Hyaluronan at the Brain-Environment Interface

Donald M. Thevalingam

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HYALURONAN AT THE BRAIN-ENVIRONMENT INTERFACE

by

DONALD THEVALINGAM

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2019
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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

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Advisor: Dan P. McCloskey, Ph.D.

Hyaluronan (HA; Hyaluronic Acid), a primary scaffolding component of the brain extracellular matrix, serves as an integral structural component to the brain extracellular space (ECS). The fossorial African naked mole-rat (Heterocephalus glaber; NM-R), a mammal which lives in a low-oxygen environment and is capable of tolerating hypoxia and hypercapnia, has been shown to synthesize and sustain a unique high-molecular-mass variant of hyaluronan macromolecule (HMM-HA). This body of work highlights HA’s role in mediating the interplay between brain ECM composition, ECS structure, and cell viability. Here we employ the NM-R as a unique animal model to observe the role of the extracellular matrix in maintaining brain microstructure and regulating diffusion in the brain ECS in health and disease states, such as ischemia. This portion of the work has produced a number of key findings, notably: 1) NM-R brain ECM expressing HMM-HA increases the hindrance to diffusion of macromolecules, and cleavage of HA further enhances this hindrance; 2) The ECS microstructure of the NM-R is remarkably resilient to focal ischemic insult, resisting significant changes in its tortuosity measure; 3) Hypo-osmotic stress differentially alters the ECS microstructure of the NM-R brain, suggesting an alternative mechanism of maintaining tissue viability. The size of HA has the potential of influencing cell behavior, notably viability. Thus, we also explore HA’s potential, based on various sizes, in conferring tolerance and enhanced cell viability in wide extracellular pH excursions. Tissue acid/base balance is compromised during prolonged hypoxia or an acute ischemic insult, shifting towards severe acidosis, and ultimately influencing cell viability. Key find-
4) Titration experiments indicate that the presence of HA differentially alters buffering dynamics in solution depending on the molecular weight of HA. 5) Under neutral pH conditions, NM-R neuronal viability is enhanced in a concentration-dependent manner when a high-molecular-weight HA species is added exogenously to primary neuron cultures; 6) Acute manipulation of extracellular pH in slice and primary neuron cultures did not stimulate HA production on the time scale investigated. Together, these findings highlight the contribution of a unique HA variant in the organization of the ECS microstructure and buffering against dramatic brain metabolic shifts. The primary goal of this research is to posit that ECM composition, and HA in particular, dynamically influences the intracellular and extracellular compartments of the brain microenvironment, and ultimately operates at the interface of brain physiology and external environment of the organism.
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CHAPTER 1

Introduction

1.1 General Introduction

The collective contributions of intra- and extracellular milieu to the brain microenvironment establishes the normal and pathological states of the central nervous system (CNS). The brain extracellular space (ECS), along with the functional network of neurons and glia, and the brain extracellular matrix (ECM) dictates the dynamic nature of this microenvironment. Changes within one of the compartments influence the status of the others, and can potentially shift the entire system across the spectrum between health and disease. The external environment, notably oxygen content, provides a critical cue for modulating the brain environment and can substantially alter both the intra- and extracellular compartments of the CNS. Ultimately, the sensitivity with which these compartments operate influences the feedback loop mechanism(s) utilized by the brain microenvironment to establish and re-establish physiological homeostasis.

The brain ECM warrants special attention on the account of its unique composition and occupation within the well-defined, microstructure of the brain ECS. The brain ECM is composed of a rich tapestry of proteins, proteoglycans, and glycoproteins, supplemented by growth factors and other soluble signals (Cragg, 1979; Syková and Nicholson, 2008). These ECM components are produced intracellularly and extruded out into the extracellular space, occupying the brain parenchyma. The ECM serves in both a structural and signaling capacity. The structural contribution provides an anchor site for cellular components to attach to and establishes organized networks within distinct CNS regions. The signaling component is attributed to the diverse set of matrix proteins, glycans, and lectins which serve as biological cues for cellular activity, growth, and survival (Bonneh-Barkay and Wiley, 2009; Lau, Cua, Keough, Haylock-Jacobs, and Yong, 2013; Vecino and Kwok, 2016). During CNS development, fine temporal and spatial control of expression levels for specific ECM
components is exerted to facilitate neurogenesis, cell migration, differentiation, as well as the diffusion of critical growth factors (Bandtlow and Zimmermann, 2000; Margolis, Margolis, Chang, and Preti, 1975). In adulthood, the ECM primarily serves to stabilize the extracellular space, including synaptic structures. As such, it plays a crucial role in regulating synaptic plasticity and discouraging rogue synaptic remodeling (Dityatev and Schachner, 2003; Lau et al., 2013).

The expansive reach of the brain ECM cannot be understated, and the extent of its influence continues to be a topic of active research. Of particular interest is a seemingly unassuming ECM molecule, Hyaluronan (Hyaluronic Acid; HA). HA, a major component of the ECM, is a large unbranched, nonsulfated glycosaminoglycan composed of repeating disaccharide units of N-glucuronic acid and N-acetylglucosamine (Toole, 2000, 2004). HA can have distinct biological functions depending on its localization, size, and concentration. These functions include altering tissue hydration and elasticity of the ECM and creating open space for cell migration. HA can also serve in a signaling capacity, by initiating cell signaling pathways via transmembrane HA receptors (Sherman, Matsumoto, Su, Srivastava, and Back, 2015).

