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ARTICLE

Hyaluronic acid and neural stem cells: Implications for biomaterial design

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While in the past hyaluronic acid (HA) was considered a passive component with a primarily structural role in tissues, research over the past few decades has revealed its diverse and complex biological functions, resulting in a major ideological shift. HA is abundant during normal central nervous system (CNS) development and, although down-regulated, remains ubiquitous in adult extracellular matrix (ECM). Significant changes in HA content are associated with pathological conditions, including stroke, traumatic injury and multiple sclerosis, and these changes likely disrupt repair by endogenous neural stem cells (NSCs). In this review, we describe recent findings in HA biology relevant to NSCs–focusing on the potential of HA-NSC interactions to mediate CNS regeneration. Currently, HA biomaterials are being developed to counteract matrix changes associated with CNS injury and disease, thereby promoting NSC survival and directing differentiation. In parallel, HA-based biomaterials engineered to mimic the native CNS microenvironment are being used to investigate the relationship between NSCs and their HA-rich surroundings within a controlled experimental space. As our understanding of HA-NSC interactions improves, so will the therapeutic potential of HA-based biomaterials. Efforts to better understand the relationship between HA bioactivities and biomaterial design parameters are already underway. Although significant progress has been made improving techniques for controlled fabrication of HA-based hydrogels with precisely defined features, there is still much work to be done. Ideally, future designs will incorporate multiple types of microenvironmental cues – orthogonally tuned in time and space – to direct differentiation of NSCs into various specialized lineages within a single biomaterial platform.

I. Introduction

Hyaluronic acid, also known as hyaluronan (HA), – a non-sulphated glycosaminoglycan (GAG) and major constituent of the extracellular matrix (ECM) – is composed of repeating disaccharide units of N-acetylglucosamine and glucouronic acid. HA levels are particularly high in the ECM of central nervous system (CNS), which includes the brain and spinal cord. The molecular weight of HA, which ranges from less than a thousand to a few million Daltons (Da) naturally, determines its physiological functions, which are remarkably diverse[1]. In the embryonic ECM, HA is present in relatively large quantities and decreases as development progresses. Total HA content in rodent neonatal brain is only around 25% of its levels in the embryo – illustrating the central role of HA in CNS development[2].

As the organizing center of the ECM, HA interacts with proteins and other GAGs via unique binding sites and with various "linker" proteins forming a complex mesh. Although HA has this organizational function in many tissues, the specific composition of the accompanying ECM components are unique to each tissue. For example, the brain and spinal cord ECM lacks the fibrous components, such as collagen I, that dominate non-CNS tissues and instead is enriched in the sulphated GAGs, such as neurocan, brevican, versican, tenascin-C during development and tenacin-R in the adult CNS (**Figure 1**). More detailed descriptions of the HA-rich ECM in the CNS can be found in reviews by Rauch[3] and Zimmerman and Dours-Zimmerman[4]. For an in-depth review of hyaldherins other HA-binding linker proteins, see Day and Prestwich[5]. In addition to being part of the organizing centers for the ECM, it is now well-established that HA and other hyalectins are involved in formation of perineuronal nets (PNNs) – specialized, dense ECM structures found around neuronal cells bodies and proximal dendrites (for reviews of PNNs see Celio and Blümcke[6], Celio et al.[7], Dityatev et al.[8], Kwok et al.[9], and Jäger et al.[10]). PNNs do not completely mature until after birth – 21 days old in a rodent[11], suggesting a gradual change in components with the ECM during the development of the CNS.

In addition to its structural functions, HA directly participates in diverse biological processes, including development, inflammation, angiogenesis and regeneration. In general, HA levels increase during periods of rapid cell proliferation, cell migration and ECM remodeling[12]. HA affects proliferation and maturation of many cell types, including neural stem cells (NSCs), whose functional relationship with HA is a major focus of this review.

Within the CNS, high molecular weight HA is reported to decrease proliferation of astrocytes[13, 14] and HA-dependent activation of toll-like receptor (TLR)-2 on immature oligodendrocytes has implications for remyelination in multiple sclerosis[15]. Beyond the CNS, it appears that HA influences the proliferation and maturation of a multitude of

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cell types[16], including endothelial cells[17-19], macrophages[20, 21], and specialized kidney cells[22].

HA asserts its biological functions via several non-integrin cell surface receptors, which include CD44, the receptor for hyaluronan-mediated motility (RHAMM), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), intracellular adhesion molecule 1 (ICAM-1) and TLRs 2 and 4 (**Figure 2**). In the CNS, increased astrocytic expression of CD44 appears to be an important response to insult or injury[23-31]. HA levels in the ECM are regulated by a balance of HA degradation by hyaluronidases, receptor-mediated endocytosis of extracellular HA, and direct deposition of new HA into the ECM by HA synthases[11, 32-34].

HA has an effect on proliferation and maturation of many cell types, including NSCs, whose functional relationship with HA is a major focus of this review. For a broader review of HA biology, see Dicker et al., 2014[35]. In this review manuscript, we first describe the functions of HA in the normal brain and spinal cord ECM. Next, we discuss the relationships between HA and NSCs in CNS pathologies, including spinal cord injury and cancer. There is a large body of evidence that suggests that pathological mutations in NSCs are responsible for initiation of brain tumors[36, 37], whose ECM is highly enriched in HA and likely contributes to cancer progression[38-41]. Finally, we describe development of HA biomaterials engineered to recapitulate these relationships and look forward to innovative strategies for development of bioactive materials that leverage the effects of HA to direct NSC phenotype.

II. HA and NSC in the CNS

Embryonic Development of Neural Tissues

HA levels peak in the ECM of rat brain 7 days after birth and decline rapidly to about 51% of this peak by 18 days as the brain matures[2]. HA is thought to be instrumental to CNS development. For instance, during development of the chick spinal cord, HA expression is observed around ventricular zones, which are hypothesized to be an NSC "niche", and around differentiating neurons, including motor neurons[42]. Although HA content continues to decrease with age[11, 43, 44], HA-NSC interactions remain vital for proper function and repair of the CNS throughout adulthood. Many of the interactions and biological effects of HA are mediated by CD44, which is highly expressed by NSCs[45-49]. For a broader reviews of the role of CD44 in the nervous system, see Dzwonek and Wilczynski 2015[50] and Ruppert et al., 2014[51]. TLR-2, another receptor for HA, is also expressed by proliferating, NSC-like cells during mouse brain development[52].

HA is also highly expressed in PNNs. Although PNN formation and its importance for proper maturation of neuronal networks is well studied, the mechanism for initiation of PNN formation remains unclear. Recent findings suggest that HA may be essential to PNN formation during later stages of development[10, 53]. In an elegant study, Giamanco, et al. 2010 demonstrated that when primary rodent cortical neurons isolated from embryonic rodent brains were cultured in the absence of glial-derived ECM components, aggrecan and HA were sufficient for normal PNN development to continue *in vitro*[53]. A separate study showed that co-expression of HA synthase 3 (HAS-3) and an HA-binding linker protein by cultured kidney cells induced formation of PNN-like dense ECM[54]. Taken together, these finding suggest that HA, aggrecan and HA linker proteins are essential components for the formation of compact PNNs and maybe early nucleating factors.

