:** 4-5% (v/v) normal donkey serum + 0.1% (v/v) Triton-X in TBS

6) **Primary antibody dilution buffer:** 0.5% (v/v) normal donkey serum + 0.1% (v/v) Triton-X in TBS

7) **Secondary antibody dilution buffer:** 0.5% (v/v) normal donkey serum in TBS

Protocol

(i) Fix the neuron-bead cultures (on coverslips)

1. Remove cells from incubator and rinse 1X with 0.1M PB.
2. Fix cells in 4% PFA in 0.1M PB for 25min at room temperature.
3. Rinse 1X with PB.
4. Rinse 2X with TBS. (Note: Can place cells at 4°C at this time if you wish to pause the protocol, or for long-term storage provided that the cells were fixed under sterile conditions.)

(ii) Block nonspecific sites and incubate with primary antibody

5. Transfer coverslips to be stained into the wells of a 12-well plate containing TBS.
6. Be sure to add 1-2 extra coverslips for the control staining conditions (without primary antibody).
7. Aspirate TBS and add 400µL/well of blocking buffer.
8. Incubate for 30min at room temperature on a rotating shaker with gentle shaking.

9. During step 8, prepare the primary antibody solution and add desired combinations of antibodies at the appropriate dilution. Be sure to reserve some solution without antibody for the control wells.
10. Following blocking step, remove blocking buffer and add 400μL/well of antibody or control (no primary) antibody solutions.
11. Wrap plates in parafilm and place on a rotating shaker at 4°C.
12. Incubate overnight with gentle shaking.

(iii) Wash coverslips and add secondary antibody

13. The next day, rinse cells 3X10min with TBS at room temperature on a rotating shaker with gentle shaking.
14. Remove TBS and add secondary antibodies diluted to 1:200 (v/v) in secondary antibody dilution buffer (400μL/well).
15. Incubate at room temperature for 60min on a rotating shaker with gentle shaking.
16. Rinse cells 3X10min with TBS at room temperature on a rotating shaker with gentle shaking.

(iv) Mount and store coverslips

17. Mount the coverslips onto glass slides, inverting each coverslip over a drop of mounting medium applied to the slide.
18. Gently press flat and aspirate excess mounting medium/TBS that accumulates around the sides.
19. Let dry overnight at room temperature, covered.
20. When dry, apply clear nail polish around edges to seal.
21. Can store slides at 4°C for several months or at -20°C indefinitely.

BASIC PROTOCOL V: Imaging parameters and quantification of bead-neuron cultures fixed for immunocytochemistry.

This section outlines the details of the confocal microscope and the imaging parameters used for the examination of all immunostained slides, as well as the quantification methods used.

Microscope and imaging parameters

For imaging of fixed coverslips, we used an Olympus (Tokyo) Fluoview FV1000 laser scanning confocal microscope with a 60x PlanApo oil immersion objective [1.4 numerical aperture (NA)] on an IX81 inverted microscope. The lasers used include a multiline-Argon (457/488/515nm) and/or Helium-Neon (543nm and 633nm) lasers.

Fields of interest were selected using the brightfield (differential interference contrast, or DIC) channel. For double or triple immunostaining, fluorescence images were acquired via sequential scanning of each individual channel along with the corresponding DIC image. For each coverslip, optimal parameters were carefully adjusted to avoid image saturation. All imaging parameters were manipulated using the Olympus Fluoview software accompanying the microscope.

Images of single optical sections through the neuritic plane were acquired with 1x digital zoom and 4x Kalman averaging. Where z-stacked images were acquired, stacks of at least one and up to several microns in depth were performed beginning with the focused neuritic plane and moving up in 0.2-0.3 micron steps. Stacks were typically acquired with 2x digital zoom and 2-3x Kalman averaging. The depth of the stack was dependent on the persistence of fluorescence (i.e. synaptophysin-positive immunolabeling) surrounding the bead. For every experiment, a control condition was performed in which neuron-bead cultures were processed for immunocytochemistry in the absence of primary antibody. This typically resulted in little to no fluorescence accumulation either within neurons or beneath the beads, as shown in the example below (Figure 1.1).