This exposition is designed to expand on the potential role that HA plays in bridging the structural and signaling capacity of the brain ECM. It will begin with an elaboration of the biochemical characteristics of HA; including the structural and cellular signaling aspects of the molecule, as well as evidence that pathophysiological states indeed influence its signaling capabilities. This will be followed by a modest overview of the brain ECS and how its geometry influences the ease with which molecules can diffuse through this space. The African Naked mole-rat will serve as a novel animal model to explore the interplay between the external environment, brain ECM, ECS, and physiology. Naked mole-rats (*Heterocephalus glaber*) are a fossorial rodent species native to northeastern Africa which harbors unique heavy variant of HA, high-molecular-mass Hyaluronan (HMM-HA). Thus, a brief overview of the biology of the naked mole-rat, as well as their unique contribution to this particular question of interest will be covered. Next, this exposition will provide evidence that the NM-R’s unique physiology, notably its resilience to hypoxic stress, is recapitulated within the domain of the extracellular space, and that this resiliency extends beyond a structural contribution of the extracellu-
lar matrix. To understand how the brain microenvironment maintains homeostasis, a deeper appreciation of the feedback system employed by the intracellular and extracellular compartments of the CNS is essential. This body of work aims to highlight the sensitivity of such compartments to the external environment, the instructive potential of the composition of the extracellular matrix, and provide additional clues on how to intervene during anomalous insult and facilitate repair and recovery.

1.2 "Hyaluronan, is a simple but unusual polysaccharide" – Brian Toole, 2000

1.2.1 HA as a polymer

Hyaluronan, present in nearly all extracellular matrices, is a high-molecular-weight un-branched glycosaminoglycan (GAG) composed of repeating N-acetylg glucosamine (GlcNAc) and glucuronic acid (GlcUA) disaccharide subunits (Bernard Weissmann and Meyer, 1954; B Weissmann et al., 1954). HA polymers typically consist of 2000 - 25,000 disaccharide subunits, conferring molecular weights ranging from 106 - 107 Da. GAGs are linear and heterogenous sulfated glycans, whose chains can link to a protein core-forming proteoglycans (Toole, 2004). Despite its loose classification as a proteoglycan, HA does not contain sulfated groups, epimerized uronic acid residues, and lacks a covalent linkage to a protein backbone (Toole, 2000). HA maintains a polyanionic charge under physiological conditions due to the carboxyl residues present on the GlcUA subunit. HA polymers are generally of high-molecular-weight and occupy a large hydrodynamic volume in dilute solution via random coiled configurations.

Molecular dynamics simulations and nuclear magnetic resonance studies have demonstrated that the average conformation of HA molecules in a dilute solution resembles a left-handed 4-fold helix (Almond, Deangelis, and Blundell, 2006). The large hydrodynamic volume is attributed to in part by mutual repulsive interactions between the carboxyl groups (Balazs, 1970; Toole, 2000). Amino, carboxyl, and hydroxyl groups on the polymer form transient hydrogen bonds with water molecules forming water bridges on adjacent groups of neighboring saccharides. A single water molecule can potentially form hydrogen bonds with 2 different HA molecules (as depicted in Figure 1.1A). The transient nature of these hydrogen bonds confers enhanced flexibility to the HA polymer (Almond,
2005; Heatley and Scott, 1988; Průšová, Conte, Kučerík, and Alonzo, 2010).

At higher concentrations, HA molecules entangle to form continuous, porous networks which are conferred with unique rheological properties such as shear-dependent viscoelasticity, high hydration capacity, is able to retain flow, and resists gel-formation (Cowman, Schmidt, Raghavan, and Stecco, 2015; Krause, Bellomo, and Colby, 2001). HA polymers also demonstrate excluded volume effects in that one part of a long chain molecule cannot occupy space that is already occupied by another part of the same molecule. This results in the ends of the polymer chain in solution on average to be further apart than if there was no excluded volume. The polyelectrolyte nature of HA compounds amplifies these properties and as a result, are affected by ionic strength (Hayashi, Tsutsumi, Nakajima, Norisuye, and Teramoto, 1995). Taken together, HA functions within a biological system as a mechanical buffer and lubricant.

1.2.2 HA Biosynthesis

The biosynthesis of HA differs from other glycosaminoglycans, such as chondroitin sulfate, in a few fundamental respects. The biosynthesis of HA occurs directly without a protein core and subsequent linkage to the oligosaccharide (Mason et al., 1982a, 1982b). The synthase(s) responsible for producing HA resides at or near the plasma membrane (Prehm, 1984). Elongation of the nascent HA chain occurs at its reducing end by a proposed 2-site mechanism in which the growing chain is stays attached to the UDP moiety of the newly added sugar residue (Prehm, 1983). Finally, the growing chain is extruded into the extracellular space through the plasma membrane of the cell (Prehm, 1984).

