Engineering a Stable Synaptogenic Coating for Brain-Computer Interfaces

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AUTHORS CONTRIBUTION

Jean-Pierre Clement (Kennedy lab, McGill University)- Wrote thesis and manuscript text and figures. Performed all the experiments and analysis apart from those mentioned below.

Dr. Tim Kennedy (Principal investigator, McGill University) – Supervisor, developed the rationale and experimental design. Contributed to and edited the manuscript and thesis.

Dr. Laila Al Awan (Kennedy lab, McGill University)- Performed the initial experiment regarding the fluorescent microscopy characterization of the synaptic markers around dPGA coated microbeads in figure 1b,d and e, figure 2a-d, figure 4a and b, and figure 5b and c, including most of the quantification and analysis for those experiments.

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ABSTRACT

Intracortical microelectrode implants hold great promises for paralyzed patient to regain autonomy through the development of brain-computer interfaces. However, the clinical application of such technologies has been hampered by the instability and the progressive decay of the neuronal signal recorded by the electrode. In this thesis, we propose to develop a synaptogenic electrode capable of directing the formation of synaptic connections with the surrounding neurons to form a truly bio-integrated interface. While studies of the molecular cues involved in synaptogenesis have shown that "synapse-like" elements can indeed form *in vitro* onto synthetic surfaces, usually polymer microbeads coated with synaptogenic peptides, when implanted into the brain synaptic specializations formed but were resorbed within 2 weeks. This lack of stability is fundamentally problematic for the development of a synaptogenic electrode coating that may need to remain functional for years or even decades in patients. Using the same *in vitro* model employed in those studies we show that the stability of synaptic elements formed around microspheres coated with synaptogenic polymer corelates with the polymer's resistance to proteolysis. Our findings suggest that the synapse loss observed *in vivo* may be due to proteolytic degradation of the coating. With the aim of addressing this limitation, we describe the synaptogenic properties of a novel polymer, a dendritic polyglycerol amine (dPGA) nanoparticle, that is completely devoid of peptide bonds, and thus highly resistant to proteolysis. We show that microbeads coated with this polymer recruit more synaptic elements and maintain them for longer than the well characterized synaptogenic polymers poly-L-lysine and poly-D-lysine in cultures of rat cortical neurons. Our findings suggest that a dPGA-based coating possesses ideal characteristics to ultimately support the development of a functional chronic intracortical synaptogenic microelectrode.

RÉSUMÉ

L'utilisation de microélectrodes intra corticales permettrait de redonner une certaine autonomie au patient atteint de paralysie totale ou partielle via le développement d'interface entre le cerveau et les ordinateurs. Néanmoins, l'instabilité et la détérioration progressive du signal neuronal capté par ces électrodes empêchent leur utilisation dans un milieu clinique. Cette thèse propose le développement d'une électrode synaptogenique, soit une électrode capable de former des connections synaptiques avec les neurones à proximité afin de permettre une bio-intégration de l'électrode avec le system nerveux. Malgré que plusieurs études, portant sur les signaux moléculaires impliqués dans la synaptogenèse, ont démontré que des synapses peuvent en effet être formées sur des surface artificielles tel que des microbilles de plastique recouvertes par des molécules synaptogeniques *in vitro*, lorsque les chercheurs tentèrent d'implanter dans un cerveau de tel microbilles, les spécialisations synaptiques se forment, mais ne durent pas plus de 2 semaines avant de se désagréger. Ce manque de stabilité est problématique pour le développement de microélectrode synaptogeniques, car ces électrodes doivent rester fonctionnelles durant des années voir des décennies une fois implantées dans un cerveau humain. En utilisant le même modèle *in vitro* utilisé par les études mentionnées plus haut, nous avons démontré ici que la nature éphémère des spécialisations synaptiques formées sur des microbilles de plastiques couvertes de polymères synaptogeniques est corrélée avec la résistance à la protéolyse du polymère synaptogenique utilisé. Notre découverte suggère que la disparition de synapses observée *in vivo* est possiblement causée par la dégradation protéolytique du recouvrement synaptogenique de l'implant. Pour adresser ce problème concernant la dégradation des recouvrements synaptogeniques, nous avons identifié un nouveau polymère synaptogenique, une nanoparticule dendritique à base de poly-glycérol aminé (dPGA) qui ne possède aucun lien peptidique, ce qui la rend particulièrement résistante à la protéolyse. De plus, nous avons démontré que des microbilles recouvertes de ce nouveau polymère recrutent plus d'éléments synaptique et maintient ces éléments pour plus longtemps que d'autre polymères synaptogeniques connues tel que poly-L-lysine et la poly-d-lysine dans des culture de neurones corticaux. Notre découverte suggère qu'un recouvrement à base de dPGA possède les caractéristiques

nécessaires au développement d'une microélectrode synaptogenique pour les interfaces neuronales.

LITERATURE REVIEW

CHAPTER 1 – INTRODUCTION

INTRACORTICAL ELECTRODES

From early work on deep brain stimulation (DBS) to treat Parkinson's disease (Benabid et al., 1991; Gardner, 2013) to more recent applications for patients suffering from severe paralysis that allow them to operate a robotic arm with their thoughts (Hochberg et al., 2006; Kim et al., 2008; Chadwick et al., 2011; Simeral et al., 2011; Hochberg et al., 2012; Collinger et al., 2012) including recent advances in transmitting visual information directly to the brain of blind patients (Niketeghad and Pouratian, 2019), chronic intracortical microelectrodes capable of recording and/or stimulating electrophysiological activity from surrounding neurons in the brain are increasingly employed to treat neurological conditions in humans

CLINICAL APPLICATIONS OF INTRACORTICAL ELECTRODES

Initial applications for DBS relied on implanting a few (1-2 microwires with 4-8 electrode contacts each) relatively bulky electrodes (1.5 mm in length x 1.3 mm in diameter) (Amon and Alesh, 2017) to modulate the activity of brain regions to mitigate the movement disorders of patients suffering from Parkinson's disease who are no longer responsive to drug-based therapy (Benabid et al., 1991; Gardner, 2013). These devices are now widely used clinically with an estimated 160,000 patients who have received DBS implants as of 2019 (Lozano et al., 2019). Furthermore, a variety of clinical trials are currently investigating their potential for the treatment of other neurological diseases such as major depressive disorder (Dougherty et al., 2014), epilepsy (Fisher et al., 2010, *Salanova et al., 2015*), obsessive-compulsive disorder (Kohl and Kuhn, 2017) and Tourette's syndrome (Baldermann et al., 2015).

While DBS holds great promise for novel therapies that require neuromodulation of relatively large brain areas, there has been a recent emergence of novel clinical trials using microelectrode arrays with much higher spatial resolution. These include the Braingate 1 (Hochberg et al., 2006) and 2 clinical trials (clinicaltrials.gov, NCT00912041), trials carried out by Dr. Boninger's groups in Pittsburgh (clinicaltrials.gov, NCT01364480), the Gennaris and the CORTIVIS projects (Niketeghad and Pouratian, 2019), and recently, the company Neuralink (Musk, 2019), all of which aim to record, decode and activate motor and sensory functions using intracortical microelectrodes.

As opposed to DBS, these new initiatives, use high density silicon-based neural electrodes, such as the Utah Array (Maynard and Normann, 1997), or microwires (Musk, 2019) to record activity from single neurons (single unit) or small populations of neurons (local field potential) in order to decode motor intention from the primary motor cortex of paralyzed or "locked-in" patients. This information can then be transmitted to an electronic aid such as a wheelchair controller, a robotic arm or a computer cursor. Similar devices are also investigated for the stimulation of the primary visual cortex in a spatially restricted manner to provide visual input from a glass-mounted camera to patients suffering from blindness due to damage to the visual pathway (Niketeghad and Pouratian, 2019).

CLINICAL LIMITATIONS OF CURRENT NEURAL ELECTRODES

Although these electrode arrays can provide functional benefits for the patients, it has become clear, through studies in animal models (Barrese et al., 2013, Black et al., 2015) and ongoing clinical trials, that certain key challenges arise from trying to record reliably the small currents produced by individual or small population of neurons (Kim et al., 2018). These include high day-to-day signal variability (Vaidya et al., 2015) and gradual decay of the signals recorded by the electrode(Barrese et al., 2013), which limits the useful lifespan of such devices to a few months or years at most. These limitations pose fundamental challenges to microelectrodes that must be addressed before these devices can be approved for widespread clinical use.

DAY TO DAY VARIABILITY

The amplitude of the electrical signal recorded by electrodes follows an exponential decay as a function of the distance of the current source from the electrode (Mechler and Victor, 2012). This becomes problematic when trying to record from single neuronal units as the signal to noise ratio is extremely dependent on the electrode being in close proximity to the target neuron's cell body (usually within 150-200 μm from the recording site) (Buzaki, 2004).

This leads to high day-to-day (Dickey et al., 2009; Fraser and Schwartz, 2012) and even intra-day (Perge et al., 2013) signal variability due to electrode displacement relative to the target cell body caused by brain/electrode micromotions (Perge et al., 2013). For instance, over a 15 day period, only 39% of the electrodes at one site actually continued to recorded from the same units (Dickey et al., 2009; Vaidya et al., 2015) and in 40% of the sessions evaluated, the recordings show signs of array micromovement within the session (Perge et al., 2013). The unreliability of the signal adversely affects the decoding performance of the algorithms trying to extract the motor intention from the neural recordings as the electrode may be recording from completely different neurons from one recording session to the other (Jarosiewicz et al., 2015).

In turn, this results in major day-to-day performance variability (Dickey et al., 2009, Perge et al., 2014), that require extensive calibration every day, and even within the same day, by a trained technician to be corrected for the devices to be usable (Brandman et al., 2018). This alone renders the current generation of devices impractical for daily uses by paralyzed patients.

PROGRESSIVE SIGNAL DECAY

Another fundamental obstacle for clinical implementation of intracortical microelectrodes is the progressive signal decay experienced by the devices over a period of months to years. While there are some reports of micro-electrodes remaining functional for up to 7 years in a non-human primate (Kruger et al., 2010) and up to 5 years in a human (Hochberg et al., 2012), almost all devices experience a progressive decay in the electrode impedance, signal amplitude and the number of detectable active units from each electrode channel (Chestek

et al., 2011; Prasad and Sanchez, 2012; Barrese et al., 2013; Perges et al., 2014). In humans, this translates to about a 2-5%/ month decrease in device performance (Perge et al., 2014) and 3-5% in non-human primates. When extrapolated, this gives a maximum useful lifespan of about 8 years before the devices can no-longer extract meaningful information (Barrese et al., 2013). This decrease in performance poses a clear issue for the clinical uses of such devices, which should be able to remain functional for decades if not the entire lifespan of the patients since the implantation and removal procedure require extensive surgeries and involves major patient risks.

While surface biofouling, mechanical failure, and insulation delamination likely contribute to the loss of functional channels over time (Barrese et al., 2013), the progressive glial ensheathment and neuronal cell death/displacement around the electrode, commonly referred to as the "foreign body response" is generally regarded as the primary driver of this signal decay (Guo, 2016, Szostack et al., 2017).

THE FOREIGN BODY RESPONSE

This foreign body response is characterized by the rapid migration and proliferation of the surrounding astrocytes and microglia, the resident macrophages of the central nervous system, to the implant site within 24-72h after implantation (Kozai et al., 2012). This is followed by the encapsulation of the implant by a dense network of glial processes to isolate the implant from the surrounding healthy tissue over the following 2-3 weeks, often referred to as the "glial scar" (Szarowski et al., 2003). The glial scar formation appears to plateau after 6-12 weeks forming a permanent biological barrier around the electrode (Kozai et al., 2014). This glial response is accompanied by a progressive loss of neurons cell bodies around the implant site (McConnell et al., 2009; Biran et al., 2015).

The exact mechanism that links the glial scar to the electrode's signal decay remains a question of debate. Some researchers argue that the thick glial sheath around the electrode acts as an electrical insulator (Williams et al., 2007; Nolta et al., 2015; Salatino et al., 2018), while other argue that the gradual loss of signal is due to the progressive loss of neuronal cell bodies or processes around the electrode (Biran et al., 2005; McCreery et al., 2016; Eles

et al., 2018; Solarana et al., 2020). Regardless of the cause, this progressive loss of signal coming from the implanted electrodes limits the useful lifespan of the implant to a few years in patients who received the electrode.

APPROACHES TO TACKLE THESE ISSUES

Extensive efforts to tackle these issues have been deployed in recent years. To compensate for high day to day signal variability in recorded signal, techniques have been developed to improve decoding performances including better signal processing (Fraser et al., 2009; Jarosiewicz et al., 2013), constant algorithm correction (Gurel and Mehring, 2012; Homer et al., 2014) and the use of machine learning (Li et al., 2011; Brandman et al., 2018).

Several approaches have been investigated to reduce the foreign body response, with the aim of preventing signal decay over time. These include soft (Harris et al., 2011; Kolarcik et al., 2015; Du et al., 2017,) or flexible electrodes (Luan et al., 2017) to reduce the electrode-tissue mechanical mismatch, ultrasmall electrodes to reduce the electrode's footprint (Kozai et al., 2012; Luan et al., 2017; Yang et al., 2019) or electrodes with novel geometries (Liu et al., 2015; Yang et al., 2019).

Furthermore, researchers have investigated biologically active coatings made of recombinant proteins (Cui et al., 2003; He et al., 2006; Azemi et al., 2011; De Faveri et al., 2014, Taub et al., 2014; Golabchi et al., 2020), hydrogels (Sommakia et al., 2014; Shen et al., 2018; Bourrier et al., 2019), small molecules (Zhong and Bellamkonda, 2007; Grand et al., 2010; Wang et al., 2015; Boehler et al., 2017) or even embedded cells (Purcell et al., 2009; Azemi et al., 2010) that either mimic the natural composition of neural tissue and its extracellular matrix or aim to mitigate the inflammatory response caused by the neural implant.