An examination of ECM components in adult human brain tissue reported that while aggrecan and linker proteins showed regional specificity, HA expression was ubiquitous – emphasizing the widespread influence of HA in the CNS[55]. Likewise, examination of adult human spinal cord tissue revealed an abundance of HA throughout the white matter and HA confinement to PNNs in the grey matter, which is typically associated with long-range projection neurons[10]. In the adult rodent spinal cord, fully developed PNNs containing HA have been reported in approximately 30% of motor neurons, 50% of large interneurons, and 20% of dorsal horn neurons[11]. Degradation of HA in PNNs results in enhanced fiber sprouting and plasticity[56, 57] – providing compelling evidence that HA in PNNs acts to stabilize mature synaptic connections[10, 11, 58-63].

Adult Neurogenic Areas & HA Expression

ECM molecules have significant effects on cell division, differentiation and migration – as evident in neurogenic areas of the adult brain. Similar to the developing CNS, these neurogenic areas in adult CNS express specific spatial and temporal patterns of ECM that dictate cell function, division and eventual differentiation (**Figure 3**). Recently, there have been exciting advances in our understanding of the role of ECM – and in particular of HA – in neuronal differentiation and synapse maturation and plasticity[64-68]. A recent article found that digesting HA within the ECM using hyaluronidase within the gerbils auditory cortex can increase the plasticity of that brain region[69]. Below we focus on the influence of HA in the ECM on maintenance of NSCs in neurogenic niches.

Brain: SVZ and SGZ

Like in the developing brain, high amounts of HA are observed within neurogenic regions of the adult brain[70-72]. Both cell-cell and cell-ECM interactions are thought to be essential for NSC maintenance in these neurogenic regions[33]. Currently, there are two known neurogenic regions in the adult rodent brain: (1) the subventricular zone (SVZ) (see **Figure 3A**), from which NSCs migrate along the rostral migratory stream (RMS) and produce new neurons, and (2) the subgranular zone (SGZ) (see **Figure 3B**), which is part of the dentate gyrus within the hippocampus[73-76].

In addition to NSCs, multiple cell types reside in neurogenic niches – including astrocytes, ependymal cells of the CNS and endothelial cells and pericytes of nearby blood vessels – and each cell type is critical to niche function and maintenance. Interestingly, high levels of HA

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are present in the SVZ and RMS[72] and in the hippocampus[77], where HA mediates NSC migration via RHAMM[72]. However, the expression pattern of HA in hippocampus is lamina-specific. For example, the CA1 region and the strata oriens and radiatum display high levels of HA, but the stratum lacuunosum-moleculare does not appear to contain HA[71]. Discrete deposition of HA has also been observed adjacent to glia fibrillary acidic protein (GFAP)-positive NSCs in the SVZ and SGZ[33]. This persistent, localized expression of HA in neurogenic niches of the adult brain suggests the important role of HA in NSC maintenance.

Recent data examining cultured NSCs suggest that the presence of high molecular weight HA within the niche may direct the ultimate fate of NSCs that have migrating beyond the niche[78]. *In vitro*, NSCs derived from mouse embryonic stem cells have been reported to express high levels of HA during the neural induction phase that co-localized with developing neurons and oligodendrocytes, but not astrocytes[78]. Although the exact role of HA in close proximity to NSCs is still an active area of research, it may be that HA regulates and/or slows NSC proliferation within adult neurogenic niches. This idea is supported by evidence that RHAMM[79] and CD44[80], both cell surface receptors for HA, can each affect mitosis.

Spinal Cord

The central canal, or ependymal region, of the adult spinal cord is also considered to be an NSC niche[81] (see **Figure 3C**). However, much less is known about the function of this niche in the spinal cord compared to the niches within the SVZ and SGZ. HA is detected throughout the white and grey matter of the spinal cord through development into adulthood; however, in the grey matter, HA appears to be concentrate around PNNs[11, 82]. Several distinct differences exist between the stem cell niches in the brain and the central canal of the spinal cord: 1) sustained neurogenesis is not observed in the adult spinal cord as it is in the adult brain and 2) although HA is present throughout the parenchyma, no HA deposition is apparent along the ependymal face of the central canal[83]. However, analogous to NSC migration along the RMS, spinal cord NSCs respond to injury or chronic inflammation by proliferating and then migrating along HA-rich white matter tracts toward the lesion[84-86].

Although the NSCs in the central canal do not appear to generate new neurons in adults, they do develop into new myelinating oligodendrocytes and astrocytes[87, 88]. The majority of actively proliferating NSCs in the central canal niche line the ependymal surface and co-express vimentin and Sox9, but do not typically express GFAP[87]. The central canal also contains GFAP$^+$/Sox9$^+$/vimentin$^-$ NSCs, which have been commonly referred to as radial glia; however, these cells are also scattered throughout the parenchyma[87]. A third type of NSC, termed by Barnabe-Heider et al., as oligodendrocyte progenitors, is also found throughout the adult parenchyma[87]. Taken together, these observations suggest that in the spinal cord HA may be important for supporting NSC differentiation into oligodendrocytes or even motor neurons over astrocytes.

III. HA and NSCs in CNS Pathology and Repair

Many pathological conditions in the CNS are accompanied by significant changes to the ECM: in particular the spatial distribution, molecular weight and overall amount of HA[40, 89, 90]. The presence of low molecular weight HA in the acute phase of injury in the CNS is vital to NSC recruitment, immune response and angiogenesis[12, 15, 33, 46, 91-93]. In chronic pathologies, high molecular weight HA is also overexpressed in the brain after ischemia[31], in multiple sclerosis lesions [46], Alzheimer's disease[94, 95], epilepsy [96, 97], amyotrophic later sclerosis (ALS)[27], in many types of brain tumors[40, 98-104], and after spinal cord injury[46, 91, 105]. Likewise, HA synthases, CD44 receptors and hyaluronidases are upregulated in these conditions[29-31, 40, 46, 90, 91, 106-109]. These pathological changes in the CNS ECM are likely to play significant roles in the reparative processes by compromising survival of neurons and oligodendrocytes, altering functional outputs of these cells and preventing NSC-mediated tissue regeneration.

NSC Response to HA Matrix Alterations

Endogenous NSCs in both the brain and spinal cord can migrate towards regions of injury during the acute inflammatory phase where they begin to proliferate[86, 110-113]. Lindwall, et al., recently demonstrated that HA deposition and expression of RHAMM by migrating NSCs were both up-regulated in the RMS after ischemic injury in adult mice – emphasizing the central importance of NSC-HA interactions to the CNS injury response[72]. Interestingly, some degree of inflammatory response is required for NSCs to accumulate in large numbers near an injury in the spinal cord, and differentiate into oligodendrocytes to remyelinate denuded axons[87, 114, 115]. Delivery or over-expression of mitogenic and chemotactic factors at the injured area can further increase NSC numbers[116-120]. Despite the presence of large numbers of NSCs that may benefit recovery via production of neurotrophic and other survival factors[121], differentiation of NSCs in or near CNS lesions is highly inefficient and fails to mediate functional tissue repair[113, 122, 123]. Instead, most of these cells remain undifferentiated or differentiate into astrocyte-like cells that contribute to the inhibitory glial scar[87, 105, 124]. A major contributing factor to this differentiation "block" could be the lack of appropriate microenvironmental cues – in particular those provided by the ECM[86, 125-128].

Changes in HA Molecular Weight and Receptor Binding

HA chains of different molecular weights can elicit vastly different responses from the same cells and many cell types appear to be sensitive to HA molecular weight[1]. Although still not well understood, differential biological effects via high and low molecular weight HA is likely due to size-dependent changes in its three-dimensional (3D) conformation that lead to altered affinity for and/or clustering of CD44 and other HA receptors[1, 129, 130] (**Figure 2**). The cellular response to HA is further complicated by evidence that

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both low and high molecular weight HA induce distinct responses dependent on whether they interact with CD44, RHAMM, TLR-2, or another HA receptor[52, 131-133].