A family of glycosyltransferases, termed HA synthases, with two catalytic activities: one for the glucuronic acid transfer and N-acetylglucosamine transfer is responsible for the synthesis of HA. The family of HA synthases consists of three isoforms, HAS1, 2, and 3. These isoforms are evolutionary conserved and are highly (55-70% identity) homologous. However, each of the three isoforms differs in half-life, stability, rate of HA synthesis, and affinity for HA substrates, thereby affecting the regulation of HA synthesis (Weigel, 2015; Weigel, Hascall, and Tammi, 1997).
HA synthases synthesize HA of varying molecular masses. Shorter polymers ($1.0 \times 10^5 - 1.0 \times 10^6$), are typically synthesized by the HAS3 isoform. HAS1 and HAS2 isoforms synthesize larger polymers ($2.0 \times 10^5 - 2.0 \times 10^6$), with HAS2 tending to produce polymers on the larger end of the spectrum (DeAngelis, Papaconstantinou, and Weigel, 1993; Itano and Kimata, 2002). Of the three isoforms, HAS2 is expressed during all stages of embryogenesis and is a critical HA synthase in development. Of the three isoforms, only HAS2 is necessary and sufficient for producing viable and fertile mice. HAS2 +/- mice resulted in severe cardiac and vascular defects, leading to midterm embryonic lethality; whereas HAS1 and HAS3 deficient mice (including double knockouts) were viable and fertile (Camenisch et al., 2000; Tien and Spicer, 2005).

### 1.2.3 HA Degradation and Turnover

Hyaluronidases, HYALs, are responsible for the enzymatic degradation and subsequent turnover of HA. HYALs are a family (HYAL1-5, PH20, and HYALP1) of highly homologous endoglycosidases which specifically hydrolyze the $\beta 1,4$ linkage of the HA molecule (Csoka, Frost, and Stern, 2001; Stern and Jedrzejas, 2006). Mammalian HYALs vary in localization, the range of activity, specificity, and are not necessarily limited to HA digestion, as they can also degrade Chondroitin Sulfate (CS) proteoglycans. Prokaryotic HYALs however specifically act on HA (Jedrzejas, Mello, Groot, and Li, 2002; Kelly, Taylor, Li, and Jedrzejas, 2001; Rigden and Jedrzejas, 2003). HYALs can be further broken down into distinct pH-dependent groups, with HYAL1-3 active in the acidic pH, and PH20 optimally active at a neutral pH (Oettl, Hoechstetter, Asen, Bernhardt, and Buschauer, 2003). In somatic tissues, HYAL1 and HYAL2 are common isoforms which act in concert to catabolize HA (Sherman et al., 2015; Stern and Jedrzejas, 2006). Two presumed hyaluronidases have been identified as having their catalytic activity operate in the extracellular space. CEMIP, a cell-migration inducing HA-binding protein (also known as KIAA1199), is a secretory protein which has both HA binding and degradation activities (Yoshida et al., 2013). A recently identified novel cell surface hyaluronidase, TMEM2 (transmembrane protein 2), has its hyaluronidase activity residing in the extracellular domain (Perkins, Arranz, Yamaguchi, and...
The concentration of HA varies between tissue types; ranging from 2-4 g/L in the synovial fluid to 0.1-0.01 mg/L in normal serum. The largest contribution of total HA content comes from musculoskeletal tissues and the extracellular matrix of the skin. The rate of catabolism of HA is tissue-dependent, but it is typically on the order of days (Laurent and Reed, 1991; Weigel and Yik, 2002). Aside from enzymatic digestion of HA in the extracellular space, and non-enzymatic depolymerization, HA can be degraded and internalized within tissues locally or released from the extracellular matrix, transported through drainage routes into the vasculature, through the lymph nodes, and cleared out to the liver and kidneys (Monslow, Govindaraju, and Puré, 2015).

With approximately 50% of the total HA body content residing in skin tissues, only a small portion (20-30%) is internalized and degraded locally within the tissue. The metabolic half-life of HA is approximately 1.5 days, with HA sourced from the epidermis having a much shorter half-life of 2-3 hours (Tammi, Säämänen, Maibach, and Tammi, 1991). In a receptor-mediated pathway, rat epidermal keratinocytes internalize newly synthesized HA polymers into non-clathrin-coated endosomes, which are then transported into the endo-/lysosomal system for degradation (Tammi et al., 2001). HA oligosaccharides undergo endocytosis through bulk phase pinocytosis, while larger HA molecules require multivalent interactions with several of cell-surface receptors (Cyphert, Trempus, and Garantziotis, 2015).

Prevailing evidence suggests that larger HA molecules (~107 Da) undergo partial degradation (to ~106 Da) prior to being unlinked from the extracellular matrix, followed by subsequent entry into the lymphatic system (Fraser, Kimpton, Laurent, Cahill, and Vakakis, 1988). Additional metabolic degradation occurs in the lymphatic tissues as HA polymers are transported from the peripheral tissues into the bloodstream. Lymph nodes are able to extract and degrade 50-90% of circulating HA content from the peripheral lymph, as demonstrated by injecting radio-labeled HA into afferent lymph vessels. The remaining HA (~105 Da), re-enters the bloodstream and eliminated by sinusoidal endothelial cells in the liver (Fraser et al., 1989).