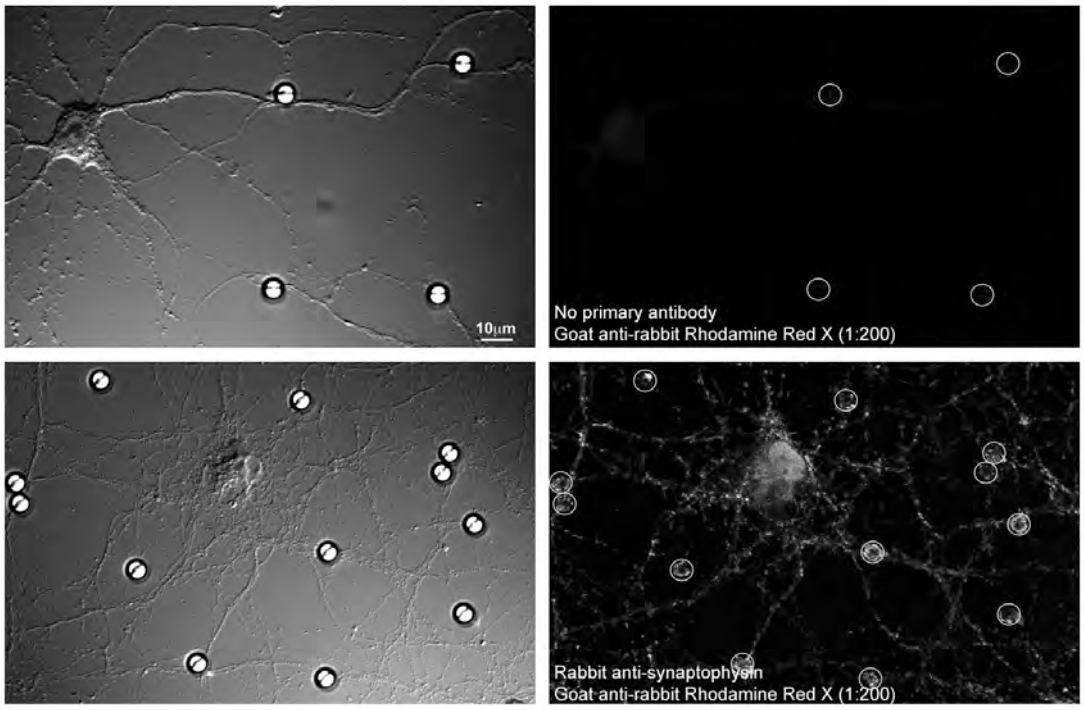


Figure 1.1. Specificity controls. Representative image panels of cultures fixed and incubated in goat anti-rabbit Rhodamine Red X (secondary) antibody following incubation with buffer alone (top panel) or a solution of anti-synaptophysin (primary) antibody in buffer.

Experiments where we observed fluorescence labeling in the absence of primary antibody were discarded.

Image Quantification

This section outlines in detail the methods used for quantification of all fixed samples processed for immunocytochemistry. Quantification of all immunocytochemistry and colocalization was performed using NIH ImageJ software (to download, go to the website http://rsbweb.nih.gov/ij/). The necessary plugins that were added to the basic software were the 'Multi Measure' (http://www.optinav.com/Multi-Measure.htm), 'Intensity Correlation Analysis' (http://www.macbiophotonics.ca/imagej/installing_imagej.htm), and 'Measure Stack' (http://www.optinav.com/MeasureStack.htm) plugins. Unless otherwise stated, all image quantifications were calculated for at least 50 beads per condition per experiment and

averaged across at least 3 separate experiments per condition. The method of quantification will be explained using the following series of images as an example.

These images are derived from neuron-bead cultures triple stained for actin (Alexa 488-Phalloidin, Molecular Probes), synaptophysin (Anti-synaptophysin antibody, Zymed) and bassoon (Anti-bassoon antibody, Stressgen). (A) Represents the DIC image while (B) represents the merged fluorescence images.