While high molecular weight HA (>1 x 10^6Da) – which is normally present in the ECM of healthy CNS tissues and enriched in NSC niches – appears to maintain NSC quiescence, low molecular weight HA (<30 kDa), appears to trigger NSC differentiation[46]. Interestingly, the type of hyaluronidase enzymes expressed may determine the typical size of fragments produced, providing an additional mechanism for cellular control[1, 12]. For example, Preston et al. recently demonstrated that digestion products of a hyaluronidase known as PH20, which produces mid-sized oligosaccharides (~65 kDa), and not those of other hyaluronidases investigated, acted to inhibit differentiation of CNS progenitors into oligodendrocytes[109]. Moreover, they reported that progenitor cells and reactive astrocytes in the vicinity of multiple sclerosis lesions specifically overexpressed PH20.

These and many other reports have supported the concept that high molecular weight HA promotes tissue stasis and cell quiescence, while low molecular weight HA promotes ECM remodeling and tissue inflammation. Over the last decade, data reported by the Sherman group at Oregon Health and Science University suggest that high molecular weight HA is degraded from normal ECM after injury and the low molecular weight HA contributes to the proliferation of astrocytes[14]. Moreover, high molecular weight HA has also been shown to inhibit the differentiation of oligodendrocyte precursors and is found in demyelinating lesions in humans[46] (**Figure 4**). However, in reality, the role of HA and its degradation products in CNS injury and repair may be even more complex. For example, a recent study demonstrated that the presence of very low molecular weight HA oligosaccharides (specifically tetrasaccharides, or HA4) in the spinal cord after injury reduced accumulation of macrophages and microglia in the lesion, enhanced motor neuron survival and increased sprouting of motor neurons in the corticospinal tract[134]. It has been suggested that the neuroprotection provided by HA4 may be from perturbation of apoptotic pathways induced by oxidative species, which are produced in abundance after injury[135]. In astrocyte cultures, HA4 induced an increase in production of regenerative factors, brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), which are also up-regulated during the acute phase of spinal cord injury[135].

Overall, it has become obvious that HA likely affects multiple cell types in the CNS and that a balance of HA content and its molecular weight distribution in the ECM is vital to the injury response and functional regeneration. Indeed, the biological effects of HA and its fragments are reminiscent of other neuropeptides in the CNS where both the parent molecule and the resulting peptides have biological activities. In this manner, HA can be thought of as a resident precursor within the normal ECM towards different bioactivity via its many possible by-products.

HA, NSCs and Inflammation

HA can have a direct role in the inflammatory environment of CNS tissues after traumatic injury. For example, high molecular weight HA exhibits direct antioxidant effects and indirect affects on production of inflammatory cytokines, which confer protection from secondary injury after spinal cord injury[12, 136, 137]. Conversely, low molecular degradation products of the HA matrix up-regulate the inflammatory response through TLR-2 and TLR-4 on macrophages and dendritic cells[92, 138-140]. Although TLR-2 activation has been reported to be necessary for NSC-mediated remyelination *in vivo*[114], expression of TLR-2 has also been associated with an inhibition of oligodendrocyte maturation in experimental models of multiple sclerosis[15]. Okun *et al.*, reported that engagement of TLR-2 on progenitor cells by low molecular weight HA prevents proliferation[52]. Taken together, these studies indicate that low molecular weight HA likely interacts with TLR-2 on NSC-derived progenitor cells to suppress proliferation and induce differentiation. Identification of these opposing effects of HA on NSCs in acute (low molecular weight HA) and chronic (high molecular weight HA) injury support the idea that while the initial, acute inflammatory response provides some benefit to repair, progression to a sustained, hyper-inflammatory state (as in chronic lesions) leads to pathological changes in the ECM that ultimately inhibit regeneration.

IV. Advances in HA-Based Biomaterials

The majority of studies investigating the biological effects of HA have used experimental models where HA is solubilized in the culture medium[130, 133, 141, 142]. However, the solubilized state is not necessarily reflective of HA presentation *in vivo*, where HA exists as an insoluble, hydrated meshwork with other ECM proteins (**Figure 1**). For example, Schizas, et al. reported that only insoluble HA – and not solubilized HA – increased survival of motor neurons in spinal cord slice cultures[143]. This finding, in addition to the fact that HA experiences rapid turnover *in vivo*[144], have motivated researchers to develop methods to create crosslinked HA hydrogels that can provide biological interactions analogous to native HA and in which kinetics of enzymatic degradation can be controlled.

HA can be chemically modified by a variety of methods and made into hydrogels, which have a high water content (>90%) similar to natural tissue (**Figure 5**). Furthermore, the molecular weight and density of base HA polymer and crosslinking arms can be varied to impart precise control over the physical properties of the resulting hydrogels, including swelling, porosity and mechanical resistance[145, 146]. HA hydrogels can also be used to present other insoluble microenvironmental cues, such as integrin-binding peptides[147-150], and for controlled release of soluble drugs, proteins and genes[151-155].

HA hydrogels have also shown immense promise as 3D culture microenvironments for primary, CNS-derived cells[147, 156-159] and as delivery vehicles for NSC transplantation therapies[149, 151, 154]. In spinal cord[13, 151, 154, 160-162] and traumatic/ischemic brain injury[148, 150, 163-168] models, HA hydrogels significantly reduce the inflammatory response, secondary injury and glial scar formation, while actively

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promoting angiogenesis, wound healing and survival of transplanted NSCs.

Chemical Modifications of HA and Hydrogel Fabrication

As the majority of clinical injuries in the CNS are contusions, injectable hydrogels are desired to avoid the risk of further injury and inflammation of spared tissue and to guarantee that the material fits into irregularly shaped injury defects with good apposition to spared tissue[34, 160, 169, 170]. Likewise, encapsulation of NSCs for 3D culture and/or therapeutic delivery to the CNS requires that hydrogels be formed from non-cytotoxic precursors in physiological conditions and the hydrogel product, as well as any side products or unreacted moieties, must be biocompatible. HA is an ideal base material for these applications because of it is non-immunogenic, shear thinning properties in solution and has multiple chemical groups that can be easily functionalized using aqueous, biocompatible chemistries to create crosslinking sites[145, 146]. A few major strategies exist for chemical modification of HA that target either the carboxyl groups of the glucuronic acid unit, the multiple primary hydroxyls present on the carbon rings of each saccharide, or by oxidation of saccharide rings to reactive aldehyde groups. Typically, a functional group is introduced through which the HA chains can be crosslinked into a hydrogel and/or functionalized with other bioactive molecules. A number of functional groups have been investigated as mediators of biocompatible crosslinking – many of which form spontaneously under aqueous conditions at physiological temperature and pH. For a detailed review of chemical strategies to modify HA and form biocompatible hydrogels, see articles by Burdick and Prestwich[145, 146, 171].