While many of these approaches have achieved some improvements in electrode-tissue integration, there are still no completely satisfying solutions to date (Kim et al., 2018). This is possibly best exemplified by the fact that even the most recent clinical trials continue to

use the same silicon-based Utah arrays developed in the 1990's (clinicaltrials.gov, NCT00912041& clinicaltrials.gov, NCT01364480).

SYNAPTOGENIC SURFACES AS A NOVEL APPROACH TO ENHANCE NEURAL ELECTRODE FUNCTIONALITY

The Kennedy lab, in collaboration with other groups at McGill, are investigating approaches to reduce the foreign body response to neural implants, for example, developing ultra-soft neural implants (<20 KPa) (Zhang et al., submitted). In this thesis, specifically, we explore a fundamentally different approach to biologically active coatings:

Instead of relying on the proximity of an electrode to target neurons, we aim to develop devices capable of directing neurons to form stable connections with the electrode itself. Employing bioactive coatings for electrode surfaces and molecular biomimicry to leverage existing mechanisms in the CNS, we aim to develop a **synaptogenic electrode** capable of directing neighboring neurons to form synaptic connections directly onto the electrode surface. An electrode able to direct the formation of synaptic elements onto its recording site could in theory offer major advantages over traditional recording devices.

ADHESIVENESS

The synapse is a surprisingly strong point of adhesion due to the high local density of multiple types of cell adhesion proteins (Zuber et al., 2005; High et al., 2015). These include NCAMs, SynCAMs Neuroligins, Cadherins, Netrins and other CAM and ECM components (Missler et al., 2012). Indeed, work on the biochemical isolation of synapses has shown that the separation of pre- and postsynaptic elements resists high salt treatment, high centrifugal forces and require protein denaturing treatment (i.e. Urea) combined with high concentrations of detergent to dissolve the complex (Phillips et al., 2001).

Moreover, daily human activity can impose up to a 5% deformation of the brain tissue, seemingly without causing significant damage to the synaptic network (Bayly et al., 2005). As a result, a robust synaptic connection formed onto the surface of a neural implant may

withstand daily brain micro- and macro-motion, without loss of the connection between the two partners. We speculate that forming a direct and stable connection between a neuron and electrode may greatly improve day-to-day signal stability.

Furthermore, synapses have been observed to last for a significant portion of an animal life *in vivo* (Trachtenberg et al., 2002) and, in principal, may last for the entire lifetime of an individual. Thus, synaptic connections between a specific neuron and a synaptogenic neural implant could potentially remain stable over months or even years.

PROXIMITY

A second potential advantage is that a synaptogenic electrode may provide unique capacity to directly record from and stimulate synapses. As described above, electrophysiological signal amplitude decreases exponentially as a function of the distance of the target cell from the recording site (Buzsàki, 2004; Mechler and Victor, 2012). Based on electron microscopy analysis, the typical size of a synaptic cleft (the distance between the pre- and postsynaptic element) is ~12-40 nm (Savtchenko and Rusakov, 2007). Individual action potentials (AP) are readily recorded from axons with existing extracellular microelectrode technologies both *in vitro* (Bakkum et al., 2017) and *in vivo* (Bartho et al., 2014). The close proximity between a synaptogenic electrode and synaptic specializations formed onto its surface could provide unparalleled conditions to stimulate and record electrophysiological activity from individual synaptic elements.

Similarly to other bioactive coatings that have been developed, we predict that a coating that mimics natural synaptogenic proteins that results in a surface covered with synaptic elements may reduce the foreign body response, by effectively masking the implant surface and resulting in improved tissue integration.

POSSIBLE LIMITATION OF A SYNAPTOGENIC ELECTRODE

While a synaptogenic electrode may, in theory, have several advantages as described above, there are many unknowns relative to the reaction of neural tissue to a synaptogenic coating. First and foremost, is it even possible to develop an electrode capable of forming synaptic connections with surrounding neurons? Early work by Richard Bury seem to suggest that it is indeed possible to form synaptic-like elements onto synthetic surfaces, namely latex microbeads, both *in vitro* and *in vivo* (Burry 1980; Burry 1982; Burry 1983; Burry 1985). It remains to be determined if this can be accomplished onto an electrode surface and if these synaptic elements will persist for extended periods of time.

There are other concerns regarding the feasibility or usefulness of a synaptogenic electrode that need to be addressed. For instance, the synaptogenic coating on the surface of such an electrode may become biofouled by proteinsand other macromolecules within the extracellular space or by cellular debris and blood released from the trauma caused by the initial insertion of the electrode (Kozai et al., 2015), the glial sheath surrounding the electrode may physically mask the electrode surface, create a milieu unfavorable for synapse formation either by degrading newly formed synapses on the electrode surface (Hong et al., 2016) or repel the neurites from which synapses would arise (Solarana et al., 2020). Furthermore, the extracellular potential produce by the activity of individual synapses will likely be smaller than cell body from which recordings are typically derived. As a result, despite their close proximity to an electrode, synaptic signals may be too small to be picked up by an electrode over the background noise. Finally, the strong molecular adhesion at the synapse surface may not be sufficiently strong to withstand the micromotions caused by the mechanical mismatch between the relatively stiff material of an electrode (typical stiffness in the order of 3-400 GPA) (Du et al., 2017) and the softer nervous tissue (3-10 Kpa) (Miller et al., 200).

However, before testing any of these hypotheses regarding the potential benefits or limitations of a synaptogenic electrode, we must first need to develop a coating capable of recruiting and maintain stable synaptic connections onto the synthetic surface of an electrode. This thesis investigates the synaptogenic properties of a new class of polymer

and evaluate its suitability as a synaptogenic coating for chronic intracortical microelectrode implants.

CHAPTER 2: BRIEF REVIEW OF SYNAPSE BIOLOGY

Synapses are the primary site of information transfer between neurons in the central nervous system. As one of the most common structures in the CNS (there are an estimated 100 trillion synapses in the brain alone (Yang et al., 2001)), it comes as no surprise that synapses have incredibly diverse shapes, sizes, molecular compositions, cellular locations and types (Hell and Ehlers, 2008, Südhof, 2018). The canonical CNS synapse, modeled after the glutamatergic synapse, the most common synapse type in the brain (Südhof, 2018), is composed of 3 compartments, the presynaptic specialization, the synaptic cleft and the postsynaptic specialization.

PRE-SYNAPSE

The presynaptic side is typically located in the axonal compartment of a neuron and forms a protrusion or an enlargement of the axonal segment. This enlargement contains a dense pool of neurotransmitter-filled vesicles docked at the membrane facing the postsynaptic side (Burette et al., 2015) ready to be released upon axonal depolarization via calcium influx from voltage-gated calcium channels that are also located at the active zone (Dolphin and Lee, 2020). The RIM protein complex anchors the synaptic vesicles to the active zone, where the vesicles are released, as well as voltage-gated calcium channels and the molecular machinery necessary to release and maintain this pool of vesicles (Südhof, 2012). There are a variety of other protein complexes that play key roles in the function of a presynaptic terminal, including recycling endosomes (Goldenring, 2015), neurotransmitter transporters/recycling (Edwards, 2007), 'autocrine' neurotransmitter receptors (Miller, 1998), and the SNARE complex (Rizo and Xu, 2015). Although synapse function is increasingly well understood, it is likely that others remain to be discovered and their function identified.

SYNAPTIC CLEFT

Between the pre- and postsynaptic specializations, there is a thin extracellular space where neurotransmitters are released from the presynaptic terminal to bind to receptors on the postsynaptic side (Burette et al., 2015). Although the synaptic cleft is often illustrated as

empty extracellular space, it is known to be occupied by a number of transmembrane protein complexes that span the entire width of the cleft and are involved in maintaining synaptic adhesion, synapse's structure and also regulating synaptic function (Missler et al., 2012). Recent advances in cryo-electron microscopy have allowed for the visualization of these protein complexes at high resolution to provide impressively detailed insight into the structure of the synaptic cleft (Tao et al., 2018). This study found that at least 5% of the extracellular space was occupied by these protein complexes (High et al., 2015). Proteomics studies have also begun to catalog the identity of the proteins that occupy the synaptic cleft. Not surprisingly, these studies have revealed an enrichment of cell adhesion proteins (Loh et al., 2016; Cijsouw et al., 2018).

POST-SYNAPSE

Across the synaptic cleft and directly opposed to the pre-synaptic terminal is the postsynaptic specialization. It is composed of a densely packed band of proteins located at the intracellular edge of the postsynaptic membrane (Liu et al., 2019). On the basis of ultrastructural studies this band was named the postsynaptic density (PSD) due to its electron-dense appearance in transmission EM micrographs (Palay, 1956). The PSD is composed of scaffolding proteins such as the Shank protein family, Homer proteins, MAGUK proteins and Gephyrin that are thought to serve as anchors via their multiple protein-protein interaction domains. The domains in these synaptic adaptor proteins act as docking sites for proteins important for the postsynaptic function, organizing clusters of neurotransmitter receptors and their downstream effector proteins (Verpelli et al., 2012).

SYNAPTOGENESIS

In human brain, the vast majority of synaptogenesis occurs during late embryonic development and early post-natal maturation (Huttenlocher et al., 1982). The dense synaptic network is then refined later in development through activity-dependent plasticity to reinforce meaningful connections and prune unnecessary synapses (Shatz, 1996). In fact, over the next 2 decades of life, approximately 40% of the synapses that were

initially formed will be pruned from the circuitry (Huttenlocher et al., 1982; Bourgeois and Rakik, 1993). This is mechanism is thought to ensure the adaptability of the neuronal network in the face of different task requirements imposed by the environment (Goodman and Shatz, 1993). The number of synapses then stabilizes in adulthood (Südhof, 2018), but relatively recent evidence suggests that a certain amount of synaptogenesis is still maintained throughout life (Zito and Svoboda, 2002; Zuo et al., 2005) and is believed to play critical roles in learning and memory (Xu et al., 2009; Yang et al., 2009) and brain plasticity (Zito and Svoboda, 2002; Holtmat et al., 2006).

CELLULAR-MOLECULAR MECHANISM OF SYNAPSE FORMATION

During development, the ends of axons and dendrites elaborate highly motile structures (axonal and dendritic growth cones) that explore the local environment with the ultimate goal of finding a synaptic partner (Shen and Cowan, 2010). Upon axo-dendritic contact, receptors binding their ligands localized on the extracellular surface of axons and dendrites initiates axo-dendritic adhesion and activates intracellular signaling (Südhof, 2018). The activation of downstream signaling initiates multiple processes that include the recruitment of additional cell adhesion protein to stabilize the initial axodendritic contact (Abe et al. 2004; Kayser et al. 2008), cytoskeleton remodeling (Biederer and Südhof, 2001; Wegner et al., 2008; Goldman et al., 2013) and the molecular machinery that will form the nascent synapse (Dean and Dresbach, 2005; Biederer and Stagi, 2008). This machinery includes cell adhesion proteins that align and hold the pre- and postsynaptic elements together, and that form the neurotransmitter release sites, as well as the various intracellular adaptor scaffolding proteins described above (see synapse biology section) that will organize and anchor additional functional components within the pre- and postsynaptic specializations (Südhof, 2018). Activity-dependent mechanisms then determine whether the synapses will be pruned, maintained or strengthened during the lifetime of the individual (Shatz, 1996; Stein and Zito, 2018).

SYNAPTOGENIC PROTEIN

The proteins on the surface of an axon or dendrite that are responsible for the initiation of the synaptogenesis process are called synaptogenic cues. An increasingly large number of proteins have been identified that contribute to synapse formation. The compliment of synaptogenic cues and receptors expressed by a searching axonal growth cone or dendritic filopodium, their localization along the neurite and the synaptogenic cues expressed by the cellular-targets determines whether or not a synapse will be formed and what kind of synapse it will be (Shen and Cowan, 2010; Südhof, 2018).

Functional study of synaptogenic cues has identified many that are extremely potent signaling molecules. Notably, the formation of an active pre- or postsynaptic terminal does not require the presence of a synaptic partner; certain synaptogenic proteins are sufficient to induce synaptic differentiation even when expressed by a non-neural cell (Scheiffele et al. 2000) or coated on a non-biological surface (Dean et al., 2003; Goldman et al., 2013). Indeed, pre- and/or postsynaptic specializations can form quickly at sites of neurite adhesion to non-neuronal cells expressing recombinant protein such as neuroligins/neurexins, SynCAMs/Necls (Biederer et al., 2002), EphBs/ephrinBs (Aoto et al., 2007; Kayser et al., 2006), netrin G ligands (NGLs/LRRC4s) (Kim et al., 2006) and LRRTMs proteins (Linhoff, 2010) or even synthetic microbeads coated with extracellular proteins such as netrin-1 (Goldman et al, 2013). Since these synaptic-like elements are composed of a pre- or postsynaptic-like specialization adhered to a synthetic surface, in the absence of a corresponding cellular synaptic partner, they are termed “hemi-synaptic specializations”.