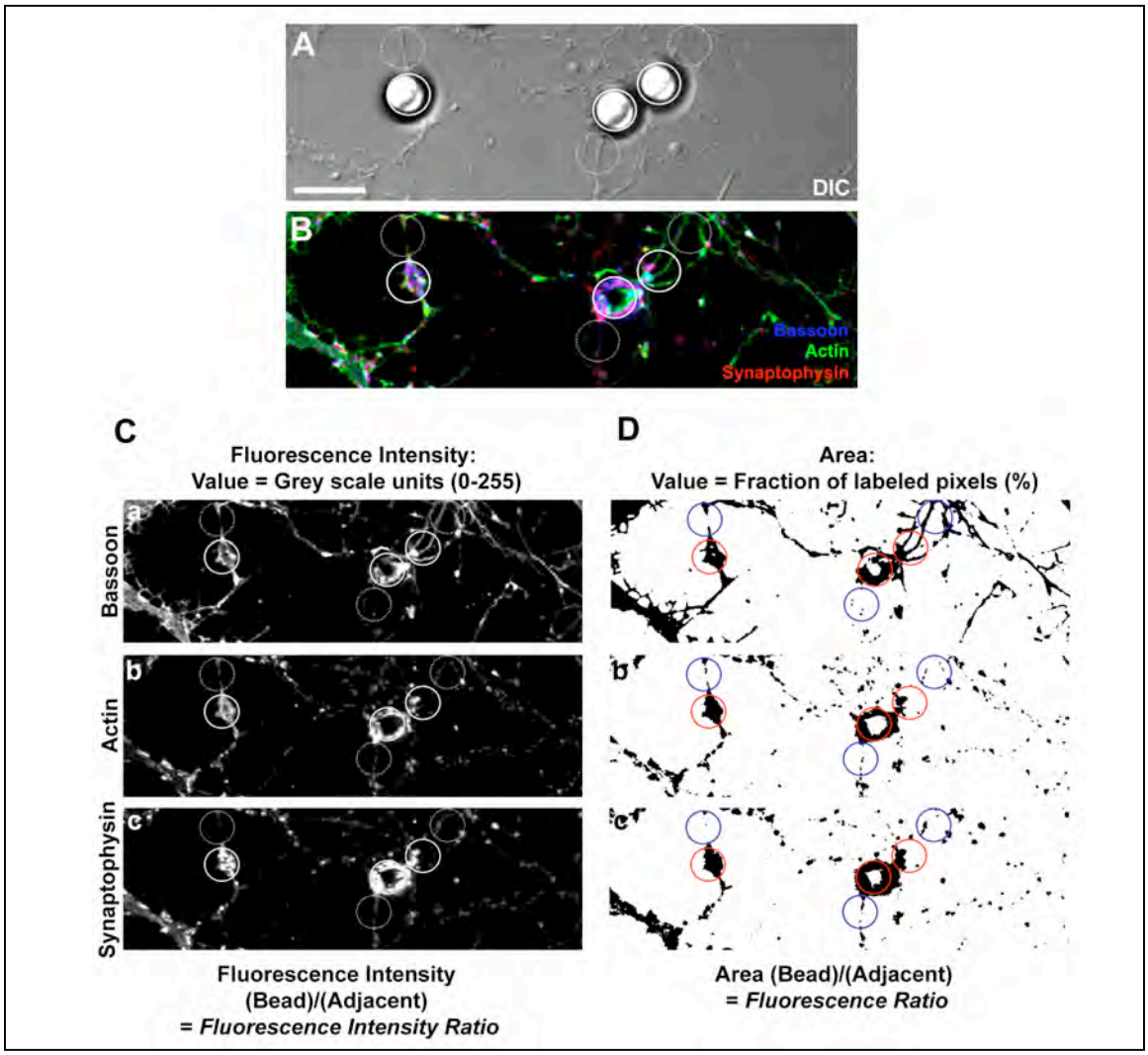


Figure 1.2. Representative image panels of neuron-bead cultures analyzed for fluorescence intensity and area (previous page). (A), DIC image of the field to be analyzed. Scalebar, 10μm. (B) Corresponding merged fluorescence image of the field shown in (A). (C) Greyscale images of the three fluorophores corresponding to the immunolabeled proteins shown in (B). These images will be analyzed for fluorescence intensity. White circles in (B,C) correspond to be bead (solid) and adjacent (dashed) sites to be analyzed. (D) Thresholded versions of the images shown in (C) to be analyzed for area. Circles correspond to the bead (red) and adjacent (blue) sites to be analyzed.

(i) Select the bead and adjacent sites to be analyzed

1. Open the DIC image in Image J (Figure 1.2A).
2. Open the Multi Measure panel from the Plugins toolbar (Plugins → Multi Measure). This will open a separate panel in which you can create a list of all the ROIs that are generated in a given DIC image.
3. Create an appropriately sized region of interest (ROI). The ROI should be slightly larger than the diameter of the bead (to account for puncta that extend beyond the bead diameter). The area size of the ROI must be kept the same within each image. *(Note: The "bead ROIs" are displayed as the solid white (Figure 1.2B,C) or red (Figure 1.2D) circles.)*
4. Select a desired bead site and on the Multi Measure panel click 'add and draw'.
5. For each bead ROI, select a region immediately adjacent to the bead to as a control. This region should follow the length of the same axon contacting the bead as much as possible. If the bead is contacting more than one neurite, the control site should also contain a similar number of neurites or should encompass the same neurites contacting the bead. This is done to ensure accurate representation of the changes in intensity/area. *(Note: The "adjacent ROIs" are displayed as the dashed (Figure 1.2B,C) or blue (Figure 1.2D) circles.)*
6. Select an appropriate adjacent site and on the Multi Measure panel click 'add and draw'.