The high turnover rate of HA in circulation (1 mg/kg a day), leads to low concentrations of
HA within those compartments (10 - 100 ng/mL), with the daily turnover of HA on the order of one-third of the total body content (Fraser and Laurent, 2007). Cell surface receptors, hyaluronan receptor for endocytosis (HARE) in particular, bind with HA fragments in the extracellular space and internalize them. Upon endocytosis, these HA fragments are transported to lysosomes which contain hyaluronidases for total degradation into monosaccharide subunits (Zhou, Weigel, Fauss, and Weigel, 2000).

1.2.4 HA Interactions with Cell Surface Receptors

Hyaluronan interacts with many proteins which are also known as hyaladherins, many of which act as cell-surface receptors. Of these, CD44 and RHAMM (Hyaluronan-mediated motility receptor) have been previously established as signal-transducing receptors that influence a range of cell behaviors, including cell proliferation, survival, and motility (Day and Prestwich, 2002; Turley, Noble, and Bourguignon, 2002). CD44 is a cell-surface glycoprotein containing a cytoplasmic, transmembrane, and extracellular domain (Aruffo, Stamenkovic, Melnick, Underhill, and Seed, 1990; Ponta, Sherman, and Herrlich, 2003; Stamenkovic, Amiot, Pesando, and Seed, 1989). The extracellular domain houses the amino terminal of the hyaluronan-binding residue, which is structurally related to the linking modules of HA-binding proteoglycans and link proteins (Day and Prestwich, 2002).

Numerous combinations of spliced exons products are sourced from the region which encodes the extracellular domain on the CD44 gene. Thus, although HA is the primary ligand for CD44, several other molecules (i.e. fibroblast growth factors and matrix metalloproteinases) are capable of binding to the carbohydrate side residues attached these “spliced-in” regions (Ponta et al., 2003). Depending on the context of activation, the cytoplasmic tail of CD44 interacts with a number of regulatory molecules, such Rho GTPases, SRC kinases, ezrin, GAB1, VAV2, and ankyrin, which are critical for oncogenic signaling and cytoskeletal activation (Bourguignon, 2001; Thorne, Legg, and Isacke, 2004). CD44 is also involved in the cellular uptake and degradation of hyaluronan, which thereby influences tissue integrity and regulation of cell growth (Kaya, Rodriguez, Jorcano, Vassalli, and Stamenkovic, 1997).
Alternative splicing of RHAMM results in the varied distribution of the protein product, which can be found both inside the cell and on its surface. Like CD44, RHAMM transduces cellular signals which influence growth and motility (Turley et al., 2002). Lacking a link-module domain, RHAMM contains an HA-binding motif which is also present in several hyaladherins. This HA-binding motif contains a sequence of approximately 7 non-acidic amino acids, sandwiched by either arginine or lysine residues (Yang, Yang, Savani, and Turley, 1994). Intracellular RHAMM binds with a number of cytoskeletal components and signaling proteins, including actin, microtubules, SRC, and ERK1 (Hall, Lange, Prober, Zhang, and Turley, 1996; Zhang et al., 1998).

Several studies have demonstrated that HA activates the PI3K-AKT signaling pathway, as well as phosphorylates FAK and BAD, all of which promote cell survival (Itano et al., 2002; Misra, Ghatak, Zoltan-Jones, and Toole, 2003; Sohara et al., 2001). Inhibition of constitutive HA-CD44 interactions reverse these cell survival effects. Both CD44 and RHAMM can activate FAK through HA interactions (Fujita et al., 2002; Hall, Wang, Lange, and Turley, 1994). Recent work has demonstrated that HA promotes the interaction of CD44’s cytoplasmic domain with the p110 subunit of P13K via GAB1, thereby activating the P1K3-AKT pathway. This highlights a direct mechanism by which HA-CD44 interactions mediate cell survival signaling (Bourguignon, Singleton, Zhu, and Diedrich, 2003). Activation of ERK1 through the HA-RHAMM interaction results in the phosphorylation of BAD73, which also contributes to cell survival (Zhang et al., 1998). Thus, the biosynthesis and degradation of HA is associated with rapid remodeling of the extracellular matrix, extending beyond serving a structural role in various tissues.

1.2.5 Size Specific Effects of HA

It is important to note, HA-CD44 interactions have a differential signaling effect based on HA size. HA polymer length can potentially influence the binding features and downstream consequences. High and low-molecular-weight variants of HA are capable of inducing anti- or pro-inflammatory effects upon binding to CD44, and consequently, provide cellular instruction for anti-proliferative or proliferative signals in various cell types (Gao et al., 2010; Noble, 2002).
Low-molecular-weight HA interactions with CD44 have been associated with physiological stressors such as induction of angiogenesis, inflammation, and cancer (Jiang et al., 2005; Scheibner et al., 2006; Tremmel et al., 2009). HA size influences cell behavior even at the level of the receptor. The addition of exogenous high-molecular-weight HA to CD44-transfected COS-7 cells induced dynamic CD44 clustering, which was subsequently disrupted by the presence of exogenous HA oligosaccharides of smaller mass. Also, naturally expressed CD44 receptors underwent a similar clustering distribution in HK-2 (human renal proximal tubule cells) and BTS49 (human breast cancer cell line), both of which expressed high levels of high-molecular-weight HA (Yang et al., 2012).