Most commonly, formation of covalent crosslinks between HA chains proceeds via Michael-type addition[149, 159], cycloaddition (i.e., "click")[172-175] or condensation[148, 150, 157, 176] reactions. Covalent crosslinks have also been produced using photo-chemistry initiated by UV or visible light[13, 158]. Although photo-activated mechanisms provide the ability for fine control of spatial features and the possibility for dynamic tuning of hydrogel properties[146, 177], their use as *in situ*-forming materials or 3D cell scaffolds is limited because of free radical generation and difficulty of removing unreacted components after gelation *in vivo*. Even in the presence of cytocompatible photoinitiators, exposure to UV light and the generation of free radicals are damaging to cells. CNS-derived cells are especially sensitive to external stressors; for example, dopaminergic neurons are highly susceptible to oxidative stress imposed by photochemistry-generated free radicals[178, 179]. Photochemical crosslinks that are activated by visible light and strategies to spatially confine and/or quickly terminate free radical generation would substantially enhance the feasibility of these scaffolds as clinical implants in the CNS.

Non-covalently crosslinked HA hydrogels have also been developed. In this case, crosslinking may occur via physical segregation of hydrophobic/hydrophilic regions, ionic/electrostatic interactions, or disulphide bonds. Most strategies for injectable, physically crosslinked hydrogels utilize formulations so that gelation is triggered by exposure to conditions within the CNS, such as osmolarity, temperature and pH. The Shoichet laboratory at the University of Toronto has developed injectable hydrogel blends of HA and methylcellulose (HAMC), which are more liquid-like at colder temperatures but form physical crosslinks via hydrophobic domains within methylcellulose at physiological temperature[160, 162]. These hydrogels have also been used to successfully deliver NSC transplants to the rodent spinal cord after injury[151]. For a review of hydrogel formulated for injection into the CNS, see Pakulska and Shoichet[169].

Drug and Biomolecule Delivery

HA hydrogels can be engineered to deliver specific bioactive factors, including chemo-attractant growth factors that recruit endogenous NSCs to the implant area, such as stromal cell derived factor-1α (SDF-α)[180, 181], platelet-derived growth factor-AA (PDGF-AA)[117, 151] and fibroblast growth factor-2 (FGF-2)[153]. HA hydrogels have also been used for local delivery of growth factors to enhance cell survival. Growth factors could be loaded into hydrogels by physical encapsulation[154] or into polymeric (e.g., poly(lactide-co-glycolide)) microspheres[152]. Alternatively, non-covalent tethering of PDGF-AA to injectable HAMC hydrogels resulted in enhanced oligodendroglial differentiation of transplanted NSCs and a significant increase in functional recovery after SCI in rodents[151]. A group from Tsinghua University in Beijing has conjugated Nogo66 blocking antibodies to the backbone of HA hydrogels via a pH-sensitive hydrazone linkage so that the antibody is released in an acidic environment[182]. Implantation of these hydrogels, which contained poly-L-lysine to facilitate cell adhesion, into an infarct area in a rat stroke model resulted in significantly enhanced behavioural recovery[183]. Incorporation of enzymatically susceptible linkages into HA hydrogels could be a valuable tool to achieve biologically activated delivery of drugs or regenerative factors[154, 155].

Release of bioactive oligosaccharides is intrinsic to HA hydrogel materials as they are enzymatically degraded by native hyaluronidases. For example, Purcell *et al.,* demonstrated that degradation of HA hydrogels implanted in cardiac infarcts mediated the release of SDF1-α[155]. Furthermore, they showed that HA fragments locally released from degrading hydrogels acting synergistically with SDF1-α to recruit bone marrow stromal cells (BMSCs) to the infarct site. This finding is supported by previous reports that HA-CD44 interactions mediate BMSC migration[184] and that HA can "prime" cells to respond to concentration gradients SDF1- α[185]. Given that low molecular weight HA oligosaccharides act as chemo-attractants for several different cell types, including endothelial cells[141, 142], it may be that degradation of larger HA chains in the hydrogel implants to smaller fragments mediated this phenomenon.

These and other studies (*discussed in section II*) demonstrate that the molecular weight of HA fragments produced as the hydrogel degrades should be considered when designing HA-based biomaterials. For example, as the native ECM of healthy CNS is

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composed of high molecular weight HA, it is often considered the preferred base for HA hydrogels used in CNS. Although this high molecular weight (>5 x 10^6Da) HA has anti-inflammatory effects, HA around 200 kDa induces production of inflammatory cytokines while those around 4 kDa stimulate angiogenesis[1]. The size profile of fragments released from HA-based biomaterials as they degrade will depend on the hydrogel formulation, but may also be unique to the site of implantation, which defines the local composition of hyaluronidases[186]. To promote spinal cord injury repair, it may be desirable to prepare a biomaterial that would release neuroprotective HA4 during the acute phase, mid-sized fragments in the late acute/early chronic phase to encourage angiogenesis and high molecular weight HA during all stages of injury progression to reduce astrocyte and macrophage activation. At the same time, the concentration of HA may have substantial impacts on biological outcomes. In particular, the prolonged presence of concentrated, high molecular weight HA in hydrogel materials may actually be detrimental – as evidenced by the overexpression of high molecular weight HA that occurs in chronic CNS disease. Thus, regenerative strategies based on HA biomaterials would benefit from temporal control over HA presentation.

HA Presentation and Receptor Binding in Hydrogel Constructs

When considering HA hydrogels for biomedical applications, the presentation of HA and subsequent signal transduction to cells or tissue of interest should be carefully considered. Although there are multiple groups per HA disaccharide repeat available for chemical modification, it is likely that over-functionalization inhibits the intrinsic bioactivity of the HA backbone. For example, there is evidence that the extent of chemical modification of the HA backbone in hydrogel systems has greater effects on NSC differentiation than properties that have been more widely studied, such as stiffness and HA concentration[187-189]. *In vivo*, it has been reported that HA could be filtered through the liver when approximately 35% of the carboxyl groups were modified, while HA that was approximately 68% modified accumulated in tissue and could not be processed[187].

Modification of the HA backbone can interfere with interactions between CD44 and other receptors[129, 187, 190, 191]. In particular, modification of the carboxylic acid site on glucuronic acid can alter hydrogen bonding and thus the 3D conformation of HA in solution. It has been hypothesized that this 3D structure is what allows high molecular weight HA to induce different phenotypic responses, but not low molecular weight HA[1, 5, 129]. Ring opening modifications also likely interfere with 3D conformation. Thus, extensive chemical crosslinking of HA backbones may alter CD44-HA interactions – triggering physiological responses in an analogous manner to its lower molecular weight forms – or eliminate HA bioactivity altogether. Effects of HA modification on NSCs and other cells of the CNS have yet to be extensively characterized; however, performance of these studies in the future will be necessary for improved design of engineered HA hydrogels for CNS repair.

In commercially available HA hydrogel systems (*e.g.,* Hyaff-11®, HyStem®), the HA backbone may be extensively chemically modified and have significantly lower than the native high molecular weight form[192, 193]. Notably, non-covalently crosslinked HA hydrogels, such as the HAMC formulation[151, 153, 160, 162], often do not require any chemical modification of HA itself, which may improve biological activity. Current strategies for non-covalent HA crosslinking will be improved by developing new strategies that mimic crosslinking modes of native ECM via linker proteins. For example, hybrid hydrogels of HA and other GAGs may be crosslinked via small linker proteins, several of which have been previously identified to have a similar structural role in native CNS (**Figure 1**)[5, 194]. While physically crosslinked HA may better mimic its native interactions with receptors, covalent crosslinking of HA can yield hydrogels with several advantages, including increased mechanical strength and slowed, controllable degradation rates. However, biological activity must be evaluated when developing covalently crosslinked HA hydrogels. For example, Hachet, *et al.,* demonstrated that substitution of up to 30% HA chains still allowed their binding to CD44 and RHAMM receptors on multiple cell types[189].