DEVELOPING A SYNAPTOGENIC COATING

COATING PROTEOLYSIS

While a number of synaptogenic proteins have been shown to be sufficient to direct the formation of synaptic specialization onto synthetic surfaces (e.g. microbeads), and it is certainly feasible to coat an electrode with recombinant proteins (Aregueta-Robles et al., 2014), the peptidic nature of those proteins makes them sensitive to brain-derived proteases (Wang et al., 2007; Bai and Pfaff, 2012) which will eventually degrade the

bioactive coating. This is less of an issue for natural synapses which constantly renew their surface proteins (Alvarez-Castelao and Schuman, 2015; Jin et al., 2017), but to engineer stable synthetic synapses, this poses a major challenge as the engineered surface may rapidly lose its bioactivity due to degradation of the surface coating.

SYNTHETIC SYNAPTOGENIC POLYMERS

A primary reason that we turned our attention to synthetic polymers, with potential synaptogenic properties, was the aim of enhancing biostability compared to protein-based coatings. Well before the identification of the first synaptogenic protein, experiments performed by Richard Burry and Benjamin Peng in the early 1980's, demonstrated that certain synthetic peptides, such as the cationic polypeptides poly-lysine and poly-ornithine, when coated onto polystyrene microspheres, have the capacity to direct the formation of synapse-like elements by neurons in culture (Burry 1980; Burry 1982; Burry 1985; Peng et al., 1987).

Their findings, along with more recent work on synthetic synaptogenic polymers (Lucido et al., 2009, Gopalakrishnan et al. 2010) indicates that high density of amine groups, which provide a strong net positive charge to the polymer at physiological pH, is required for the synthetic peptide to be synaptogenic.

Extending this, a study replaced with polycationic peptide coating and instead used lipids with cationic head groups to coat microbeads, demonstrating that these beads similarly direct synapses to form on their surface (Gopalakrishnan et al. 2010). Not all cationic molecules, however, have proven to be synaptogenic, as illustrated by experiments with the cationic lipid DOTAP, indicating that the process is more constrained than simply requiring a high local concentration of positive charge (Lucido et al., 2009).

MECHANISM

The mechanism underlying the synaptogenic properties of cationic polypeptides remains unknown. An initial theory put forward to explain this phenomenon was that the positive charges of the microbead's coating interact with the negative charges of the phospholipids'

heads in the plasma membrane and that this simple electrostatic adhesion is sufficient to trigger the formation of a synaptic element (Bury, 1980). However, subsequent experiments (Lucido and al., 2009) provide evidence that positively charged amine groups and/or mechanical adhesion alone are not sufficient to trigger synaptic formation onto a microbead. Instead, the authors suggested that the local aggregation of heparan sulfate proteoglycans (HSPGs), a component of the extracellular matrix (Condomitti and Wit, 2018), may be involved in the synaptogenic effect of polycationic peptide coated beads (Lucido and al., 2009). The rationale for this argument was that the oligosaccharide components of HSPGs are large polymers with negatively charged sulfate groups, conferring a high net negative charge at physiological pH, therefore they are likely to interact strongly with positively charged coatings. Experiments carried investigating this hypothesis provide evidence that HSPG loss impedes synapse formation. While this could be due to a different, but required role for HSPGs in synapse formation, these previous findings raise the interesting possibility that polycationic peptides are not synaptogenic cues per se, but may bind and act as a scaffold for other synaptogenic molecules secreted in the extracellular space. The local enrichment of synaptogenic cues at the bead's surface could then trigger the formation of a synapse upon contact with a neuron.

While all the above studies were performed in dissociated neuronal cultures, in a series of papers, Burry and his colleagues described how 50 um diameter latex beads coated with poly-lysine could direct the formation of hemi-synaptic specializations when implanted in the cerebellum of young rat pups (3-4 days post-natal). These experiments demonstrated that synapse-like specializations were also formed *in vivo* onto an artificial surface despite the obvious trauma caused to the tissue by inserting foreign objects inside the brain (Burry 1983; Burry and Hayes 1986).

Similar to other synaptogenic proteins, poly-lysine, which is a protein, is also susceptible to degradation by several commonly secreted proteases, including brain-derived trypsin (Tsuyuki et al., 1956). Consistent with this, the presynaptic specializations formed *in vivo* on the poly-L-lysine (PLL) coated beads used by Burry and his colleagues, degraded within two weeks post-implantation (Burry and Hayes 1986). Therefore, although this study

shows that PLL can direct the formation of hemi-synaptic specializations onto a coated bead *in vitro* and *in vivo*, the lack of biostability fundamentally limits its application as a synaptogenic coating.

PROTEASES RESISTANT SYNAPTOGENIC POLYMERS

Historically, the proteolytic degradation of PLL was a well-known challenge for biologists attempting to establish long-term adherent cell cultures, as poly-lysine is a widely used substrate coating to promote cell adhesion to *in vitro* culture vessels (Banker and Gosslin, 1998). Poly-d-lysine (PDL), the stereo-enantiomer of PLL, was developed to provide a cell-culture substrate coating resistant to protease degradation, and thereby enhance the longevity of adherent cell cultures (Banker and Gosslin, 1998). However, although PDL is resistant to certain proteases such as trypsin, early experiments have shown that it is still sensitive to proteolysis by other mammalian proteases (Tsuyuki et al., 1956). Since PDL is not a naturally occurring protein, it may be considered to be an engineered molecular bio-mimic of naturally occurring PLL.

DENDRIMER-BASED COATING

Extending the idea of molecular biomimicry further, here we investigated the synaptogenic properties of a coating made of dendritic polyglycerol amine nanoparticles (dPGA), a molecular biomimetic of cationic polypeptides, that is completely devoid of peptide bonds (Frey and Haag, 2002). We chose to investigate dPGA as a synaptogenic candidate because it's dendritic architecture provides a high density of basic amine group on the molecule's surface, similar to other artificial synaptogenic molecules and, in theory, its core made of branched polyglycerol monomers, renders it highly resistant, if not invulnerable, to endogenous brain proteases. The polyglycerol core of dPGA is similar in structure to polyethylene glycol polymers, a widely used, FDA approved, drug adjuvant, and food additive. Furthermore, polyglycerol dendrimers have proven to be remarkably biocompatible, even when injected into the circulation at doses of up to 1mg/kg in rodents (Calderon et al., 2010).

WHAT ARE DENDRIMERS?

Dendrimers are a class of nanoparticle polymers named after the Greek word 'dendron' which means tree, because of the unique highly branched/tree-like structure of their core (Abbasi et al., 2014). The chemical composition of dendrimers can vary widely, but their structure can be broken down into 3 main components. The core serves as a nucleus for the initiation of the polymerization reaction. Extending from the core is a periodically branched structure, that can be tuned to vary the size and shape of the molecule. These branches terminate in functionalized end-groups, that can greatly influence the interaction of the dendrimer with other molecules. The core geometry, degree of branching, and end group chemistry can all be varied to modify the chemical properties of the polymer. The branching pattern allows for an increase in the number of functional groups within each molecule, while their tunable size allows them to be tailored for a variety of different applications (Lee et al., 2005).

The specific type of dendrimer used here has a tris(hydroxymethyl)propane core and is composed of branched poly-glycerol structure with amine-end groups that give it a net positive charge at physiological pH. Due to the steric hindrance of the different "branches" and the electrostatic repulsion of the end groups, dPGA molecules adopt a spherical shape in solution (~8 nm in diameter) with a molecular weight of ~100 KDa. The specific size used, amongst this type of polyglycerol aminated dendrimer, was selected-based on a previous study examining the biocompatibility of different size polyglycerol dendrimers as the most biocompatible candidate that still retained a high density of amine end-groups (Hellmund et al., 2015).

BIOMEDICAL APPLICATION OF DENDRIMERS

Polyglycerol dendrimers were originally developed as nano-drug carriers for tumor-targeted delivery and have been shown to be remarkably biocompatible and biostable *in vivo* (Ofek et al., 2010, Calderon et al., 2010). Non-modified dPGA (non-aminated) are being investigated as soluble drug carriers (Kurniasih et al., 2015), and anti-biofouling technologies (Siegers et al., 2004), while negatively charged versions of the polymer are being investigated as a small molecule anti-inflammatory drug (Rades et al., 2018). The

only reported application of aminated (positively charged) dPGA described in the literature is as a DNA/RNA nanocarrier for gene transfection into cells (Ofek et al., 2010; Hellmund et al., 2014).

SUMMARY OF THE THESIS

This thesis aims to test the hypothesis that, based on its structure and high density of amine end groups, dPGA may act as an effective highly-stable synaptogenic coating. To test this, we used coated microspheres, an assay applied previously in the literature, to investigate synaptogenic polymers that cannot be expressed in non-neuronal cells (Bury, 1980; Lucido et al., 2009). We coated 7 um polystyrene microspheres with dPGA and added them to cultures of E18 rat cortical neurons, and tested for the presence of neuronal elements with properties similar to bona fide synaptic elements. We used a variety of techniques to measure the presence/amount of hemi-synaptic elements associated with the surface of the coated microbeads, including immunocytochemistry for canonical synaptic markers, transmission electron microscopy to examine synaptic ulturastructure, and FM1-43 vesicle labeling to assess presynaptic release. To evaluate the synaptogenic properties of our dPGA-based coating, we compared it to two known synaptogenic polymers, PLL and PDL, as well as uncoated beads as a control surface.

RESULTS

CHAPTER 3- MANUSCRIPT

Molecular Biomimicry and the Synthetic Synapse: Engineering a Stable Synaptogenic Extracellular Matrix.

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ABSTRACT

Synaptic specializations can form quickly at sites of neuronal adhesion to synthetic surfaces coated with certain artificial polypeptide such as poly-lysine. This raises the possibility to engineer designer surfaces that could direct the formation of functional hemi-synaptic connections at desired sites for electrophysiological recording and tissue engineering. Although, earlier studies have shown that hemi-synaptic specializations formed onto poly-lysine coated microspheres implanted in brain, they degraded within a few weeks likely due to poly-lysine proteolysis. Here we describe a novel class of highly stable synthetic poly-cationic dendritic polyglycerol amines (dendrimers) that exhibit superior synaptogenic capabilities and promoted synapse stability over an extended period. We propose that such dendrimers can be used to engineered surfaces to act as molecular biomimics of synaptogenic extracellular matrix resistant to proteolysis for biomedical use.

INTRODUCTION

Microelectrodes capable of recording and stimulating neural activity from the brain have enabled human quadriplegic patients to control a computer and even a robotic arm with their thoughts (Hochberg et al., 2006; Simeral et al., 2011; Hochberg et al., 2012).

However, the clinical implementation of this technology has been impeded by the limited lifespan of current devices that is in part due to the inflammatory response triggered by the implant. This leads to glial ensheathment around the implant site and the progressive decay of the neuronal signals recorded by the electrode (Guo, 2016, Szostack et al., 2017).

To mitigate this issue, researchers have investigated different types of biologically active coatings made from recombinant proteins (Cui et al., 2003; He et al., 2006; Azemi et al., 2011; De Faveri et al., 2014, Taub et al., 2014; Golabchi et al., 2020), hydrogels (Sommakia et al., 2014; Shen et al., 2018; Bourrier et al., 2019), small molecules drugs (Zhong and Bellamkonda, 2007; Grand et al., 2010; Wang et al., 2015; Boehler et al., 2017) or even live embedded cells (Purcell et al., 2009; Azemi et al., 2010) in order to either mimic the natural composition of neural tissue and its extracellular matrix and/or reduce the inflammatory response caused by the neural implant. None of these approaches have been completely successful and efforts to enhance the biocompatibility of neural explants are ongoing.

Here, we aim to develop a bioactive synaptogenic coating that will direct neurons to form a synaptic-like connection directly onto the electrode to enable true bio-integration of an implant into the neuronal/synaptic network.

Indeed, many extracellular proteins involved in the recruitment, organization, and maintenance of synapses have been identified and the molecular biology of synaptogenesis is increasingly well understood (Südhof, 2016). In fact, the formation of an active pre- or postsynaptic terminal does not require the presence of the opposite synaptic partner and synaptic specializations can readily be directed to form on the surface of non-neural cells engineered to express synaptogenic proteins such as Neuroligins/neurexins (Scheiffele et al. 2000), Ephrins (Aoto et al., 2007; Kayser et al., 2006) and SynCam (Biederer et al., 2002) and even on synthetic surfaces coated with recombinant synaptogenic proteins (Goldman et al., 2013; Kim et al., 2016) to form hemi-synaptic specializations.

However, peptide-based coatings, such as a coating composed of one of the synaptogenic proteins described above, are susceptible to degradation by common proteases secreted by neural cells, such as brain-derived trypsin and cathepsin (Waley and Watson, 1953). Proteolytic degradation severely limits the useful lifespan/bioactivity of such coatings to at most few weeks after implantation. Importantly, this is not an issue for natural synapses, which constantly renew their adhesive and extracellular matrix proteins, but for a synthetic synaptogenic coating, a steady loss of bioactivity over time due to proteolytic degradation poses a major challenge to maintaining the functionality of the implants surface. To be useful in vivo, and potentially clinically relevant, a synaptogenic coating should remain stable for months or even years. This requires a synaptogenic coating that is capable of effectively recruiting synaptic element onto an engineered surface in vivo that will remain stable for a clinically relevant timescale.

Before the first synaptogenic protein were identified, work done in Richard Bury and Benjamin Peng's laboratories demonstrated that microspheres coated with cationic polymers, such as poly-lysine and poly-ornithine were sufficient to direct neurons in cell culture to form hemi-synaptic pre-synaptic specializations directly onto the microspheres' surface (Burry 1980; Burry 1982; Peng et al., 1987). Burry's group then extended these findings *in vivo*, showing that pre-synaptic specializations readily formed onto coated micro-beads transplanted into the cerebellum of 3-4 day old rat pups (Burry 1983; Burry and Hayes 1986).