Physiological stressors, such as inflammation, increase HA fragmentation, resulting in higher levels of HA biopolymers of lower molecular weights (Stern and Jedrzejas, 2006). Consequently, the effects of HA in various pathologies are often associated with variations in polymer mass (Monslow et al., 2015). There is no real consensus on the delineations of HA mass categories. Many groups have put forth various descriptions of mass, i.e. what constitutes high-molecular weight and how does that differ from a low-molecular weight HA. An attractive size hierarchy was described Bohaumilitzky et al.: high-molecular-weight HA (HMW-HA, > 1000 kDa), medium molecular weight HA (MMW-HA, 250-1000 kDa), low-molecular-weight HA (LMW-HA, 10-250 kDa), and oligomeric HA (oligo-HA, < 10 kDa) (Bohaumilitzky et al., 2017).

### 1.2.6 HA and the Central Nervous System

In the central nervous system (CNS), HA serves a major scaffolding component of the ECM and perineuronal nets (PNNs) (as depicted in Figure 1.1B). PNNs are specialized, sieve-like structures, that encase certain neuronal cell bodies and proximal dendrites, with exposed openings for synaptic boutons (Sorg et al., 2016; Zimmermann and Dours-Zimmermann, 2008). The major ECM components of PNNs include HA and chondroitin sulfate proteoglycans, hyalecticans (brevican, neurocan, versican, and aggrecan) and phosphacan, along with tenascin-R and other various link proteins [Deepa et al. (2006); Yamaguchi2000-we]. In the cortex and hippocampus, PNNs are primarily expressed on parvalbumin-positive GABAergic inhibitory interneurons but are also present on various
types of excitatory and inhibitory neurons throughout the CNS (Celio and Chiquet-Ehrismann, 1993; Frischknecht and Gundelfinger, 2012).

The appearance of the PNN seems to signify the maturation of the CNS that is concurrent with a decrease in plasticity. PNNs are involved in the stabilization of existing synapses, the prevention of new synapses on mature neurons, the linkage of the ECM with the cytoskeleton, and may facilitate neuron-astrocyte interactions (Frischknecht et al., 2009; Kwok, Carulli, and Fawcett, 2010). PNNs may also inhibit growth and synapse formation, and their perisynaptic localization around interneurons suggests a potential role as synaptic stabilizers (Dityatev and Schachner, 2003; Hensch, 2003). The disruption of PNNs restores ocular dominance plasticity in the adult visual cortex and facilitates recovery from early monocular deprivation (Pizzorusso et al., 2006). The onset of PNN expression is indicative of closing the critical period in barrel cortex (McRae, Rocco, Kelly, Brumberg, and Matthews, 2007; Nowicka, Soulsby, Skangiel-Kramska, and Glazewski, 2009).

1.3 "The ECS has been regarded as a quiet street for many years but is now seen as bustling and indispensable thoroughfare" – Charles Nicholson & Sabina Hrabětová, 2017

1.3.1 The Brain Extracellular Space

In a study by Margolis et. al., 1975, HA content in the postnatal rat brain was characterized from P0-P30 pups as well in a 40-day old adult rat. There was a 51% drop in HA content compared to the peak value 7 days after birth, with adult levels attained by 18 days. Early on, the postnatal brain was characterized as well hydrated, with over 90% of the HA content able to be extracted from the brain. The role of HA-enriched extracellular matrix (ECM) in the developing brain is believed to confer a high degree of hydration to the tissue in order to facilitate migration, diffusion of substances, and differentiation processes critical for development (Margolis et al., 1975). The brain ECM then becomes a critical component in regulating the extracellular space (ECS) as it relates to the diffusion of small molecules and substances within and around the brain.

Individual channels within this intricate system of interconnected narrow spaces, separated cellular membranes, spanning 30-60 nm are filled with ionic solution and ECM macromolecules, in-
cluding glycosaminoglycans and proteoglycans (as depicted in Figure 1.2) (Thorne and Nicholson, 2006; Xiao, Nicholson, Hrabe, and Hrabětová, 2008). The ECS serves as the conduit for transport of neurotransmitters, metabolites, neuromodulators, nutrients and therapeutic compounds, all of which influence the brain microenvironment. These substances travel through the ECS primarily by diffusion since there is no dedicated active transport mechanism (Syková and Nicholson, 2008). The biophysical process of diffusion can be interrogated experimentally in living tissue to quantify and explore the macroscopic properties and structure of the ECS. The ECS is characterized by two fundamental parameters: extracellular volume fraction ($\alpha$) (Figure 1.3) and tortuosity ($\lambda$) (Figure 1.4). The volume fraction represents the volume of the tissue is occupied by the ECS and is quantified as $\alpha = \frac{V_{ECS}}{V_{Tissue}}$. Tortuosity describes how well can substances diffuse through the ECS relative to an obstacle-free medium, and is described by: $\lambda = \sqrt{\frac{D}{D^*}}$ (Syková and Nicholson, 2008).