HA Hydrogels for 3D NSC Cultures and Vehicles for NSC Delivery

HA constructs can be useful as 3D culture microenvironments in which to investigate NSC biology[147, 149, 156-159] or as protective vehicles for transplantation of NSCs[145, 149, 151, 167]. NSCs derived from multiple CNS regions and of various ages have shown excellent viability when cultured in 3D, HA-based hydrogels[147, 151, 156-159] (see **Figure 5 B&C**). HA hydrogels are optically transparent so that encapsulated cells can be readily imaged using a variety of optical microscopy techniques – providing an accessible tool for studying NSCs *in vitro*. Furthermore, HA hydrogels can potentially be formulated to mimic native fetal tissues or adult NSC niches. Several researchers have reported that HA-encapsulated NSCs remain undifferentiated unless key adhesive cues, such as tethered integrin-binding peptides, were added[33, 46, 147, 159].

When stimulated, NSCs that undergo differentiation in 3D HA hydrogel environments display mature phenotypes that more closely resemble their *in vivo* counterparts than those cultured on 2D substrates[147, 151, 152, 156-159]. This phenomena has also been explored by culturing human embryonic stem cells in 3D HA hydrogels, resulting in the differentiation of cells from all three germ layers[195] (**Figure 5B**). Similar benefits have been observed when NSCs derived from multiple sources – including rodent embryonic midbrain[158], forebrain[152, 157], cortex[156, 167] and SVZ[156], adult rodent cortex and SVZ[156], and NSCs derived from human induced pluripotent stem cells (iPSCs)[147] – have been cultured in HA hydrogels. Only in a 3D environment can cell densities matching those in the native CNS be cultured, a prerequisite for re-creating similar numbers of cell-cell contacts and synapses to that observed *in vivo*[196, 197].

In addition, HA hydrogels can be tuned to exhibit varying moduli to influence NSC fate. There is substantial evidence that softer hydrogels with stiffness similar to native CNS tissue (<2 kPa) promote

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neuronal differentiation, neurite branching and migration[198, 199], while harder hydrogels (>5 kPa) bias differentiation towards astrocytes[158, 200-203]. There is evidence that this response is due to increased activity of Rho GTPases, which suppresses neuronal differentiation, when NSCs are exposed to stiffer substrates[204]. One research group reported that culture of oligodendrocyte progenitors on substrates with elastic moduli around 500 Pa maximized proliferation[149]. Similarly, application of mechanical stimuli may contribute to oligodendrocyte differentiation[205].

Survival of NSC transplants was significantly improved when delivered via HA hydrogels[149, 151, 152, 167, 206, 207]. This benefit is likely due – at least in part – to attenuation of the immune response and free radical scavenging by HA itself[12]. HA-CD44 interactions may also provide anti-apoptotic signalling to NSCs, similar to HA-CD44-dependent resistance to apoptosis that occurs in many cancers[41, 208]. As discussed in the previous section, the majority of NSC that survive transplantation remain undifferentiated or differentiate into astrocyte-like cells that contribute to an inhibitory glial scar[105, 123, 124]. HA hydrogels may serve to alter the extracellular environment in these lesions to promote differentiation of transplanted and endogenous NSCs. This strategy is particular relevant to remyelination therapies, where HA-CD44 mediated migration is required for remyelination by transplanted progenitor cells in models of inflammatory demyelination[209]. A few recent studies have demonstrated that HA hydrogels can support NSC survival, oligodendrogenesis, and subsequent myelination in rodent models of stroke[167], spinal cord injury[151] and multiple sclerosis[149]. When transplanted into the brain, neurogenesis was also observed[167].

Addition of ECM-Derived Adhesive Cues

Several studies have demonstrated that implantation of scaffolds that include high molecular weight HA into the brain or spinal cord after injury results in a significant reduction in glial scar deposition and inflammatory cell prescence[13, 148, 150, 151, 154, 160-167]. In particular, benefits have been observed when mechanical modulus of hydrogel implants was matched to native spinal cord tissue[13, 149, 210]. Despite these promising results, infiltration of endogenous cells and axon regeneration through pure HA implants is generally poor[13, 151, 160]. To overcome this limitation, researchers have functionalized HA hydrogels with integrin-binding sites by direct modification of the HA polymer[147, 148, 150, 164] or addition a second backbone component (*e.g.,* polyethylene glycol (PEG) or other ECM-derived biomolecules). ECM-derived molecules commonly incorporated into HA hydrogels include gelatin, such as in the commercially available HyStem® formulation [149, 159, 211, 212], poly-L-lysine[158, 163, 176], and other GAGs/proteoglycans[167].

The most widely used peptides for CNS applications include the IKVAV sequence, derived from laminin, and the RGD sequence, which is present in many ECM proteins[147, 150, 164]. Lam *et al.,* reported an optimal mixture of the laminin-derived peptides IKVAV and YIGSR with RGD for induction of neurons from human iPSCs-derived NSCs cultured in HA hydrogels[147]. However, further studies will be required to identify optimal concentrations and presentation of ECM-derived components that drive differentiation of NSCs down specific lineages. Beyond adhesion, cell infiltration into hydrogels also depends on the ability of cells to either squeeze through microscopic pores or remodel hydrogels via hyaluronidase production. Park, et al. reported that addition of matrix metalloprotease (MMP)-susceptible peptides into HA hydrogels, in addition to IKVAV peptide, enhanced cell migration through hydrogel matrices, leading to increased neuronal differentiation of transplanted BMSCs and better functional recovery after spinal cord injury[154].

V. Features of Advanced HA Hydrogels and Future Directions

Ultimately, HA hydrogel implants are being developed to display CNS tissue-appropriate microenvironmental cues, including stiffness, adhesive peptides and the capacity to be remodelled by native enzymes. Incorporation of multiple microenvironmental cues into a single biomaterial platform – where each cue can be varied independently – will be essential to elucidating the relative contributions of each cue to CNS tissue development, physiology and pathology. Several studies have emphasized that coordination of multiple types of extracellular cues, including 1) chemical cues from insoluble ECM and soluble growth factors and 2) mechanical cues from the surrounding tissue matrix[213-215]. Specifically, ECM interactions appear to be crucial for NSC differentiation into oligodendrocytes. Therapies aimed at regenerating CNS tissue by driving differentiation of either transplanted or endogenous NSCs into various specialized cell types of interest will require fine-tuning of the local microenvironment (**Table 1).**

Orthogonal Tuning of Micro-environmental Cues

A particular challenge to creating this type of modular biomaterial platform is achieving independent control of HA concentration, mechanical modulus, HA molecular weight and hydrogel porosity. Typically, mechanical modulus has been altered by simply increasing total HA content in the hydrogel[145]. Alternatively, modulus can be changed independently of HA concentration by varying degree of HA modification[13, 158, 189, 216] or by adding a non-bioactive polymer (*e.g.,* PEG) to the hydrogel formulation[149, 159, 194] to increase mechanical strength. While these strategies maintain HA concentration when modulus is varied, the porosity of the material, which has marked effects of diffusion of oxygen, nutrients and waste and ultimately differentiation of encapsulated NSCs[217], may change dramatically. The Anseth laboratory at the University of Colorado-Boulder has demonstrated the use of orthogonal “click” chemistries to independently control chemical and physical cues presented by PEG hydrogels[174]. Ultimately, application and development of new methods to orthogonally tune HA concentration, HA conformation, HA molecular weight, mechanical properties and hydrogel porosity will be necessary to achieve 3D culture platforms that provide fine control over NSC phenotype.