Unfortunately these synthetic poly-cationic proteins, poly-lysine and poly-ornithine, are effectively degraded by endogenous proteolytic enzymes (Tsuyuki et al., 1956) and, consequently, the presynaptic specializations formed onto poly-lysine-coated microbeads implanted *in vivo* were not stable and degraded within 2-3 weeks post-implantation in the brain (Burry 1983; Burry and Hayes 1986). Although long-term stable synapses could not be maintained *in vivo*, at least two critical findings were derived from these experiments: First, they show that synthetic polymers other than endogenous proteins can support synapse formation, and second, that such a synaptogenic coating can effectively induce synapses formation onto a foreign object/synthetic surface when implanted into the CNS.

Extending these early findings, additional synthetic polymer coatings have since been shown to be synaptogenic *in vitro* (Lucido et al., 2009; Gopalakrishnan et al. 2010).

One such polymer, poly-D-lysine (PDL), the d stereo-enantiomer of PLL, was initially developed for use as a protein resistant "biomimetic" alternative to poly-L-lysine in order to increase the functional longevity of the cell culture substrate (Banker and Gosslin, 1998). Although PDL is partially resistant to proteases such as trypsin (Li and Yeung, 2008) and PDL coated microbeads are synaptogenic (Lucido et al., 2009), early experiments have shown that PDL is still sensitive to enzymatic degradation by mammalian proteases (Tsuyuki et al., 1956), limiting its practicality as a long-term bioactive implant coating.

Further extending the idea of molecular biomimicry, we investigated the potential application of a polycationic dendritic polyglycerol amine (dPGA) nanoparticle (Ofek et al., 2010, Calderon et al., 2010) as a highly stable synaptogenic coating. Critically, dPGA is entirely devoid of peptide binds and therefore highly resistant to proteolysis (Frey and HAg, 2002).

We demonstrate that microbeads coated with dPGA are potently synaptogenic, with synapses formed in culture maintained for extended periods compared to PLL or PDL coated microbeads. Our findings support the idea that synaptogenic extracellular matrices may be engineered to promote the stable formation and long-term maintenance of synaptic specializations onto synthetic surfaces. The identification of a highly stable synaptogenic coating is a significant step toward seamless bi-directional communication with neurons *in vivo*.

MATERIAL AND METHODS

Animals

All procedures with animals were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research and approved by the Montreal Neurological Institute Animal Care Committee and the McGill Animal Compliance Office.

Sprague-Dawley rats and CD1 mice were obtained from Charles Rivers Laboratory (St-Constant, QC, Canada).

Embryonic Cortical Neuron Cultures

Cell cultures were prepared from cerebral cortex of embryonic day 16–18 (E16–E18) CD1 mice or E18 Sprague Dawley rats as previously described (Goslin and Banker, 1998). Briefly, cortices from multiple embryos were pooled and dissociated cells were plated at high density (~40,000 cells/cm2). The cells were seeded in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% pen-strep for 12 hrs to allow for cell adhesion before being switched to Neurobasal medium containing 1% B27, 2 mM glutamax and 0.5% N2. All cultures were maintained for 7-29 *days in vitro* (DIV) at 37°C in a humidified 5% CO2 incubator. 50% of the media was changed every 7 days.

Preparation of Microsphere Beads

Carboxyl-functionalized polystyrene microspheres (7.3 µm diameter, Bangs Laboratories Inc.) were washed 3× in PBS (sterile, pH 7.4) before use, then incubated overnight with (50 µg/ml) PLL, PDL 150-300 kDa (Sigma Aldrich) or dPGA. Beads were then washed 3x in PBS, pelleted by centrifugation, 7 min at 6500 rpm, and resuspended in culture medium before addition to cultures. Control beads were treated similarly, without coating. Beads were added to cultured neurons at 11 or 28 DIV and maintained for an additional 3, 7, or 14 days of incubation (DOI).

Adhesion Assay

Bead-cell adhesion assays were carried out essentially as described (Goldman et al 2013). Following incubation of the beads with the neurons in cell culture, wells were washed three times with 37°C sterile PBS, pH 7.4, and the cells then fixed with 4% PFA, 0.1% glutaraldehyde for 5 min. The number of beads was counted in washed and unwashed conditions and presented as the percentage beads remaining [# of beads per field in washed conditions]/[average # of beads per field in unwashed conditions] * 100.

Immunocytochemistry

For immunolabeling, cells were fixed in 100% MeOH for 8–10 min at −20°C, permeabilized using 0.25% Triton X-100 in PBS for 10 min then blocked using 5% bovine serum albumin (BSA) in PBS for 1 hr at room temperature (RT). Cells were incubated overnight with primary antibodies in blocking solution (3% BSA and 0.1% Triton X-100 in PBS). The following antibody were used, rabbit anti-PSD95 1:200(Cell Signaling), guinea-pig anti-PSD95 1:1000 (Synaptic System), mouse anti-Synaptophysin 1 1:200 (Synaptic System), guinea-pig anti-VGAT 1:500 (Synaptic System), rabbit anti-Gephyrin 1:300 (Synaptic System), guinea-pig anti-VGLUT 1 1:500 (Synaptic System), rabbit anti-Neurofilament M 1:1000 (Millipore). After washing 3x15 min in PBS, cells were incubated with the appropriate secondary antibody at 1:500 : donkey anti-rabbit with Alexa 488 (Invitrogen), goat anti-guinea-pig with Alexa 555 (Invitrogen) and donkey anti-mouse with Alexa 674 (Invitrogen), then washed 3x15 min in PBS before being mounted with Fluoro-Gel (Electron Microscopy System).

Data Acquisition and Analysis

Images were captured using an Olympus FV1000 confocal microscope. Sister cultures were used in all experiments in which comparisons were made. At least 60 Beads were quantified per condition using ImageJ (NIH; Schneider et al., 2012). Corrected total cell fluorescence intensity (CTCF) for the area surrounding the bead and an adjacent section of the neurite contacting the bead were calculated from a single optical section (Z slice) as: Integrated Density - (Area of selected cell X Mean fluorescence of background). The fold change in CTCF between the area surrounding the bead/adjacent area of the neurite was then plotted. Data are expressed as mean ± SEM and were analyzed using GraphPad Prism 5 for windows. One or two-way ANOVA (for data containing more than one comparison) followed by a Bonferroni's multiple comparisons *post hoc* test was used to calculate *p* values for the relevant comparisons.

Colocalization

Confocal z-stacks corresponding to the entire height of the bead + the distance to the coverslips were acquired using an Olympus FV1000 confocal microscope. Images stacks were binarized using a threshold value for each stack equal to (average fluorescent

intensity + 2 times the standard deviation of an area of the background picked within the same stack). Pixels that were positive for both markers within the same optical section were considered as co-localized voxels.

3D Image reconstruction

Confocal image-stacks for 3D reconstruction were collected using a Zeiss LSM 710 confocal microscope with a 63x oil objective. A DIC channel was used to visualize the bead itself. The images were reconstructed using Imaris 3D reconstruction software (Bitplane) with the "filament tracer" tool for the pre-synaptic (red) and postsynaptic (green) neurites, transparency was set to 60% to better visualize the synaptic puncta. The "volume reconstruction" tool was then used the reconstruct individual synaptic marker puncta (Synaptophysin 1 in magenta and PSD95 in yellow) and the polystyrene bead reconstructed based on the DIC images. Only neurites in close proximity to the bead were reconstructed for clarity.

FM1-43 Vesicles Labeling

Embryonic rat cortical neurons were grown on 35 mm glass-bottom dishes (MatTek Life Sciences) using the method described above.10 µl of dPGA, PDL-coated, or uncoated beads were added to the media at 11 DIV and left for 7 days. Before imaging, the culture medium was replaced by FluoroBrite phenol red-free DMEM for 10 min (Thermo Fisher Scientific). The solution was then changed to 10 µM of FM-143 (Molecular Probes) and 100 mM KCl in FluoroBrite DMEM for 90 sec to induce depolarization and dye loading in neurotransmitter vesicles. The dishes were washed 2 times for 5 min each using FluoroBrite DMEM with 1 µM tetrodotoxin and once for 5 min using only FluoroBrite e DMEM. Images were taken continuously for 6 min using an LSM880 inverted confocal microscope (Carl Zeiss). After localizing an area of interest, stacks of 5 optical sections (0.75 µm) were acquired using Airyscan Fast Mode at 30-sec intervals. At the 2 min mark, KCl solution was added to the dish for a final concentration of 100 mM for a second depolarization to track the decline in fluorescence as a proxy for synaptic vesicle release, and recorded for an additional 4 min.

FM1-43 Analysis

Airyscan files were processed in Zen Black and then exported to ImageJ for analysis with a custom script. Image analysis was carried out as follows: The intensity across the entire z-stack was summed and the average fluorescent intensity in a 1 μm band around the bead was measured. The background fluorescent intensity was then subtracted, and the resulting value normalized to the average intensity around the beads in the first 90 sec of baseline acquisition (prior to KCl depolarization). Every 3 consecutive time points were binned into 90 sec intervals and a mixed model two-tailed ANOVA with the Bonferroni posthoc test was used to compare to normalized fluorescent intensity around beads between each condition within the same timepoint. Representative images are shown using the ImageJ/FIJI "Fire" LUT applied to the collected 16-bit images.

Transmission electron microscopy

Rat cortical neurons at 11 DIV were incubated for 14 days with dendrimer coated beads on 8-well permanox™ slide chambers and then fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1M sodium cacodylate buffer (Electron Microscopy Sciences) overnight at 4° C. Samples were then fixed in a solution of 1% OsO4 (Mecalab) + 1.5% potassium ferrocyanide for 2 hr. The samples were then dehydrated with ethanol and infiltrated in Epon resin (Mecalab) over 2 days. The Resin was polymerized at 60 degrees for 48 hr. The Resin was then trimmed and cut into 100 nm thick sections in the "en face" orientation with a Leica EM UC6 ultramicrotome (Leica Microsystems) and transferred onto a 200-mesh Cu TEM grid (Electron Microscopy Sciences). Sections were then stained with uranyl acetate (Electron Microscopy Sciences) for 8 min, and then with Reynold's lead (Electron Microscopy Sciences). Transmission electron micrographs were then obtained using a FEI Tecnai G2 Spirit transmission electron microscope (TEM) equipped with Gatan Model 895 Ultrascan 4000 CCD camera at either 30,000x or 45,000x. Brightness and contrast was slightly adjusted using ImageJ/Fiji to improve clarity of the image.

Substrate coating (supplementary figure 1)

For experiments using dPGA-coated substrates, 12 mm German glass coverslip were incubated overnight in a sterile PBS solution of either 1, 10, or 100 ug/ml of PDL 150-300

kDa (Sigma Aldrich) or dPGA at 37° C. The plates were then washed 3x with PBS before the addition of the neurons.

RESULTS

Here, we investigated dPGA as a candidate synaptogenic coating, because, similar to other synthetic synaptogenic polymers like poly-lysine and poly-ornithine, it possesses a high density of basic amine groups on its surface. Moreover, its complete lack of peptide bonds renders it highly resistant to endogenous proteases (Calderon et al., 2010).

dPGA is a spherical (~8 nm diameter) dendritic polyglycerol-amine nanoparticle with a molecular weight of ~100 KDa. The inner core of the particle is composed of a branched polyether structure, synthesized via controlled anionic ring-opening polymerization of glycidol (Sunder et al., 1999), with the hydroxyl terminal branch groups modified to amine functional groups (~97% of surface groups modified (Zeng et al., 2015)) (Fig.1 A). This generates a rigid spherical structure with a high density of positive charge at physiological pH. These amine-functionalized dendrimers were originally developed for applications as DNA/RNAi nanocarriers for *in vitro* and tumor-targeted delivery, and have been shown to be remarkably biocompatible and biostable *in vivo* (Ofek et al., 2010, Calderon et al., 2010). Thus, dPGA nanoparticles appeared to possess characteristics suitable to function as a highly stable synaptogenic coating.

We tested whether a dPGA coating was sufficient to recruit synaptic elements onto a synthetic surface using long-term cultures of embryonic rat cortical neurons. dPGA was coated by adsorption onto carboxy-functionalized polystyrene microsphere (diameter 7.3 um) which provide ease-of-use and strong ionic interactions with the poly-cationic polymers for efficient coating.

To assess the synaptogenic potential of dPGA, we compared it to two known synaptogenic polymers, PLL, one of the earliest molecules to be described as synaptogenic both *in vitro* and *in vivo* (Burry 1980; Burry 1985, Peng et al., 1987) and its *d*-enantiomer, PDL (Fig.1 A). The partial resistance of PDL to endogenous proteases enabled us to investigate the relationship between proteases resistance and the stability of the synaptic contacts recruited by the different polymers.

Previous publication, by us and others, have shown that the carboxy-functionalized microbeads, when uncoated, do not induce synapse accumulation *in vitro* and adhere relatively poorly to neuronal cells in culture, and thus serve as a good reference surface. In contrast, beads coated with either an endogenous synaptogenic protein (netrin-1) or a synthetic synaptogenic polymer (PDL) adhered quickly to neuronal process and synaptic marker proteins were recruited in less than 30 min to the contact between the neurons and the bead (Goldman et al., 2013; Lucido et al., 2009).

To determine if a dPGA-based coating would similarly increase bead adherence cortical neurons in culture at 12 DIV, we counted the number of beads that would withstand mechanical disruption and remain attached to the neurons after washing with PBS, following increasing lengths of incubation time with the beads. We found that over a 2-hr period, increasing numbers of beads coated with any of the cationic polymers remained adhered to the neurons, while uncoated beads exhibited little adhesion to neuronal cultures, even after 2 hrs of incubation (Fig. 1B). There was no significant difference between the poly-cationic coatings in terms of the number of beads adhering to the neurons in culture. This indicates that each of the 3 poly-cationic coatings are sufficient for the beads to adhere to neurons and are comparable in terms of their neuronal-adhesive properties.