7. Repeat for all desired bead and adjacent sites in the image. (Note: Beads that contact cell bodies are excluded from analysis.)

8. Save the ROI list in the Multi Measure panel (for later reference if needed).

(ii) Analyze images for fluorescence intensity

9. Open the fluorescence images and convert them to greyscale (Figure 1.2C).
10. Select the first ROI from the Multi Measure panel.
11. Measure the average fluorescence intensity of the selected ROI directly from the greyscale image (Figure 1.2C)(Analyze → Measure). This will give the mean pixel intensity for the selected ROI. The number values will be (in theory) anywhere from 1-255 grey scale units although the vast majority will fall within a range of 1-100.
12. Repeat for each individual bead and adjacent ROI.
13. Repeat for all fluorescence images (if multiple labeling is performed, as shown in the example figure).
14. Copy the data into an Excel spreadsheet.

(iii) Analyze images for area

15. Threshold the greyscale images (go to Image → Adjust → threshold). Thresholding converts all the fluorescence intensity values into a binary value whereby a pixel has a value of 0 (no fluorescence) or 1 (anywhere from 1-255, depending on the threshold level). Thresholding was first auto-adjusted by the software and then manually adjusted (as necessary) to achieve a distribution of labeled pixels that best represented the fluorescence images (Figure 1.2D).
16. Set the desired measurements. Go to Analyze → Set Measurements and select Area, Mean Grey Value, Limit to Threshold and Area Fraction.
17. Select the first ROI in the list and analyze (Analyze → Analyze Particles → select Summarize). The 'area fraction' value is represents the proportion (%) of the ROI that contains thresholded pixels.
18. Repeat for each individual bead and adjacent ROI.
19. Copy the data into an Excel spreadsheet.

(iv) Analyzing stacks

20. For measurements of fluorescence intensity or area using stacked images, the same procedure is followed except that for each bead ROI the intensity/area was measured

for each slice within the z-stack using the 'Measure Stack' plugin (Plugins → Stacks → Measure Stack).