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HA Molecular Weight and Release of Bioactive HA Fragments

To date, it has been challenging to incorporate HA of multiple, defined molecular weights into a single biomaterial as well as to characterize – much less precisely control – the molecular weight profile of HA fragments during degradation. Future designs of HA-based biomaterials should account for the dependence of HA bioactivity on its molecular weight and the profile of HA fragments released during degradation by hyaluronidase mixtures reflecting that of the CNS microenvironment. Optimizing biomaterial design for specific therapeutic applications will require a better fundamental understanding of the role of HA in tissue maintenance and repair.

HA Hydrogel/Cell Interface: Porosity

Incorporation of a macroporous structure (size-scale on the order of a cell, 10's to 100's of microns) into HA hydrogels provides a route for cell migration immediately after implantation without the need for enzymatic remodeling of the HA matrix. Previously, it has been demonstrated that macroporosity in non-hydrogel biomaterials significantly increased cell infiltration to establish a seamless interface between the biomaterial and the native spinal cord tissue after injury[218, 219]. The presence of interconnected pores to support cell infiltration within the first few days after implantation mediated early host-biomaterial integration and reduced glial scar deposition, which allowed regenerating axons to enter and cross the injury site via the scaffold bridges[218, 219]. Macroporosity may also provide an avenue for rapid revascularization of lesions[220], which has substantial benefits on tissue survival and functional outcomes after SCI[221]. To date, strategies to add macroporosity to HA-based hydrogels have required a freeze-drying preparation[222, 223] or exposure to harsh solvents[217, 224-227] after crosslinking, and thus are not suitable for *in situ* crosslinking. Others have reported incorporation of macropores into non-HA hydrogels by embedding a secondary, sacrificial material during gelation that will rapidly degrade (*e.g.,* gelatin), leaving structured macropores within the crosslinked scaffold[228, 229]. Alternatively, hydrogel microspheres can be crosslinked together to yield bulk hydrogels containing a highly uniform porous architecture[229, 230]. Beyond porosity, hydrogel chemistries that form flexible or dynamic crosslinks may allow for ample cell migration without the need for enzymatic remodeling[231]. However, at the time of this review these methods have yet to be investigated in models of injury or disease in the CNS.

Spatial and Temporal Control of Chemical and Mechanical Cues

The ability to spatially and temporally control presentation of microenvironmental features in HA biomaterials will likely also be necessary to direct differentiation of naïve NSCs into mature, functional CNS cells. For example, extensive cross-talk between NSCs and adjacent endothelial progenitor cells is central to maturation of both cell types in embryonic development[221]. Moreover, full maturation of myelinating oligodendrocytes requires physical contact with axons and may require supporting signals from less differentiated oligodendrocyte progenitors cells[232, 233]. For example, longitudinal alignment of inductive channels in pre-formed implants induces directional alignment and bundling of regenerating axons after rodent spinal cord injury, recapitulating the native tissue architecture[218, 219]. Spatial control of ECM-derived, adhesive cues would enable creation of co-culture systems to provide for coordinated communication between NSCs and other cells types or NSCs differentiating down parallel lineages.

Photochemistry has been the tool of choice to achieve spatial and temporal control of hydrogel stiffness and presentation of ECM-derived adhesive cues in biomaterials constructs. New technologies that take advantage of confocal and multiphoton laser scanning techniques have been enabled spatial control of these cues with 3D resolution[172, 175, 234-239] (**Figure 5C**). For a review of 3D hydrogel photo-patterning techniques, refer to Kasko and Wong[177] and Khetan and Burdick[240]. Although techniques for spatial and temporal control have advanced greatly in recent years, the reliance on photochemical methods may limit their application *in vivo*, where the implanted material may be difficult to access. Pre-patterned hydrogels with spatially defined cues prior to implantation using photochemical methods would typically prohibit using an injectable, *in situ* forming material. Development of injectable hydrogels with *in situ* self-assembling spatial architectures that can produce directional alignment of fibres for axon guidance would be beneficial. For example, synthetic protein hydrogels that self-assemble into a fiber architecture have shown promising results in brain injury models[241]. External application of magnetic or electric fields during *in situ* gelation is another potential route for forming post-injection, micro-scale alignment of hydrogel features[242, 243] (**Figure 5C**).

While temporal control of soluble growth factors can be achieved very easily *in vitro* by simply changing the culture medium, control has been much more difficult to achieve *in vivo*. Tethering of soluble factors to hydrogels via biological responsive elements – such as MMPs or hyaluronidases – may be a route to coordinating the release of factors with physiological events. However, it may be difficult to find linkage chemistries suitable to a diverse range of biological triggers with highly specific cleavage mechanisms. Alternatively, temporal control of drug release, availability of specific peptides and hydrogel mechanical properties could be achieved by external stimulation. For example, photo-activation has been used to remove or activate chemical cues and induce stiffening or softening of local hydrogel mechanics [177, 234, 239, 244-246]. Other external forces could be applied within the same hydrogel system to achieve orthogonal temporal control over two distinct biological cues. For example, ultrasound has been used to trigger release of various drugs from microparticle delivery systems[247].

Gene delivery from HA hydrogels has been demonstrated in non-CNS tissues[226, 248, 249], but would be valuable for perturbing NSC fate and NSC-mediated repair in the CNS. For a detailed review of gene delivery from hydrogel biomaterials, refer to Seidlits, *et al.,*[250]. Gene delivery is an attractive alternative strategy that avoids the need for the preservation of protein activity. In particular, viral vectors provide a means for long-term incorporation of genes into cells that

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can be reversibly activated/deactivated based on application of an external stimulus, such as light or antibiotic administration, or a change in gene expression levels of a secondary "trigger" gene in the cells carrying the transgene. For example, although PDGF-AA is an important factor for progression of NSCs to oligodendrocyte precursor cells, its expression must be down-regulated for oligodendrocyte maturation to proceed[86]. In this case, researchers could promote differentiation of NSCs to oligodendrocytes by delivering a gene therapy cassette that induces up-regulation of PDGF-AA during active transcription of an early NSC marker, such as Sox2. Once NSCs proceed to oligodendrocyte precursors and halt Sox2 transcription, PDGF-AA overexpression would also cease to allow for full maturation of oligodendrocytes.

Conclusions

Although progress has been made in the our understanding of the enormous influence of the microenvironment on NSCs and our ability to re-create these environments using HA-based hydrogels, there is still much work to be done. Currently, many tools are being developed to achieve orthogonal tuning of hydrogel properties with 3D spatial and temporal resolution, which will be useful for studying NSCs *ex vivo* in the near future. However, further innovations will be required to apply these technologies to *in situ*-forming hydrogels, which are highly preferable for applications in the CNS. For clinical applications, new strategies to establish a seamless interface of *in situ*-forming hydrogels with surrounding CNS tissues, such as incorporation of macroporous architecture, will also be beneficial.