Enhanced synapse formation induced by dPGA-coated beads

We then compared the efficacy of beads coated with PLL, PDL, or dPGA to induce the formation of synaptic specialization and promote synapse stability. Coated beads were added to developing cortical neuron cultures at 11 days *in vitro* (DIV), and the cultures

were fixed and immunolabelled after different incubation times (for up to two additional weeks) with the pre-synaptic marker Synaptophysin 1 (red) and postsynaptic marker PSD95 (green) (Fig.1 C).

The local accumulation of both synaptic markers was quantified by measuring the fluorescence intensity of the area around the bead and normalizing it to an adjacent section of the neurite to account for variations in synapse density occurring along each neurite in the absence of a bead. The values in (Fig1 D & E) represent the fold change accumulation of each marker around the beads compared to normal synaptic marker density on the neurite adjacent to the bead.

Analysis of the immunoreactive corrected total cell fluorescence (CTCF) for Synaptophysin 1 and PSD95 revealed significantly enhanced signal around dPGA-coated beads compared to Control, PLL- and PDL-coated beads for all time-points between 2 hrs and 14 days in culture. At the 7-day timepoint (the peak of synaptic marker accumulation for all coatings other than dPGA), this represented a 2-fold increase compared to PLL and PDL and a 10-fold increase compared to control beads (Fig1 D & E).

Notably, at shorter time points, dPGA exhibited significantly greater local accumulation of presynaptic and postsynaptic markers compared to the other synaptogenic polymers, even as early as 2 hrs following bead application. Enhanced local accumulation of synaptic markers was detected despite that after 2 hrs of incubation, bead adherence to cells was similar between the different cationic coatings (Fig.1B), indicating that mechanical adhesiveness alone did not directly correlate with the ability of each different coating to recruit synaptic markers.

Considering the relative stability of the differentiation induced by each polymer, dPGA maintained enhanced local accumulation of synaptic markers compared to control and the poly-lysine coatings for up to 2 weeks of incubation. These results provide evidence for more effective induction and stability of long-term hemi-synaptic specializations formed around dPGA- coated beads compared to either PLL or PDL.

PLL and PDL-coated beads show complete and partial loss of synaptic marker accumulation

Both PLL and PDL coated beads have been previously shown to be potently synaptogenic when presented to cultured neurons *in vitro* (Bury, 1980; Lucido et al., 2009). One key difference between the two enantiomers is the relative resistance of PDL to proteolysis by certain endogenous proteases such as trypsin (Tsuyuki et al., 1956).

We then compared the relative capacity of microspheres coated using the same concentration of either PLL or PDL polymers to recruit and maintain synaptic marker accumulation over an extended period of time (up to 14 days) in culture. Consistent with the findings of previous studies (Burry et al., 1980; Lucido et al., 2009), we found that both PLL and PDL-coated microspheres efficiently recruit pre- and postsynaptic markers to their surface in less than 2hr and maintain the local enrichment of these markers for at least 7 days in culture (Fig. 1D and E).

After 14 days of incubation, however, the relative levels of both Synaptophysin 1 and PSD95 associated with PLL-coated beads returned close to the levels of uncoated beads, while the level of synaptic markers around PDL-coated bead remained relatively high after 14 days of incubation. However, reduced levels of PSD95 around PDL-coated beads were detected between 7 and 14 days of incubation, albeit remaining above control levels.

In contrast, the accumulation of markers of synaptic specializations around microspheres coated with dPGA, which is completely devoid of peptide bonds, remained high following 14 days of incubation at the end of the study. These findings support the conclusion that the resistance of polymers to proteolysis significantly promotes the stability of synaptic specializations.

3D distribution of synaptic element on polymer-coated surfaces

While collecting images of beads with the confocal microscope, we observed that synaptic markers accumulated not only at the base of the beads, where the bead would initially

contact a neurite extending along the flat cell culture substrate, but were also be found higher up the bead, much higher than neurites would typically extend in a normal cell culture.

Fig.1E shows representative 3D reconstructions of high-resolution confocal Z stacks of immunostained coated beads following 7 days in culture. The 3D images clearly show that pre- and postsynaptic markers accumulate at the base of the bead and extend essentially over large portions of the entire surface to form a densely interconnected network of both pre- (red/magenta) and postsynaptic (green/yellow) elements, with dPGA-coated beads showing a more dense and elaborate network compared to PDL or control beads. In contrast, 3D reconstruction of Z-stacks acquired for uncoated beads show almost no accumulation of synaptic proteins extending up the surface of the beads, with all neural elements remaining close to the cell culture surface.

Fig.1 C and D presents analysis performed on a single confocal Z-slice, at the level at which the bead contacted the neurite, in order to prevent any bias toward beads where synaptic markers would be distributed over a larger portion of the bead. This analysis assesses the local density of synaptic markers in a single focal plane, but since it does not take into account the synaptic marker signals above the point of contact between the bead and the neurite, the measure provided is likely an underestimation of the total accumulation of synaptic markers around PDL and dPGA-coated beads.

dPGA-coated microbeads promote excitatory and inhibitory synapse formation

The studies described above used the presynaptic marker Synaptophysin 1, which does not differentiate between excitatory and inhibitory presynaptic terminals. To selectively visualize excitatory glutamatergic synapses and inhibitory GABAergic synapses, the most common excitatory and inhibitory synapse in the CNS respectively (Kandel et al., 1991), glutamatergic synapses were selectively immunolabeled for glutamatergic pre-synaptic marker vesicular glutamate transporter protein (VGlut1) and postsynaptic adaptor protein PSD95, to mark glutamatergic pre- and postsynaptic compartments respectively (Kennedy, 1997 ; Kim and Sheng, 2004; Vigneault et al., 2015). To selectively label GABAergic

synapses, cultures were immunolabeled for the GABAergic pre-synaptic marker VGAT and postsynaptic adaptor protein Gephyrin (Chaudhry et al., 1998; Choii and Ko, 2015).

Consistent with previous findings, PLL, PDL, and dPGA all promoted the local accumulation of markers for excitatory glutamatergic synapses, with dPGA being significantly more effective than PLL and PDL (Fig.2 A&C). In contrast, PLL and PDL did not differ from control uncoated beads in promoting the local accumulation of inhibitory GABAergic synaptic markers after either 3 or 14 days of incubation. However, dPGA increased the accumulation of both inhibitory pre- and postsynaptic markers compared to control beads (Fig.2 B & D). These findings further demonstrate the enhanced synaptogenic capacity of dPGA, while also demonstrating remarkable specificity in the capacity of different polymers to induce the accumulation of excitatory versus inhibitory synaptic markers, which may prove useful for specific applications.

dPGA-coated beads enhance synapse formation in "young" and "old" neuronal cultures

The majority of studies examining the function of synaptogenic cues *in vitro* (including the present study) are performed on embryonic neuronal cultures during the period of peak synaptogenesis. This period typically extends from 5-28 DIV in cortical neurons cultures (Romijn et al., 1981; Ichikawa et al., 1993; Harrill et al., 2015). It is during this phase of differentiation and development that neurons are thought to be particularly receptive to synaptogenic cues (Südhof, 2016). In contrast, most neuronal recording devices are intended to record from neurons in the mature of adult CNS. Neurons in the adult brain are likely more mature and have well-established synaptic networks, therefore they may react differently to synaptogenic substrates such as dPGA-coated beads.

To begin to assess the impact of neuronal maturity, and in particular, pre-establishment of extensive synaptic networks, we examined the capacity of more mature neuronal cultures (DIV 28) to form synapses on beads. Cultures were grown for either 11 DIV or 28 DIV at which point coated beads were added to the culture and the local accumulation of synaptic markers measured by immunolabeling after 24h of incubation (Fig. 3). For cultures at

either 11 DIV or 28 DIV, dPGA was more effective at evoking synaptic differentiation, assessed by the local accumulation of pre- and postsynaptic markers, than were either PLL or PDL-coated beads, consistent with our previous findings using less mature cultures (Fig.3 B).

Interestingly, while Synaptophysin 1 accumulation after 24 hrs of incubation was the same in both young and old cultures, PSD95 immunolabeling was consistently higher for all substrates in younger 11 DIV cultures compared to 28 DIV older ones (Fig.3 C). These results show that while neurons at 28 DIV still respond to dPGA as a synaptogenic cue, the neurons in these more mature cultures exhibit reduced potential to form PSD95 containing specializations around beads coated with any substrate, including dPGA. This was the case for all-coatings (Fig.3 C), suggesting that as the cultures mature, the capacity of the neurons to form glutamatergic postsynaptic specializations in response to poly-cationic polymers is reduced, independent of the type of polymer used. Additional studies will be required to determine if this results from reduced dendritic growth, reduced availability of PSD95 and other postsynaptic proteins, or competition from existing synapses in the established synaptic network.

Impact of dPGA vs PDL-coated substrate on synapse formation

In the previous experiments, the capacity of dPGA-coated beads to recruit synaptic proteins was compared to PDL-coated beads applied to neurons grown, in both case, on PDL substrates. To test the possibility that that the signals from the substrate may compete with signals provided by the coated beads, and consequently reduce the level of synapse formation, we tested the synaptogenic potential of dPGA-coated beads applied to neurons grown on either a PDL or dPGA-coated substrate. No significant differences were detected in the excitatory synaptic markers VGlut1 and PSD95 surrounding dPGA-coated beads applied to neurons grown on a PDL compared to a dPGA substrate (supplementary Figure 1). These findings suggest that signals provided by a dPGA substrate do not compete with signals from dPGA-coated microbeads.

Synaptic ultrastructural elements form on dPGA coated microbeads

The antibody markers used above are commonly used to label synapses *in vitro* and *in vivo,* but do not guarantee that the structures detected correspond to *bona fide* pre- and postsynaptic elements. Rather, they merely indicate the local enrichment of a canonical synaptic proteins. Synapses visualized using transmission electron microscopy (TEM) have characteristic ultrastructural elements that are used to identify them in neuronal culture and tissue (Harris and Weinberg 2012; Burette et al., 2015). PLL and PDL coated microbeads have each been previously shown to direct the formation of ultrastructural elements that are remarkably similar to presynaptic specializations using TEM (Bury, 1980; Lucido et al., 2009).

Here, we performed TEM on 100 nm thick sections of 25DIV cultured cortical neuron that had been incubated with dendrimer coated beads for 14 days. Sections were stained with osmium tetroxide, to enhance the contrast of cellular membranes and reveal the internal ultrastructure of the cellular elements around the beads.

Based on the deformation of the membrane immediately adjacent to the bead surface, cellular processes appeared to directly adhere to the bead (Fig. 3 A&B, white arrows), even though the beads had been in the culture for 14 days. Structures characteristic of pre- and postsynaptic elements were visualized in direct contact with the bead surface. We also detected pools of densely packed vesicles accumulating at the point of contact with the bead. Measuring the vesicles provided an average diameter of 45.1 nm ± 7.9(SD) nm, similar in shape and size to neurotransmitter containing vesicles pools docked at the active zone of a typical pre-synaptic terminalas detected *in vivo* (Takamori et al., 2006).

Postsynaptic glutamatergic terminals, in contrast, are defined by an electron-dense band 30-60 nm thick and 0.2-1 μm wide that corresponds to the postsynaptic density (PSD) (Harris and Weinberg 2012). A similar electron-dense band was detected (Fig. 3C) extending parallel to the contact formed between the neurite and bead surface. The measured size of the detected electron dense band (length 650 nm and thickness 46 nm ± 9.2(SD) nm) falls within the parameters of a glutamatergic PSD detected *in vivo* (Harris et al., 1992). Notably, associated with this putative postsynaptic specialization, a contact by a

putative presynaptic element is also present, to form a complete synapse (Fig.3 C, black arrow). This further supports the identity of the process directly contacting the bead as a postsynaptic specialization.

dPGA Enhances Synaptic Interactions

The detection of a pre-synaptic element forming a synapse on a postsynaptic element that forms a hemi-synapse with a dendrimer coated bead prompted us to consider another aspect of the interplay between the neurons and the coated beads: synaptic elements differentiating onto the bead may also form synapses onto other neurites in close proximity, to form an interconnected synaptic network with the coated bead at its core.

Quantification of the overlap of presynaptic Synaptophysin 1 with the postsynaptic marker PSD95, within a 3D volume around the beads (voxels) (Figure 4A) revealed a high degree of overlap between the 2 markers on the surface of the beads, suggesting that pre- and postsynaptic markers are not randomly distributed on the surface of the bead, but may interact with each other.

Closer observation of the 3D volume reconstruction support this conclusion and shows that, although there are some isolated pre- (magenta) and post- (yellow) synaptic elements detected (this is expected with Synaptophysin 1 punctate which is present in both glutamatergic and GABAergic synapse, but PSD95 is exclusively found in glutamatergic synapses), many elements are located in close proximity to each other with both pre- or postsynaptic elements appearing to be in contact with the bead surface with the corresponding synaptic partner found on the outside.

We detected similar arrangements in TEM images, where, in some cases, pre-synaptic elements were found to contact both the bead and a postsynaptic partner (Fig.4D *) while the in other cases a postsynaptic element was found to contact both the bead surface and a presynaptic element from an adjacent neurite (Fig.4E #).

Moreover, quantification indicates that a higher proportion of the total Synaptophysin 1 and PSD95 signal overlaps in dPGA samples compared to PLL and PDL, suggesting that the dPGA surface supports a high density of synaptic terminals (both pre- and postsynaptic), and also supports more interaction between terminals clustered on its surface. Together, these results suggest that dPGA has an enhanced capacity, compared to PLL and PDL, to initiate and support the local formation of synaptic specializations.