21. Copy the data into an Excel spreadsheet.

(v) Colocalization analysis

For the colocalization analysis (*see Results (A): Manuscript, Figure 1 and materials and methods)*, we used the Intensity Correlation Analysis (ICA) plugin within the NIH ImageJ program. Of all parameters measured within this analysis we selected the intensity correlation quotient (ICQ) value as the measure of colocalization between pairs of fluorescence stainings (in this case, synaptophysin-bassoon or VGlut1-GAD). The ICQ is derived from a pixel-by-pixel analysis of the product of the differences from the mean (PDM), whereby the intensity of each labeled pixel is subtracted from the overall mean intensity in a given region of interest, and expressed as a product for both stainings (Li et al., 2004).

PDM = (red intensity-mean red intensity) x (green intensity-mean green intensity)

The ICQ value is therefore the sum of all the individual PDMs in a given region of interest, the values of which are distributed between -0.5 and +0.5:

Segregated staining: $0 > ICQ \geq -0.5$

Random staining: $ICQ \approx 0$

Dependent staining: $0 < ICQ \leq +0.5$.

1. Open and threshold the two fluorescence images to be analyzed.
2. Open the ROI to be measured in both thresholded images.
3. Go to Plugins → Intensity Correlation Analysis. Select the images that will correspond to channel 1 and 2 (be sure that each individual thresholded image is represented) and check the box 'Limit to Threshold'.
4. Click 'ok'. This analysis will generate many parameters that can be analyzed; please see the Plugin information manual (available to download from the site listed above) for what each parameter means. We use the "ICQ" value as the measure of colocalization.
5. Repeat for each bead and adjacent ROI.
6. Copy the data into an Excel spreadsheet.

(vi) Statistics

For each condition, Student's t-tests comparing the average mean intensity or area of the bead ROI vs. the adjacent ROI were performed. This was done to ensure that the average intensity of the bead sites was significantly different from the adjacent sites for PDL beads ($p<0.05$).

For both intensity and area, all values were then expressed as a ratio for each individual bead and corresponding adjacent site (*fluorescence intensity or fluorescence ratio, respectively*). Ratio values were used for comparison between PDL and uncoated beads, or PDL-coated beads in the presence or absence of various pharmacological inhibitors.

Graphing and all statistical analysis was performed using GraphPad Prism software (San Diego, CA).

For comparisons of fluorescence changes between two groups we assess significance by Student's t-test.

For comparisons between multiple groups we assess significance by one-way ANOVA followed by Bonferroni's *post-hoc* test.

In figures, statistical significance is indicated by (#/n.s.) for $p>0.05$, (*) for $0.05<p<0.01$, (**) for $0.01<p<0.001$, and (***) for $p<0.001$. The values in histograms for all experiments are always expressed as mean ± SEM while the numbers in brackets refer to the total number of beads analyzed.

APPENDIX II: LIST OF PRIMARY AND SECONDARY ANTIBODIES

(I) List of primary antibodies

Antibody (Anti-)	Protein Description	Raised in:	Monoclonal /Polyclonal	Original source (now offered by)	Catalog Number	Dilution (v/v)
Bassoon	Presynaptic scaffolding molecule	Mouse	Monoclonal	Stressgen (Assay Designs)	SAP 7F407	1:500
β-Tubulin	Microtubule	Mouse	Monoclonal	Developmental Studies Hybridoma Bank	Anti-β-Tubulin, Ascites fluid	1:2000
CaV2.2 (Ab571)	Presynaptic calcium channel	Rabbit	Polyclonal	Gift of Dr. E. Stanley, University of Toronto (see Li et al., 2004)	N/A	1:200
GAD65/67	GABA-synthesizing enzyme	Rabbit	Polyclonal	Chemicon (Millipore)	AB1511	1:1000
Glial acidic fibrillary protein (GFAP)	Astrocyte marker-cytoplasmic	Rabbit	Polyclonal	Sigma	G9269	1:500
Heparan Sulfate (10E4 epitope)	Heparan Sulfate	Mouse	Monoclonal	Seikagaku	370255	1:100
Microtubule-associated protein 2 (MAP-2)	Microtubule (dendrite-specific)	Chicken	Polyclonal	GeneTex	GTX 30663	1:1000
N-Cadherin	Adhesion molecule	Rabbit	Polyclonal	Raised in Colman Lab (see Fannon and Colman, 1996)	N/A	1:200
NeuN	Neuronal marker-nuclear	Mouse	Monoclonal	Millipore	MAB377	1:500
PSD95	Postsynaptic scaffolding molecule	Mouse	Monoclonal	Affinity Bioreagents (Thermo Scientific)	MA1-046	1:200
Rab3a-	Presynaptic	Mouse	Monoclonal	BD	610906	1:500