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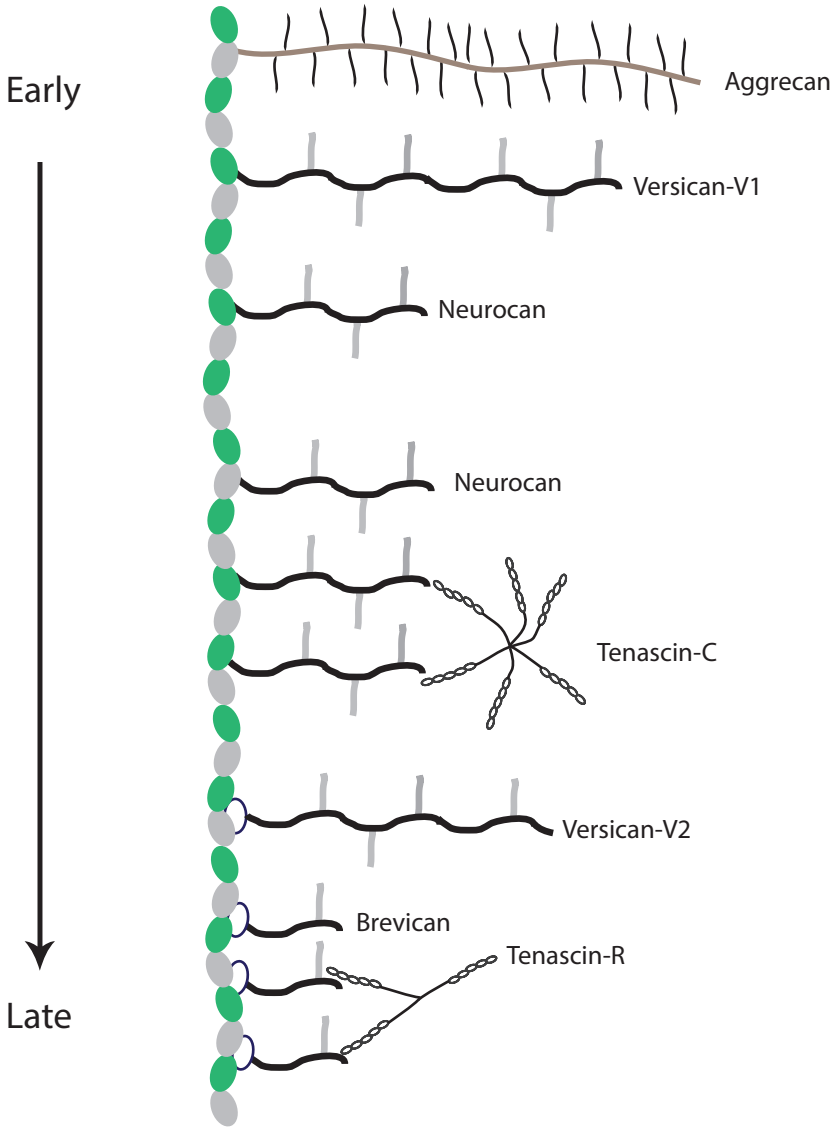
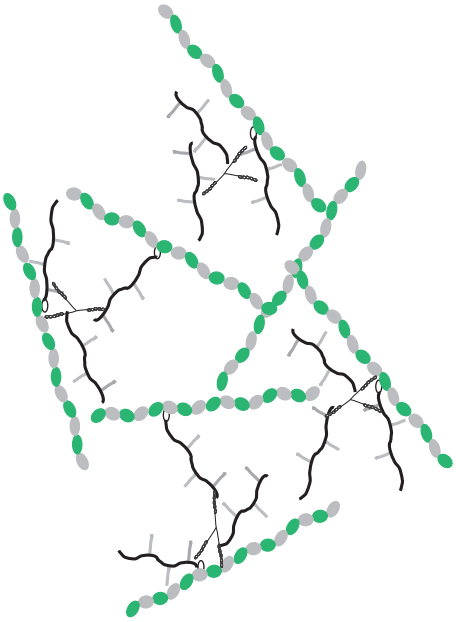
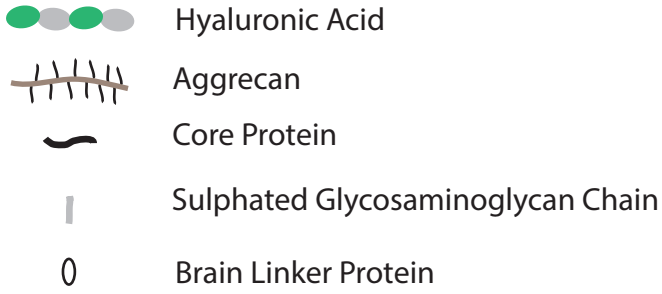
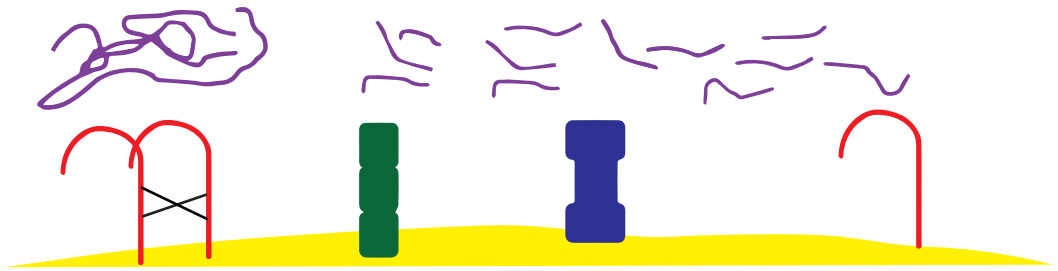


ECM Network in the Adult CNS





High Molecular Weight HA **Low Molecular Weight HA**



Clustered CD44

Maintains NSC niche
Anti-inflammatory cytokines
Regulatory T-cells
Inhibition of TLR2/4 activity
Clearance of LMW HA
Controlled degradation of bound HA
Induces NSC proliferation
Clustering of RTKs

RHAMM

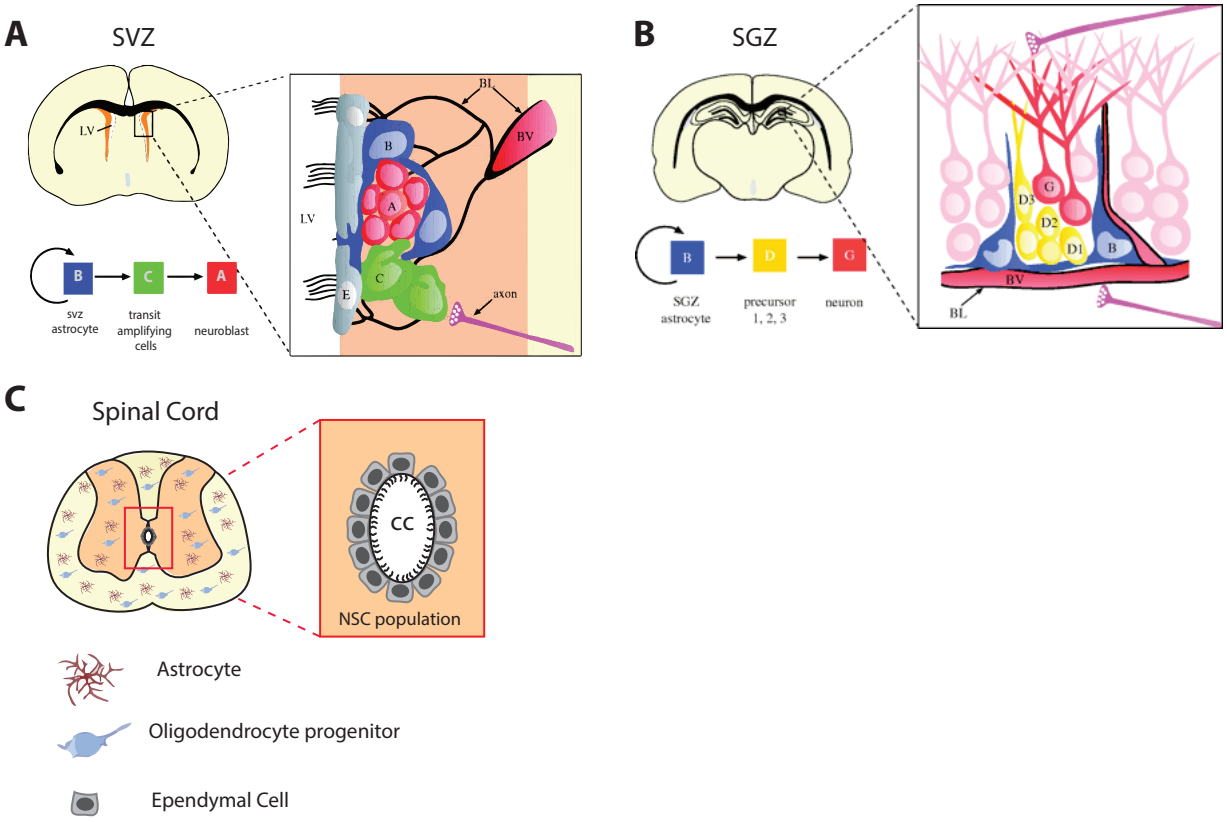
NSC migration
RTK clustering
Orientation of mitotic spindles