Moreover, our analysis supports our other observations that dPGA is more permissive for synaptogenic differentiation compared to PLL and PDL and can support a more complex network of pre- and postsynaptic elements on its surface.

Depolarization-dependent vesicle release at dPGA induced terminals

Although our immunocytochemical analysis indicates the rapid local recruitment of synaptic proteins to dPGA coated microbeads, and ultrastructural analysis identifies the differentiation of hemi-synapse like structures, the functionality of these synapse-like structures remains unclear. To investigate the functionality of the presynapse-like endings we assessed their capacity to recycle synaptic vesicles in a depolarization-dependent manner. The styryl dye FM1-43, when added to cell culture medium is internalized along neuronal processes by constitutive pinocytosis, membrane endocytosis and active neurotransmitter vesicle recycling, resulting in a local accumulation of the fluorescent dye in intracellular vesicles and compartments, including synaptic vesicles (Ryan et al., 1993). After washing away excess dye, the addition of a hyperkalemic solution causes depolarization dependent release of dye-filled synaptic neurotransmitter vesicles. A rapid drop in local fluorescence following depolarization confirms vesicle release at a particular site, consistent with synaptic vesicles release.

Cortical neurons at 11 DIV that had been incubated with dPGA coated beads for 7 days were loaded with FM-143 dye and washed. From the pre-depolarization microscope image, it is apparent that the labeled elements surrounding dendrimer beads are much brighter than the area surrounding PDL and control beads (Fig. 6 A). This suggests that these structures have taken up more dye during the loading process, consistent with the higher

synaptic marker intensity detected around dPGA-coated bead (Fig. 1 and 2). Following depolarization induced by the addition of a hyperkalemic solution (final concentration 100mM), we detected a rapid and progressive decrease in FM1-43 signal around both PDL and dPGA-coated beads over a 6-min period, while fluorescence around control beads remained largely unchanged.

The detected drop in FM1-43 fluorescence in response to neuronal depolarization suggest that neuronal elements around PDL and dPGA-coated beads have the capacity for vesicle release in a plasma membrane depolarization dependent manner, a hallmark of all chemical synapses (Ryan et al., 1993).

DISCUSSION

Early studies investigating the synaptogenic properties of poly-cationic polymers found that simple plastic beads coated with PLL could induce the formation of hemi-synaptic specializations when put in contact with neurons both *in vitro* (Burry, 1980; Burry, 1982; Burry, 1983, Peng et al., 1987; Lucido et al., 2009) and *in vivo* (Burry, 1983; Burry, 1985). However, the synapses formed did not persist and were replaced by astrocytes within a few weeks *in vivo*. Although these findings support the idea that non-neuronal surfaces, when decorated with the "correct" chemical signals could induce the formation of synaptic specializations, the short lifetime of the synapses formed was fundamentally problematic for the development of a synaptogenic coating for intracortical microelectrodes.

Here, we provide the first description of the synaptogenic properties of a dendritic polyglycerol amine nanoparticle-based coating, a highly stable cationic polymer completely devoid of peptide bonds. We show that artificial surfaces coated with dPGA nanoparticles form elements highly similar in structure and function to pre- and postsynaptic terminals when placed in contact with rat cortical neurons in cultures. We also observed that over 14-days PDL and PLL-coated beads partially or completely lose the synaptic markers that

had accumulated, which correlates with their respective resistance to proteolysis. In comparison, over the same time course, the dPGA-coated microspheres continued to recruit additional synaptic markers and maintained enhanced accumulation throughout the entire experiment.

We also show, for the first time to our knowledge, that a synthetic polymer can recruit both glutamatergic and GABAergic synaptic elements from cultured cortical neurons. Furthermore, we show that synaptogenic coatings retain the capacity to efficiently recruit synaptic markers when presented to neurons past the peak synaptogenic period in culture. Finally, through high-resolution 3D reconstruction and electron microscopy images, we show that synaptogenic coated surfaces support complex interconnected synaptic networks, with the coated bead at its core.

dPGA-coated Surfaces Promotes the Formation of Pre- and postsynaptic Specializations

Our findings show that dPGA-coated beads incubated with cortical neurons in culture promote the accumulation of cellular specializations the exhibit structural and functional features of pre- and postsynaptic elements. The bead-associated specializations we detected exhibit strong immunopositivity for canonical synaptic markers, including, synaptophysin-1, PSD95, VGLUT1, VGAT, and Gephyrin (Fig.1-2). Imaging using transmission electron microscopy revealed ultra-structural characteristics typical of glutamatergic pre- and postsynaptic elements *in vivo* (Burette et al., 2015) (Fig.3). We then assessed the functional capacity of pre-synaptic specializations to carrying out depolarization dependent vesicle recycling and release. Using FM1-43 dye, we labelled endocytosed vesicles, and demonstrated depolarization induced release in the specializations around PDL and dPGA-coated beads, consistent with neurotransmitter recycling and release at functional pre-synaptic terminals (Fig.6). These findings support the conclusion that the elements detected around dPGA-coated beads are functional pre-synaptic specializations with the capacity to recycle and release neurotransmitter.

Similar observations have been made for neuronal elements formed onto non-neuronal cells engineered to express synaptogenic proteins (Scheiffele et al., 2000) and on synthetic surfaces coated with synaptogenic proteins or peptides (Lucido et al., 2009; Goldman et al., 2013; Kim et al., 2013). Our findings corroborate substantial evidence from the literature indicating that both pre- and postsynaptic like specializations can form despite the absence of a functional (Aoto et al., 2007; Kayser et al., 2006; Kim et al., 2006; Linhoff, 2010) or even biological synaptic partner (Dean et al., 2003; Goldman et al., 2013). A model presented by Goodman and Shatz (Goodman and Shatz, 1993), argues that this reflects the apparent promiscuity of neurons to form synaptic connections during early phases of development, with the circuit later being refined by activity-dependent plasticity to reinforce meaningful connections and prune unnecessary synapses during later stages of development (Shatz, 1996). Such a mechanism ensures the adaptability of the neuronal network in the face of different task requirements imposed by the environment.

dPGA-Coated Surfaces Promote the Formation of Synaptic Specialization by Neurons in Developing and more Mature Synaptic Networks

Synaptogenesis gradually decreases in brain during later development as synaptic networks become increasingly refined and stabilized (Tau and Peterson, 2010). Yet, synaptogenesis and synaptic plasticity persist in the mature nervous system (Holtmaat and Svoboda, 2009). Similarly, new synapse formation and synaptic plasticity also occur in mature neuronal cultures (Bazarsky et al., 1994). The persistence of the capacity of a neuron to form new synapses has important implications for the application of synaptogenic coatings to neural interfaces. Brain-computer interfaces have tremendous potential for the treatment of adult patients whose mature nervous system may not be as receptive to synaptogenic cues.

Here we show that all 3 polymer coatings are effective at accumulating synaptic markers in both developing (11 DIV) and more mature (28 DIV) cortical neurons in culture. Similarly, to younger cultures, dPGA coated microspheres in the older cultures show enhanced recruitment of synaptic markers compared to the other polymers.

We did, however, observe a reduction in the accumulation of postsynaptic marker PSD95 around polymer-coated beads in mature cortical neurons, while Synaptophysin 1 accumulation remained unchanged in both cases. This suggesting that the formation and differentiation of presynaptic terminals may be more responsive than postsynaptic terminals in older neurons in response to polycationic synaptogenic cues.

While cortical neurons at 29 DIV are considered "mature" and are past their peak period of synaptogenic activity, it is not clear how representative these cultured neurons are of cortical neurons *in vivo*, neurons that may be decades old, in the adult nervous system. Thus, further studies *in vivo*, in adult brains, are required to determine the applicability of dPGA as a coating that promotes synapse formation.

Hemi-synaptic stability is linked to the polymer's resistance to proteolysis

The only *in vivo* studies to address the efficacy of a synaptogenic coating on a synthetic surface reported were carried out by Richard Bury (Burry 1983; Burry 1985). His findings demonstrated that 50 μm latex beads, implanted in neonatal rat cerebellum, supported the rapid formation of pre-synaptic specializations onto their surface, however, those elements were resorbed within 7-14 days post-implantation.

In vitro neuronal cultures have the advantage of providing a simpler model to interpret; there is no tissue injury from insertion trauma, and there is reduced presence of potentially reactive glial cells. Yet, the synaptic marker accumulation and loss that we observed around PLL-coated beads followed a remarkably similar time course to Burry's *in vivo* observations (Burry 1983), with the rapid formation of synaptic specializations followed by their complete loss and return to baseline between 7 and 14 days of incubation. In the same conditions, beads coated with PDL, which is partially resistant to proteolysis showed only a partial decrease in postsynaptic marker PSD95, while the beads coated with dPGA, a structure that lacks peptide bonds, maintained a robust accumulation of synaptic elements following up to 14 days of incubation, our longest time point in the study (Fig.1 D and E). These findings suggest that the stability of synaptic markers around cationic polymer coated beads may reflect their respective resistance to proteolysis and

provides a straightforward explanation for the loss of hemi-synaptic connections around PLL-coated beads implanted in the brain reported by (Burry, 1983; Burry, 1985).

This suggests that the use of recombinant synaptogenic protein or other peptide-based coatings would be similarly proteolyzed and rapidly lose efficacy following implantation in brain (Rao et Winter 2009). Since the dPGA-coating showed a sustained accumulation of synaptic markers over extended incubation times compared to both PLL and PDL, it may prove to be a superior alternative to other synaptogenic cues as a coating for neural implants for long-term applications *in vivo*.

Enhanced Synaptogenic Potential of dPGA Compared to PLL and PDL

Comparing the immediate synaptogenic potential of dPGA to poly-lysine, dPGA-coated microbeads showed an enhanced accumulation of both pre- and postsynaptic markers as early as 1-2 hr after adding the beads to the culture (Fig. 1 D and E). Notably, this was not simply due to enhanced adhesion/binding as all 3 polymer-coated beads showed similar adhesion to the neurons following 2 hrs of incubation (Fig. 1 B).

Our findings indicate that the dPGA-coating evokes the formation of excitatory glutamatergic synaptic terminals and inhibitory GABA terminals. It is well documented that endogenous synaptogenic cues specificity different types of synapses (Südhof, 2018). To our knowledge this is the first example demonstrating that a synthetic polymer directs the formation of both excitatory glutamatergic and inhibitory GABAergic synaptic specializations. PLL coated microspheres, for instance, effectively direct the formation of pre-synaptic elements by a variety of neuronal cell types, including motorneurons (Peng et al., 1987), cerebellar (Burry et al., 1986) and hippocampal neurons (Lucido et al., 2009). dPGA may signal via a mechanism that specifically supports the formation of excitatory and inhibitory synapses. However, we cannot ignore the interdependence of inhibitory and excitatory synapse formation mechanisms. Thus, it is also possible that the presence of GABAergic synaptic specialization is due to the enhanced formation of glutamatergic synaptic elements around dPGA-coated beads that is sufficiently dense to support the development of a local GABAergic synaptic network. Alternatively, the enhanced formation

of glutamatergic synaptic elements around dPGA-coated spheres compared to poly-lysines coated beads may be due to dPGA's unique capacity to recruit inhibitory synaptic elements.

Further investigation may reveal that dPGA or PDL may recruit other types of synapses providing further insights in the biology of synapse formation and opening new possibilities for the clinical application of synthetic synaptogenic coatings to treat neurologic disorders linked to specific types of neurotransmission such as Parkinson's disease (Lotharius and Brundin, 2002) and depression (Underwood et al., 2018).

dPGA-Coated Beads Support a Local Synaptic Network

TEM and 3D reconstruction of high-resolution confocal optical stacks of the beads in culture show that the synaptic structures that initially form onto the surface of the bead ultimately support a densely interconnected local network of synapses. The elaboration of this network covers the bead surface, makings hemi-synaptic connections with the bead surface and synaptic connections between neurites. We interpret the bead acting as a synaptic hub that initially recruits hemisynaptic elements that then form new synapses with adjacent neurites in proximity. Burry (1980, 1982) made similar observations of PLL-coated beads in culture through detailed TEM studies and similar conclusions were more recently derived from observations of hippocampal neurons interacting with PDL-coated beads (Lucido et al., 2009). Our findings indicate that the coated bead functions as a nexus for synapse formation, supporting a robust interconnected network of axon-dendrite and hemi-synapses around the engineered surface.

CHAPTER 4 – DISCUSSION

A synaptogenic electrode capable of forming direct, synapse-like, connections with the surrounding nervous tissue may, in theory, provide several benefits over traditional intracortical microelectrode interfaces, especially in terms of signal stability and interface longevity.

However, before we can test whether such an synaptogenic electrode actually brings long-term benefits to brain computer interfaces, we must first develop a molecular coating with the capacity to induce neurons to form synapses onto the non-biological surface of an electrode and capable of remaining stable for extended periods of time once implanted in the brain.

Early experiments demonstrated that synthetic poly-cationic polymers can be synaptogenic *in vivo* when coated onto biologically inert surfaces, such as latex microbeads, but in these early studies the synaptic elements only lasted for 2 weeks post-implantation (Bury, 1983; Bury and Hayes, 1986).