The ECS plays a fundamental role in brain health and disease. It serves as a reservoir of ions for neuronal electrical activity, as well as providing a dynamic microenvironment for brain homeostasis. Diffusion-mediated transport of substances spans on the order of tens to hundreds of nanometers, as in processes mediating synaptic signaling on extrasynaptic receptors, up to centimeters as with metabolite removal and volume transmission. Removal of aggregated protein plaques such as amyloid-beta and tau rely on the ECS, through a potential bulk-flow mechanism, for effective clearance. The ECS also provides a transport route for therapeutic agents entering the brain via the blood-brain or direct injection and thus has profound clinical implications for drug delivery (Hrabětová, Cognet, Rusakov, and Nägerl, 2018; Iliff et al., 2012; Syková, 1997).

1.3.2 Dynamics of the Brain ECS

The $\alpha$ and $\lambda$ values provide quantitative insight into the structure of the ECS. In a normal, isotropic, healthy brain, $\alpha$ is about 0.2 (i.e. 20% of the brain tissue volume is occupied by the ECS), and $\lambda$ is about 1.58 (a molecular probe diffusing through the brain ECS is slowed down by a factor of $\sim$2.5, compared to when it diffuses through an obstacle free-medium). These two parameters, effectively describe the percentage of the volume occupied by the ECS and the rate of diffusion.
of substances throughout the ECS (Nicholson and Hrabětová, 2017; Perkins et al., 2017). Experimental values of $\alpha$ and $\lambda$ are typically obtained with the Real-Time Ionophoretic (RTI) method (Nicholson and Phillips, 1981), where a small extracellular probe, such as tetramethylammonium ($TMA^+$; 74 Da MW), is iontophoretically released from a point source and subsequently detected by a $TMA^+$-sensitive microelectrode positioned approximately 100 micrometers away. RTI has been used to quantify $\alpha$ and $\lambda$ values in brain tissue, under physiological and pathophysiological conditions, and has provided additional information on the dynamic nature of the ECS under both conditions (Syková and Nicholson, 2008).

Rather than consider the structure of the ECS as a static entity, the ECS is both reactive and instructive in its contribution to the overall brain microenvironment. The brain ECS dynamically changes during various physiological events. For example, recent work has reported that during sleep-wake cycles in the adult mouse, the ECS widens during sleep facilitating the removal of metabolites and toxins from the brain tissue and subsequently constricts itself during awake states (Xie et al., 2013). The ECS structure change, at times permanently, during pathological states, such as ischemia, epilepsy, or brain trauma. For example, there is a significant reduction of volume fraction, from 20% to approximately 5%, and a subsequent increase in hindrance from 1.5 to about 2.1 in the rat cortex during severe anoxia or ischemia. Activity-driven reduction of ECS volume fraction, as well as an increased hindrance to diffusion due to cellular (neuronal and glial) swelling, are attributed to these deviations from physiological values (Syková, Svoboda, Polák, and Chvátal, 1994; Voříšek and Syková, 1997). It should be noted, however, that the parameters of volume fraction and tortuosity are not necessarily directly correlated values. During exposure to osmotic challenges, changes in $\alpha$ and $\lambda$ was measured using RTI-$TMA^+$ in the rat neocortex. As $\alpha$ decreases, $\lambda$ increases in hypo-osmolar conditions; however, in hyper-osmolar conditions, as $\alpha$ increases, $\lambda$ rapidly reduces to a constant value. The alterations in tortuosity may be accounted for by the composition of the brain ECM. Specifically, the presence of large glycosaminoglycans may confer a baseline value of hindrance to diffusion, which is potentiated in the event of ECS reduction (Kume-Kick et al., 2002).
1.3.3 The Contribution of the Brain ECM to the ECS

There are several potential ways with which the ECM could influence the diffusion parameters of the ECS. The presence of the ECM molecules could increase the interstitial viscosity of the ECS, and is thus no longer an analog to a free-medium. Theoretical models suggest that the presence of the ECM confers increased ECS viscosity (Rusakov and Kullmann, 1998), and experiments which added 40 kDa dextran molecules to normal brain slices demonstrated an increased ECS viscosity, which subsequently affects synaptic signaling (Savtchenko and Rusakov, 2005). AMPA receptor activation in the synaptic cleft is dependent on glutamate diffusion. Recent work using time-resolved fluorescence anisotropy imaging has provided measurements of “micro-viscosity” within a molecular environment in living brain tissue. On average small molecules move ~30% slower in the ECS of synaptic neuropil in hippocampal CA1 compared to an obstacle-free medium. This approximation reflects the hindrance of diffusion primarily in extrasynaptic spaces. Inside neuronal dendrites, this hindrance increases to 70%, possibly due to the presence of cytoplasmic macromolecules or collisions with cytoplasmic components such as organelles (Hrabětová et al., 2018; Zheng et al., 2017).

The negative charge densities present on some ECM molecules might differentially affect the diffusion of anions and cations. The potential for charge discrimination by ECM components has been demonstrated in specialized tissues such as cartilage and solutions of chondroitin sulfate and HA due to fixed negative charges partitioning ions, as described by a Donnan equilibrium process (Maroudas, Weinberg, Parker, and Winlove, 1988; Parker, Winlove, and Maroudas, 1988). Although negative charge density within the CNS is most likely far lower than in cartilage, the presence of cations such as $Na^+$, $Ca^{++}$ and $Mg^{++}$ in the ECS ionic reservoir could potentially quench the fixed negative charges on the brain ECM (Syková and Nicholson, 2008). RTI experiments have provided additional evidence suggesting that fixed negative charges on chondroitin sulfate proteoglycans (CSPGs) and other ECM components influences the local diffusion properties of calcium ions; however, digestion (via Chondroitinase ABC) of CSPGs did not alter the diffusion of $TMA^+$ ions or the extracellular volume fraction measure. This surprising finding indicates that enzyme used to digest CSPGs did not alter the ECS structure, and the diffusion of small monovalent ions, such
as $TMA^+$, was not affected by CSPGs in a normal ionic milieu (Hrabětová, Masri, Tao, Xiao, and Nicholson, 2009).