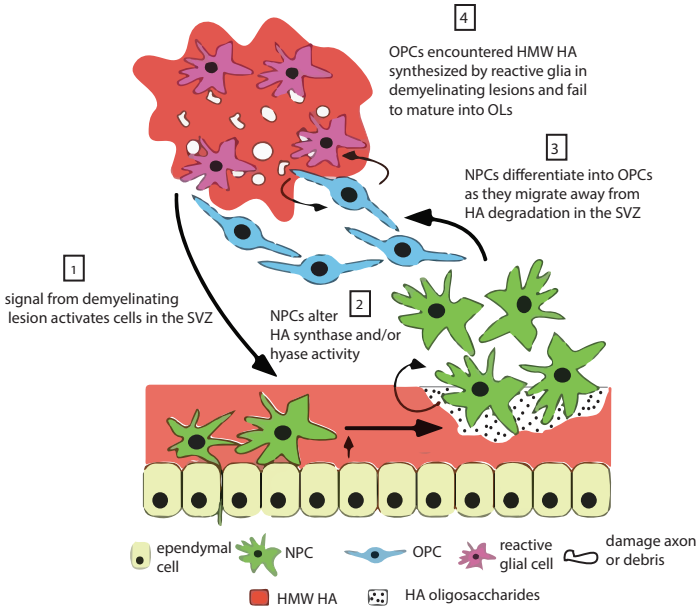
TLR2/4

Inhibition of NSC proliferation
Leukocyte homing and proliferation
Production of pro-inflammatory cytokines
Increases CD44 turnover (reduces HMW HA binding)

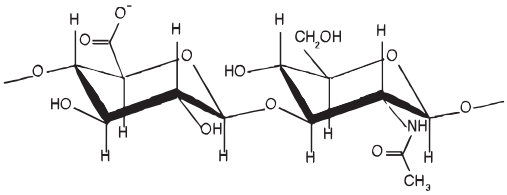
Monomeric CD44

Augmentation of TLR activation
Production of pro-inflammatory cytokines
Angiogenesis

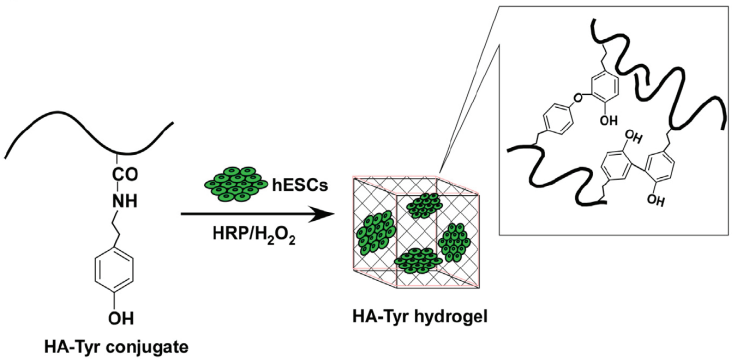

A
SVZ
LV
B
C
A
svz astrocyte
transit amplifying cells
neuroblast
BL
BV
LV
B
A
C
E
axon
B
SGZ
G
D3
D2
D1
B
BV
BL
B
D
G
SGZ astrocyte
precursor 1, 2, 3
neuron
C
Spinal Cord
CC
NSC population
Astrocyte
Oligodendrocyte progenitor
Ependymal Cell

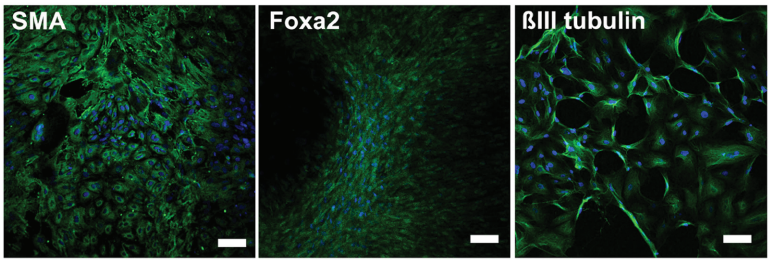

4
OPCs encountered HMW HA synthesized by reactive glia in demyelinating lesions and fail to mature into OLs
3
NPCs differentiate into OPCs as they migrate away from HA degradation in the SVZ
1
signal from demyelinating lesion activates cells in the SVZ
2
NPCs alter HA synthase and/or hyase activity
ependymal cell
NPC
OPC
reactive glial cell
damage axon or debris
HMW HA
HA oligosaccharides

A. Hyaluronic Acid Monomers



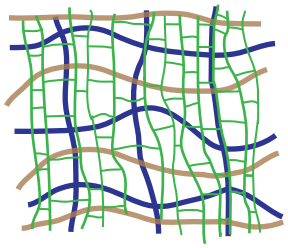
B. HA as 3D Cell Culture System





C. Adding Complexity to 3D Hydrogels

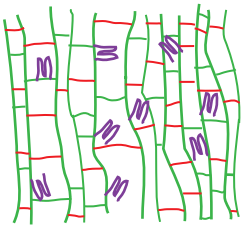
Biochemical Addition



Growth Factor

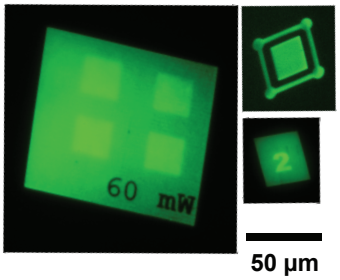
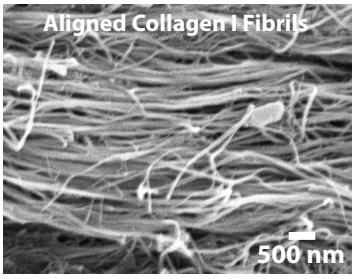
Degradable Unit

Controllable Dynamic Properties



Hyaluronic Acid

Laminin

Collagen

Micro and Nano-scale Patterning