Interacting Molecule (RIM)	active zone molecule			Transduction Labs		
RIP	Oligodendrocyte marker-cytoplasmic	Mouse	Monoclonal	Developmental Studies Hybridoma Bank	Anti-RIP, Ascites fluid	1:200
Synaptophysin, (predilute antibody)	Synaptic vesicle protein	Rabbit	Polyclonal	Zymed (Invitrogen)	08-0130	1:10
Syndecan-2	Cell-surface HSPG	Goat	Polyclonal	Santa Cruz	SC-9494	1:100
Tau1	Microtubule (axon-specific)	Mouse	Monoclonal	Chemicon (Millipore)	MAB3420	1:100
Vesicular glutamate transporter 1 (VGlut1)	Glutamate transporter	Guinea Pig	Polyclonal	Chemicon (Millipore)	AB5905	1:4000

(II) List of secondary antibodies

Fluorophore:	**Raised in:**	**Raised against:**	**Cross-adsorbed against other species (for multiple labeling):**	**Source (now offered by):**	**Catalog #:**	**Dilution (v/v):**
Alexa 488	Goat	Mouse	Yes	Molecular Probes (Invitrogen)	A11029	1:200
Alexa 488	Goat	Rabbit	Yes	Molecular Probes (Invitrogen)	A11034	1:200
Alexa 488	Goat	Guinea pig	Yes	Molecular Probes (Invitrogen)	A11073	1:200
Alexa 488	Donkey	Goat	Yes	Molecular Probes (Invitrogen)	A11055	1:500
Alexa 647	Goat	Chicken	No	Molecular Probes (Invitrogen)	A21449	1:200
Alexa 647	Goat	Mouse	Yes	Molecular Probes (Invitrogen)	A21236	1:200
Alexa 647	Goat	Rabbit	Yes	Molecular Probes (Invitrogen)	A21245	1:200
Rhodamine Red X	Goat	Rabbit	Yes	Jackson Immunoresearch	111-295-144	1:200

APPENDIX III: ANIMAL USE PROTOCOLS

ACTION	✓	DATE
CCs		
DB	✓	17/05
APPROVED		

www.mcgill.ca/research/compliance/animal/forms/

For Office Use Only:
Protocol #: 5197
Approval end date: MAY 31, 2009
Facility Committee: MNI
Renewal#: 1st 2nd

McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator: Dr. David Colman **Protocol #** 5197

Protocol Title: Engineering repair of the central nervous system **Category:** B

Unit, Dept. & Address: Neurology and Neurosurgery, MNI 3801 University street

Email: david.colman@mcgill.ca **Phone:** 514 398-5359 **Fax:** 514 398 8248

Funding source: CIHR $1385 74017

Start of Funding: Oct 1, 2005 **End of Funding:** Sept 30, 2010

Emergency contact #1 + work AND home phone #s: Anna lisa Lucido Work# 514-398-2814 Home # 514-843-1831

Emergency contact #2 + work AND home phone #s: Isabel Rambaldi Work# 514 398-8436 Home# 514-852-6328

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if the Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		