Finally, the ECM might regulate ECS width, possibly via the hydration of hyaluronan. The patent nature of the ECS may be attributed to HA and its high hydration capacity. This is especially relevant in the developing brain, where there is an abundance of soluble HA, which can potentially enhance the ECS width compared to that in the adult brain (Bignami, Hosley, and Dahl, 1993). Prior studies quantifying the polysialic acid (PSA) content of neural cell adhesion molecules (NCAM) demonstrate how specific ECM components might regulate ECS width. Enzymatic removal of PSA resulted in a ~25% decrease in intracellular distance; however, removal both chondroitin sulfate and heparan sulfate ECM components had no effect (Yang, Yin, and Rutishauser, 1992). Under global ischemia, the ECS width is estimated to be less than 10 nm, as determined by the diffusion of 3 kDa molecules and $TMA^+$ ions (Thorne and Nicholson, 2006).

Additional evidence of ECS width modification comes from mouse knockout studies, in particular, tenascin-R knockout mice exhibited a 5% reduction in ECS volume fraction in the primary somatosensory cortex, to about 15% (Syková, Vorísek, Mazel, Antonova, and Schachner, 2005). Elimination of Bral-1, a hyaladherin, as well as several other lectins in the vicinity of nodes of Ranvier, not only reduced the conduction velocity of myelinated fibers but also reduced $\lambda$ in the white matter of mice (Bekku et al., 2010). Targeted HAS3 knockout mice, which eliminate one of the genes responsible for producing HA, resulted in the reduction of HA in the mouse hippocampus and subsequent reduction of ECS volume was in the stratum pyramidale by ~40% (Arranz et al., 2014).

1.3.4 Hindrance to Diffusion

Several factors contribute to the hindrance with which a molecule undergoes while diffusing through tissue (tortuosity). As a diffusing molecule takes a more circuitous path around cellular obstruction(s), it effectively increases the traveled geometric path length (Syková and Nicholson, 2008). This is with the supposition that the ECS is well-connected; however recent experiments have alluded to the presence of local dead-space microdomains within the ECS, where diffusing molecules
are transiently delayed. Molecules which are trapped in these dead-spaces end up exploring the local domain, much like a rubber ball bouncing off the walls of a closed room, before eventually leaving the aforementioned “cul-de-sac”. This ultimately yields little change in net distance traveled, while increasing the time of travel (Hrabětová, Hrabe, and Nicholson, 2003).

Additional factors which contribute to the measure of tortuosity can be attributed to the presence of an ECM. As previously described, macromolecular components of the ECM potentially introduces interstitial, viscous drag forces on diffusing molecules. If the interstitial medium has a greater viscosity than that of a free-medium, it will contribute to the ECS tortuosity. Just exactly to what degree this contribution remains to be determined. Additional consideration must be made with regards to the size of the diffusing molecule, notably as the size of the molecule approaches that of the ECS width, it will be exposed to increased drag forces. Proteoglycan components and receptors attached to the ECM of neurons and glia may provide transient specific binding sites for diffusing molecules. Finally, fixed negative charge densities present on ECM molecules may have non-specific interactions with charge densities on diffusing molecules. Thus, the ECM itself directly modulates the speed of diffusing molecules within the brain ECS (Syková and Nicholson, 2008).

1.3.5 Integrative Optical Imaging

In order to explore the diffusion of macromolecules, integrative optical imaging (IOI) offers an alternative method allowing for the interrogation of the brain ECS structures using larger molecules (Nicholson and Tao, 1993). Briefly, a fluorescently tagged molecule of interest, of a known mass, is pressure ejected from a glass micropipette into agarose (control measures) or brain tissue in picoliter volumes, and the distribution of the fluorescence signal over the region of interest is captured over a given interval sequence using a CCD camera. The distribution of this fluorescence signal is fitted to the diffusion equation, as described by Fick’s 2nd law of diffusion, to obtain an estimate of the effective diffusion coefficient ($D^*$). Fluorophore-labeled molecules of various sizes, such as dextrans (3,000 - 70,000 Da MW), proteins (epidermal growth factor, 6000 Da MW and albumin, 67,000 Da MW), and synthetic polymers (PHPMA and polyethylene glycol (PEG) (Hrabětová and Nicholson,
The 3D distribution of the fluorescent molecules diffusing across the volume of interest ideally forms a spherical cloud that is projected on the 2D plane detected by the CCD camera. The fluorescence intensity distribution of this 2D proxy of the projected spherical cloud can be expressed by the following equation, for the $i$th image at time $t_i$ after the initial injection of the solution containing fluorophore-labeled molecules: 