Multiple possibilities may account for the loss of hemi-synaptic terminals reported by Bury: Insertion of a foreign body inside the brain recruits activated astrocytes and reactive microglia (Salatino et al., 2017) which can promote synaptic loss (Henstbridge et al., 2019). General tissue inflammation and the documented neuronal loss around the implant site (Biran et al., 2005) may also cause synaptic loss. Cellular debris and blood vessel rupture around the lesion may cause biofouling of the bead`s surface. Neuronal activity-dependent mechanisms (Stein and Zito, 2018, Südhof, 2018) may also cause neurons to prune or retract hemi-synapses. Finally, the polypeptide coating may simply be degraded by brain-derived proteases (Li and Yeung, 2008).

Here we show that the long-term stability of hemi-synaptic elements formed onto a synthetic surface *in vitro* correlates with the proteolysis resistance of the synaptogenic coating. Furthermore, we describe a new synaptogenic polymer, dPGA, with no peptide bond, thus resistant to protease enzymatic degradation. We show that dPGA-coated surfaces accumulate more synaptic markers and can maintain this synaptic marker

accumulation for longer compared to other known synaptogenic polymer coatings (PLL and PDL). Our findings suggest that a dPGA-based coating may possess suitable characteristics for a synaptogenic neural implant coating.

FUTURE DIRECTIONS

The *in vitro* results obtained to date are encouraging, yet they do have important limitations. Neural cultures do not entirely recapitulate the complex environment surrounding a brain implant (Biran et al., 2005). Therefore, even though we show that dPGA-coated beads exhibit enhanced and more persistent accumulations of synaptic markers compared to PLL and PDL-coated beads, it is possible that the loss of synaptic elements observed by Burry (1983, 1985) *in vivo* is due to a mechanism other than degradation of the synaptogenic polymer.

Furthermore, although it is possible to use contemporary extracellular microelectrodes to record from presynaptic terminals *in vitro* (Bakkum et al., 2017) and *in vivo* (Bartho et al., 2014), polystyrene beads are electrically inert, thus it has not yet been determined whether an electrode with a synaptogenic coating may provide a more informative signal from synaptic elements that have formed onto its surface. We are currently working to test functional synaptogenic electrodes coated with dPGA both *in vitro* and *in vivo*.

CONCLUSION

Our ongoing studies aim to enhance the function and stability of synapses formed onto modified synaptogenic surfaces *in vivo* and develop approaches to stimulate and record from these surfaces. We aim to promote the formation of adhesive contacts by axons and dendrites that will be inherently more stable and better positioned to record neuronal activity than is currently possible using conventional electrodes. Our findings suggest that the hemi-synaptic specializations formed onto the aminated dendritic polyglycerol surface will in-turn induce synapse formation by adjacent axons and dendrites, resulting in the development of a dense local web of synaptic connections surrounding the electrode.

Ultimately, we hope such implants would achieve seamless functional integration into the neuronal network of the surrounding nervous tissue. We envision that such implanted synaptic interfaces would be broadly applicable to extend the functionality of the injured human nervous system.

FIGURES

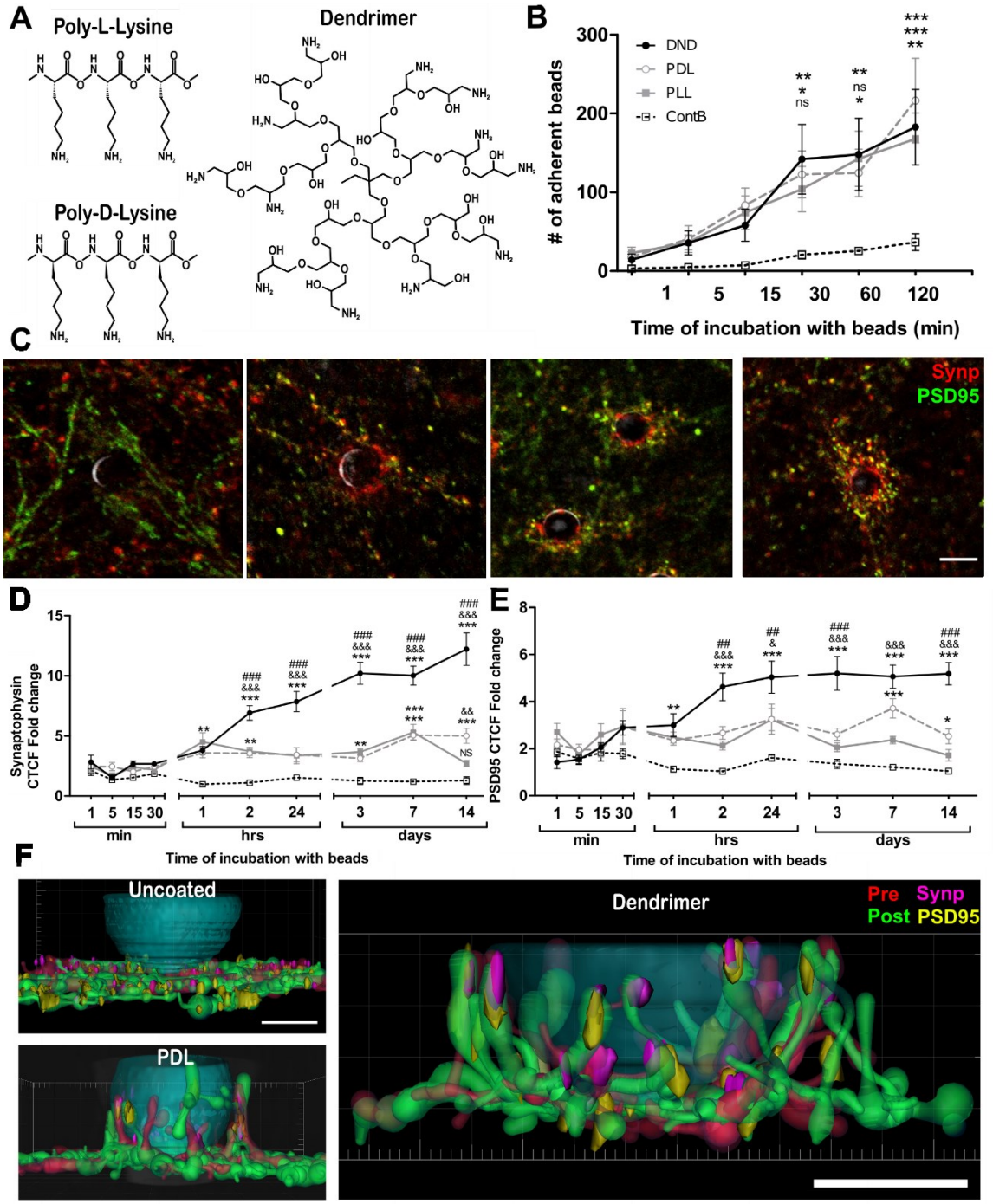


Figure 1. Enhanced synaptic marker accumulation on dendrimer coated beads. **A.** Illustration of the chemical structure of polyglycerol- amine dendrimer (right) compared to poly-L-lysine (top-left) and its enantiomer poly-D-lysine (bottom-left). **B.** Number of polymer-coated beads adhered to 15 DIV cultured rat cortical neurons as a function of incubation time. (Error bars indicate SEM, *p<0.05, ** p<0.01, ***p<0.005, 2-way ANOVA with Bonferroni's posthoc test, comparison to uncoated beads (bottom trace) are shown,

n= 9 independent cultures per condition for each timepoint). **C.** Representative images of uncoated and polymer-coated beads that were applied to 11 DIV cultured rat cortical neurons and stained for pre-synaptic marker Synaptophysin 1 (red) and post-synaptic marker PSD-95 (green) after 14 days of incubation. Scale bar corresponds to 6 µm. **D and E**. Measurement of Synaptophysin 1 (D) and PSD-95 (E) corrected total cell fluorescence intensity (CTCF) surrounding beads incubated for 1 min to 14 days with 11 DIV cultured rat cortical neurons. (Error bars indicate SEM, * denotes comparisons to uncoated beads, & denotes comparisons to PLL coated beads and # denotes comparisons to PDL coated beads (*$p<0.05$, **$p<0.01$, *** $p<0.005$, 2-way ANOVA with Bonferroni's posthoc test). For the 15 min to 14 day time points, n= 52 beads per condition from 3 independent cultures per timepoint. For the timepoints 1 to 5min, n = 15 beads per condition, per timepoint). **F.** 3D reconstruction using Imaris® software surface and filament tracing tool. The images show the side view of a representative confocal microscope z-stacks of uncoated, PDL and dendrimer-coated beads incubated with 11 DIV cultured rat cortical neurons immunostained for Synaptophysin 1 (magenta) and PSD-95 (yellow) following 7 days of incubation. Underlying neurites were assigned as pre-synaptic (transparent red) or post-synaptic (transparent green) based on detection of the corresponding synaptic marker. The 3D surface of the bead (light transparent blue) was reconstructed based on brightfield images collected with the confocal z-stack. Scale bar, 3 µm.

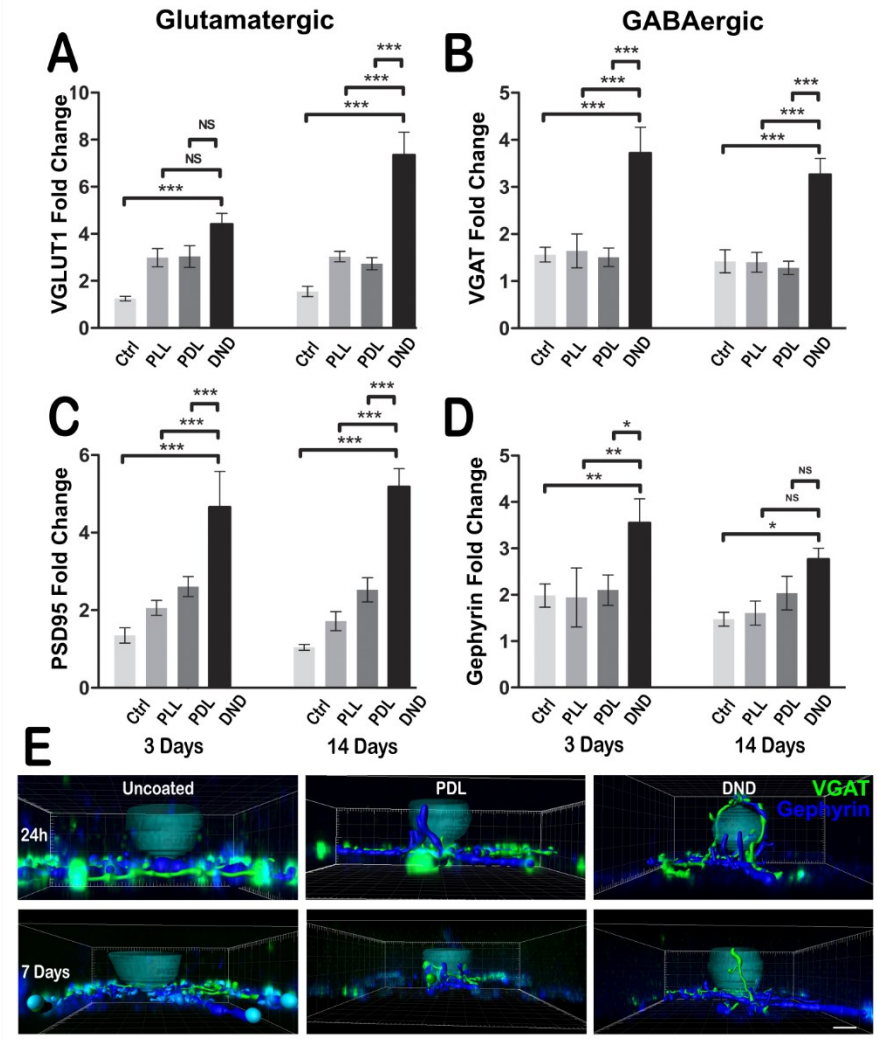


Figure 2. Enhanced recruitment of excitatory and inhibitory synaptic markers to dendrimer coated beads. **A-D.** Quantification of fold change in measured CTCF around uncoated and polymer-coated (PLL, PDL and dendrimer) beads compared to an adjacent segment of the neurite. **A and C**. CTCF fold change of excitatory pre-synaptic marker VGLUT 1 (**A**) and excitatory postsynaptic marker PSD-95 (**C**) around beads added to 11 DIV cultured rat cortical neurons for 3 and 14 days before staining. **B and D**. CTCF fold change of inhibitory pre-synaptic marker VGAT (**B**) and inhibitory post-synaptic marker Gephyrin (**D**) associated with beads added to 11 DIV cultured rat cortical neurons for 3 and 14 days before staining. (Error bars indicate SEM, NS $p>0.05$, *$p<0.05$, **$p<0.01$, *** $p<0.005$, 2-way ANOVA with Bonferroni's posthoc test, n= 52 beads per condition from 3 independent cultures, for both timepoint). **E.** 3D reconstruction using Imaris® software filament tracing tool. The images show the side view of a representative confocal microscope Z stack of an uncoated, PDL and dendrimer-coated bead added to 11 DIV cultured rat cortical neurons and stained with inhibitory presynaptic marker VGAT (green) and inhibitory post-synaptic marker Gephyrin (blue) after 24 and 7 days of incubation. The 3D surface of the bead (light transparent blue) was reconstructed based on brightfield images collected with the confocal Z stack. Scale bar, 2 µm.