11 JUIN 2008

5197

Dave Colman	PI	yes	doesn't handle animals	no	
Alyson Fournier	investigator	yes	doesn't handle animals	no	
Liliana Pedraza	investigator	yes	Zebrafish only	no	
Ziwei Li	Technician	yes	McGill course (rat and mouse) Zebrafish	no	
Ajit Dhaunchak	Postdoctoral fellow	yes	McGill course (rat and mouse)	no	
Adam Baker	Postdoctoral fellow	yes	McGill course (rat and mouse)	no	
Dalinda Liazoghli	Postdoctoral felow	yes	McGill course (rat and mouse)	no	
Isabel Rambaldi	Res. Assistant	yes	McGill course (rat and mouse)	no	
AnnaLisa Lucido	PhD student	yes	McGill course (rat and mouse)	no	
Wiam Belkaid	Ph.D. student	yes	McGill course (Rat only)	no	

* Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.

Approved by:

2. Approval Signatures		
Principal Investigator/ Course Director		Date: May 12, 2008
Chair, Facility Animal Care Committee		Date: June 11, 2008
A. C. C.		Date: June 17, 08
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		Date:
Approved Animal Use Period	Start: June 1, 2008	End: MAY 31, 2009

☐ **Renewal requires submission of full Animal Use Protocol form**

Form version May 2006

3. Summary (in language that will be understood by members of the general public)
AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).*

After injury to the CNS(as in stroke,spinal cord injury or certain disease states) axons are transected and are thus cut off from their targets. Following such injury their myelin sheath degenerate. Attempts to regenerate CNS tissue after these insults have to be framed in terms of: 1.The damaged neurons must be kept alive. To do this, an environment must be created that will encourage survival and growth(e.g. through the addition of appropiate growth factor combinations). 2. The neurons must extend processes toward the appropriate targets from which their connections have been severed. These processes must be guided to their appropriate location through the application of chemical cues and gradients. 3. New synaptic contacts must form between the regenerating axons

and their targets. 4. The axons must become re-myelinated.
This proposal is aimed at using new tools and technologies to promote these processes in vitro with a goal of translating these applications to in vivo models to promote CNS repair.

4. Has there been any animal care issues?	YES ☐ NO ☒ if yes, supply details:

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form.*
If mice expressing new phenotype have been produced, submit a *Phenotype Disclosure form.*
Blank forms at *http://www.mcgill.ca/research/compliance/animal/forms/*

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol, including amendments *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures described in the original protocol. New and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO ☐

IF NO, supply new endpoints that are different from the original protocol:

Experimental endpoints:

Clinical endpoints:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental endpoints:

Clinical endpoints:

8. Hazards **(check here if none are used: ☒)**

a) Are the hazards different from original protocol? **(infectious, radioactive, toxic, carcinogen, tumours)**

YES ☐ NO ☐ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse	rat	rat	zebrafish		
Supplier/Source	Charles river	Charles river	Charles River	own colony		
Strain	CD-1	Sprague Dawley	Sprague-Dawley			
Sex	adult female with litter	adult female with litter	female	male & female		
Age/Wt	P5-P8	P5-P8	adult pregnant	fertile adults		
# To be purchased	30	30	160	0		
# Produced by in-house breeding	0	0	0	100		
# Other (e.g.field studies)	0	0	0	0		
TOTAL# /YEAR	**30 females with litter**	**30 females with litter**	**160**	**100**		

10. Explanation of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Rodents:
We will produce one early postnatal mouse or rat culture every 2nd week for neurite outgrowth assays or for biochemical assays. Each culture will require 1 litter (for a total of 30 females with litters per year).
Also, we will need 4 pregnant rats every week for a total of about 160 animals per year. 160 pregnant rats/year will provide the number of cells necessary for our myelination studies in culture. Since neurons are postmitotic at the time we collect the tissue, they will not replenish the cultures. Therefore,we need to obtain about 2 million nerve cells/experiment.

Zebrafish:
We will collect fertilized eggs from couples mating on weekly bases.

The proposed renewal is compliant with NIH Grants Policy Statement on Terms and Conditions of NIH Grants Awards.

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

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