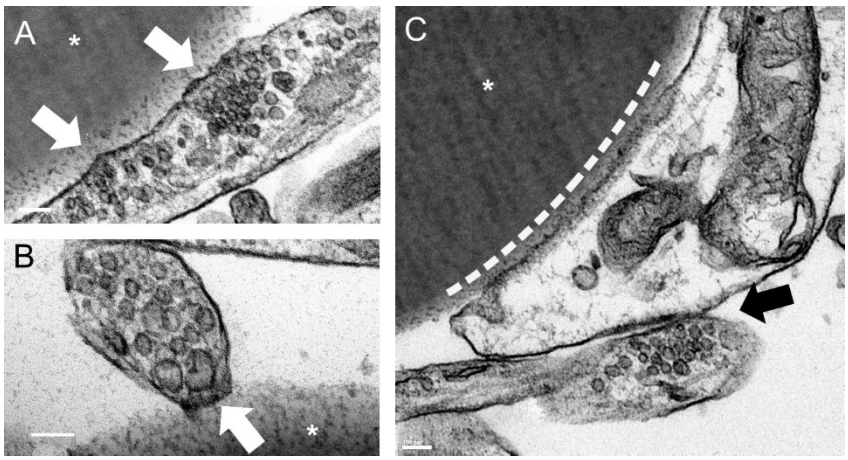


Figure 3. Transmission electron microscopy reveals contacts between neuronal processes and dendrimer coated beads that exhibit features similar to the ultrastructure of pre- and post-synaptic specializations. **A.** Pools of vesicles aggregated (white arrow), similar to pre-synaptic ultrastructure, at the point of contact with a dendrimer coated bead (marked by white *, Scale bar, 100nm). **B.** Vesicles pooled within a neurite with white arrow indicating contact with a dendrimer coated bead. White * indicates an electron-dense band within the neurite reminiscent of a pre-synaptic active zone. **C.** Putative post-synaptic element, with a thickened PSD-like structure (to the right of white dashed line), adjacent to the surface of a dendrimer coated bead indicated with white *. The putative post-synaptic element forms a neuron-neuron synapse with an adjacent neurite (black arrow).

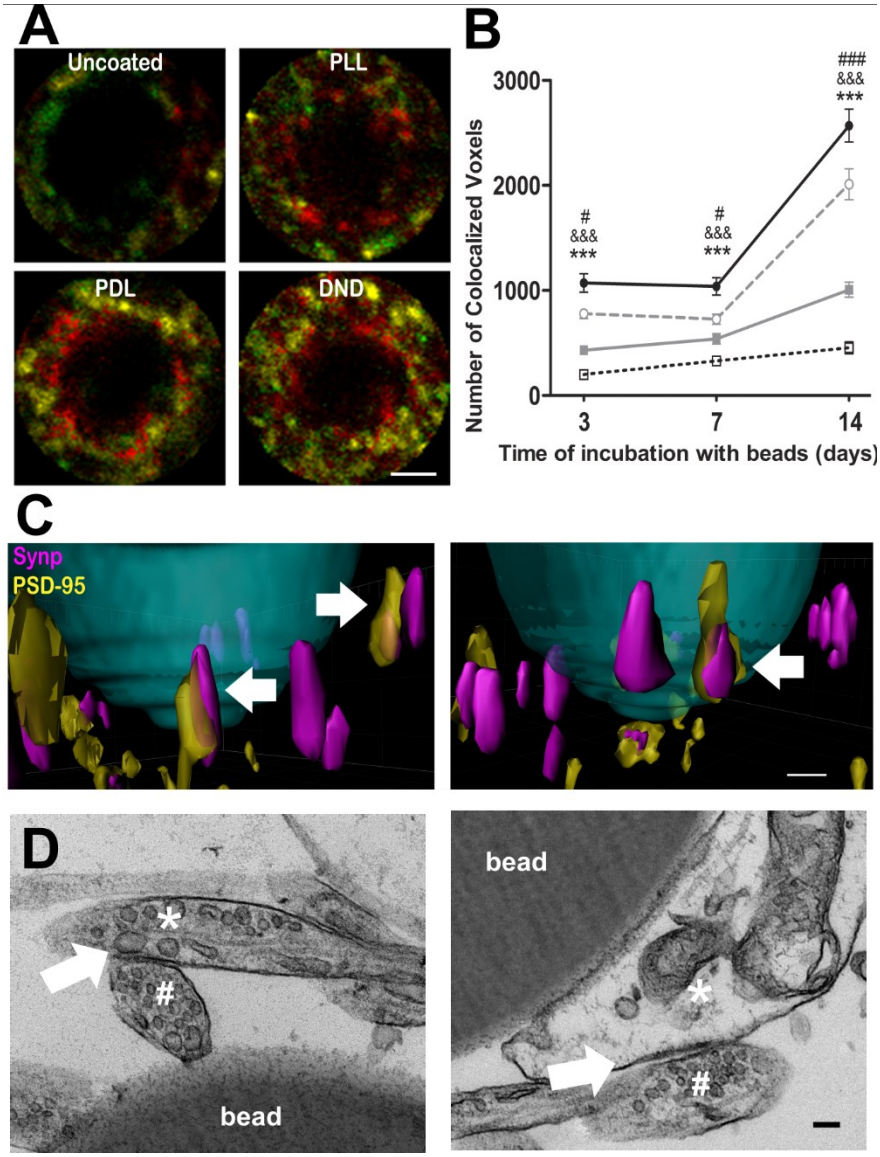


Figure 4. Dendrimer coated beads act as a nexus for neuronal synapse formation. **A.** Maximum intensity projection of representative confocal image Z stacks of uncoated and polymer-coated beads that had been added to 11 DIV cortical neurons, then cultured for an additional 14 days and then immunostained for pre-synaptic Synaptophysin 1 (red) and post-synaptic PSD-95 (green). Yellow indicates overlap of Synaptophysin 1 and PSD-95 signals within the same pixel (scale bar is 2 µm). **B.** Quantification of the number of colocalized PSD-95 and Synaptophysin 1 positive voxels around coated and uncoated beads in a confocal Z-stack (error bars indicate SEM, * denotes comparisons to uncoated beads, & comparison to PLL coated beads. and # for comparisons to PDL coated beads, *p<0.05, **p<0.01, *** p<0.005, 2-way ANOVA with Bonferroni's posthoc test, n= 52 beads per condition from 3 independent cultures, for each time point). **C.** 3D reconstruction using the Imaris® software Surface tool for Synaptophysin 1 (magenta) and PSD-95 (yellow) illustrating close proximity of positive puncta (white arrows) within a high-resolution

confocal Z stack of dendrimer coated beads added to 11 DIV rat cortical neurons and cultured for an additional 7 days (scale bar is 0.5 μm). **D.** Transmission electron microscopy images showing ultrastructure of contact between putative pre- and post-synaptic elements (white arrow) adjacent to the surface of a dendrimer-coated bead (white *, scale bar is 100 nm).

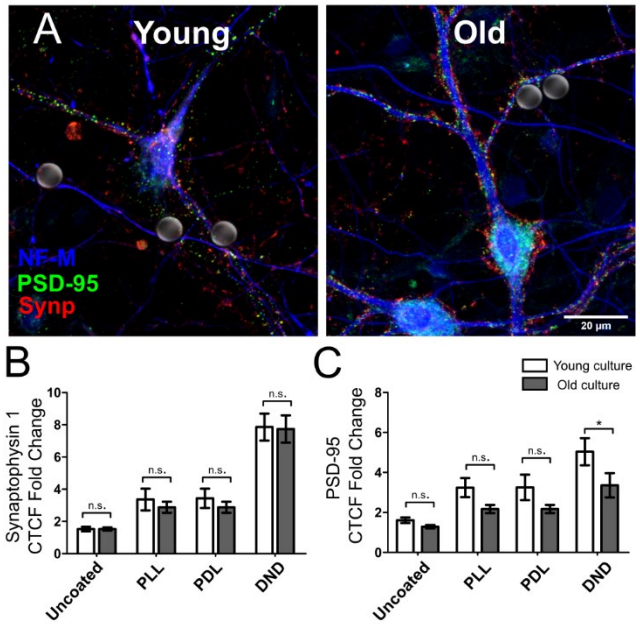


Figure 5. Dendrimer coated beads direct the formation synaptic specializations in both "young" and "old" neuronal cultures. **A.** Representative confocal image of "young" (12 DIV) and "old" (29 DIV) cultures of rat cortical neurons immunostained for the neuron-specific cytoskeletal protein Neurofilament-M (blue), pre-synaptic Synaptophysin 1(red) and post-synaptic PSD-95 (green). Cultures had been incubated with dendrimer coated beads (gray circle) for 24 hr (scale bar, 20 µm). **B and C.** Quantification of fold change in CTCF for Synaptophysin 1 (**B**) and PSD-95 (**C**), around uncoated and polymer-coated (PLL, PDL and dendrimer) beads compared to an adjacent segment of neurite of young neurons (white bars) and old neurons (gray bars), following 24 incubation with the beads (error bars indicate SEM, n.s. is $p>0.05$, * is $p<0.05$, 2-way ANOVA with Bonferroni's posthoc test, n= 43 beads per condition from 3 independent cultures, for both "young" and "old" cultures).

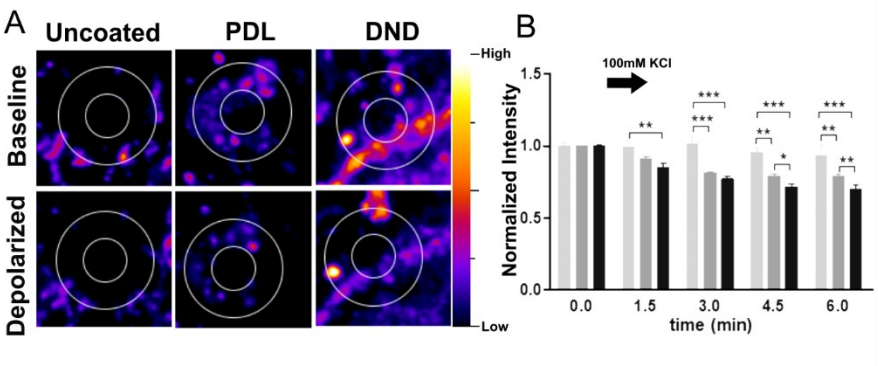
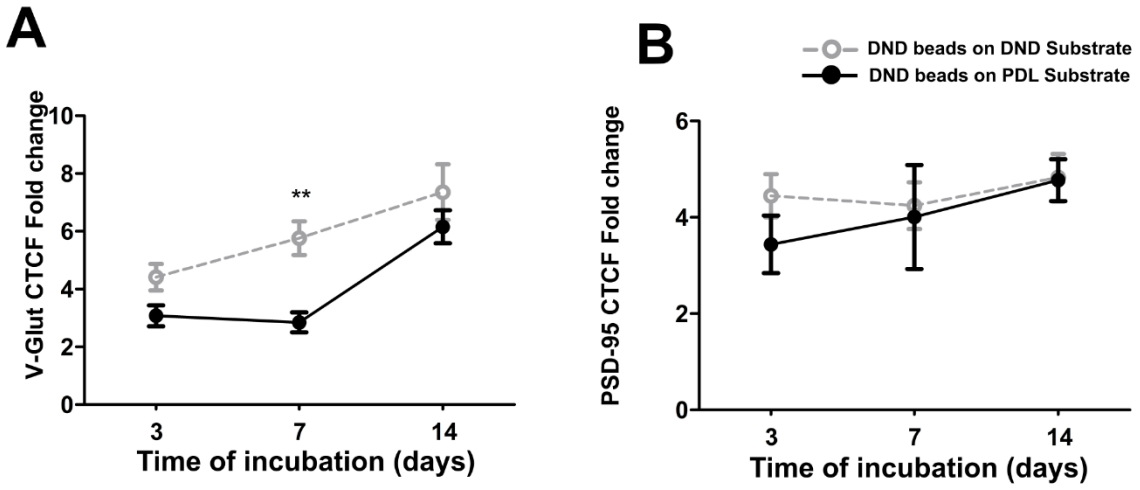


Figure 6. Putative synaptic elements associated with PDL and dendrimer coated beads exhibit depolarization dependent vesicle release and recycling, a hallmark of synaptic activity. **A.** Representative confocal images of neuronal processes containing vesicles loaded with fluorescent dye FM1-43, associated with uncoated, PDL and dendrimer coated beads that had been applied to 11 DIV rat cortical neurons and cultured for an additional 7 days. Images show fluorescence signal around the same beads before (t=0) and after (t=6min) depolarization with 100 mM KCL. Pixel values are color coded according to relative fluorescence intensity with high intensity values in warm colors and low intensity in dark/cold colors. Reference legend on right shows a linear increase in pixel intensity values from 0% to 100% (0 to 65,535 for 16 bit images, scale bar is 3μm). **B.** Normalized fluorescent intensity of FM1-43 signal associated with coated beads in 90 sec bins following depolarization with 100 mM KCL at t=0 sec (error bars represent SEM, * $p<0.05$, ** $p<0.01$, *** $p<0.005$, 2-way ANOVA with Bonferroni's post-hoc test, n=12 uncoated beads, n=56 PDL coated beads and n=65 DND coated beads.)

Appendix



Supplementary Figure 1. Dendrimer coated beads show enhanced recruitment of excitatory synaptic markers in cultures grown on both PDL and DND coated glass. **A-B.** Quantification of the fold change in the measured CTCF around dendrimer coated beads compared to an adjacent segment of the neurite of neurons grown on either a PDL coated surface (black circle) or neurons grown on a dendrimer coated surface (gray open circles). CTCF fold change of excitatory pre-synaptic marker VGLUT 1 (**A**) and excitatory postsynaptic marker PSD-95 (**B**) around beads incubated with 11 DIV cortical neuron culture for 3, 7 and 14 days before staining. (Error bars indicate SEM, NS $p>0.05$, *$p<0.05$, **$p<0.01$, *** $p<0.005$, 2-way ANOVA with Bonferroni's posthoc test, n= 30 beads per condition from 3 independent cultures, for both timepoint).

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