$$I_i(r, \gamma_i) = Ee^{-\left(r/\gamma_i\right)^2} \quad (Eq1)$$

Where $r$ is the distance from the point of injection and $\gamma_i^2 = 4D^*(t_i + t_0) \quad (Eq2)$

where the offset $t_0$ represents a virtual time origin before the actual time of injection. This allows for the use of an approximation of a theoretical volume of injection to counter the actual finite volume of injection. From the 2D projections of the diffusing molecules, intensity curves obtained from along the vertical, horizontal, and 2 diagonal axes running through the center of each image are fitted an appropriate solution to the diffusion equation. Diffusion coefficients (free diffusion coefficient, $D$, from agarose and the effective diffusion coefficient, $D^*$, for brain tissue) are obtained by fitting Eq1 to each image in the sequence to obtain a corresponding sequence of $\gamma$ values for each time point $t_i$, then a linear regression is performed on the $Eq1$ to obtain $D^*$ (or $D$) and $t_0$. The IOI method, unlike RTI, does not provide measures for volume fraction, $\alpha$, or the clearance constant, $\kappa'$ (Hrabětová and Nicholson, 2011).

It is also important to note that the diffusion coefficients are temperature dependent and that the effective diffusion coefficient, $D^*$, must be measured or adjusted to the same temperature as that which is used to determine the free diffusion coefficient, $D$ when calculating $\lambda$. This correction can be implemented by considering the hydrodynamic diameter of a compact molecule (i.e. spherical) as estimated from the the Stokes-Einstein equation 

$$d_h = \frac{k_BT}{3\pi\eta D} \cdot 10^{13} \quad (Eq3)$$

where $d_h$ is the diameter (nm), $k_B$ is the Boltzmann’s (1.3806 · 10$^{-23}$ J/°K), $T$ is temperature in degree K (°K), $\eta$ is viscosity (Pa · s), and $D$ is the free diffusion coefficient (cm$^2$/s). The viscosity measure is typically as is in pure water, and also varies with temperature. Due to the temperature dependence of viscosity, $D^*$ at one temperature can be corrected to its adjusted value at another temperature using: 

$$D_2 = \frac{T_2}{T_1} \frac{\eta_1}{\eta_2} D_1 \quad (Eq4)$$
This process becomes especially critical when correcting for $D^*$ of larger diffusing molecules (Syková and Nicholson, 2008).

1.4 ”Naked mole-rat: Nature’s Weirdest Superhero” – National Geographic, 2018

1.4.1 Overview

Naked mole-rats (*Heterocephalus glaber*; NM-R) are a fossorial rodent species native to northeastern Africa. Their subterranean habitat consists of a substantial network of tunnels sheltered from exigent climate conditions as well predation. In addition to this protected environment, naked mole rats are a eusocial species that live in cooperative colonies populated on average by 75-100 individuals (Jarvis, 1981). Naked mole-rats have become an exemplary model of longevity and aging research with their maximum life-span extending over 30 years, approximately ten times greater than other rodent species of comparable body mass (Buffenstein, 2005). A buffered fossorial habitat coupled with social cooperativity has facilitated several evolutionary adaptations to extend their longevity in their hypoxic-hypercapnic environment including lowered basal metabolic rate, acid, blunted sensitivity to acid-pain, a low core body temperature (~32°C) that is subject to change with ambient temperature (e.g. poikilothermy). The adaptations that have conferred tolerance to a hypoxic environment have also coincided with enhanced resistance to oxidative stress, neurodegeneration, and cancer (Smith, Schuhmacher, and Husson, 2015).

1.4.2 High-Molecular-Mass HA

Studies exploring HA production by NM-R fibroblasts have demonstrated that naked mole-rats accumulate a HMW-HA variant termed high-molecular-mass hyaluronan (HMM-HA) and is able to maintain relatively high levels of HA in tissue due to: i) a mutated HAS2 variant that constitutively synthesizes HA at a high rate and ii) reduced rate of HAase activity. HAS enzymes are highly conserved across vertebrate species. The HAS2 protein has a 98.7 identity and 100% similarity score between mouse and human sequences (Tian et al., 2013). Cloning and sequencing of NM-R HAS2 complementary DNA showed that two asparagine groups which are 100% conserved across
mammals were replaced with serine. The location of this particular point mutation corresponds to the cytoplasmic loop containing the enzyme’s active site (Tian et al., 2013).

Various tissue sections collected from NM-Rs were stained for HA using Alcian blue and demonstrated robust expression of HA in skin, kidney, heart, and brain tissue. Purified HA samples obtained from naked mole-rat tissues also exhibited a higher molecular weight relative to samples purified from mouse tissues. NM-R HA has a molecular mass ranging from 6-12 MDa, compared to 0.5-3 MDa synthesized by mouse and guinea-pig. The molecular mass HA extracted from NM-R brain tissue reached 0.5-2 MDa, whereas for mice the molecular mass was at ~ 0.5 MDa (Tian et al., 2013).

Recent work unraveling the material properties of NM-R HMM-HA polymers have demonstrated that this unique variant of HA is conferred with unique mechanical properties (Kulaberoglu et al., 2019). HA extracted from conditioned media of immortalized NM-R skin fibroblasts underwent atomic force microscopy topography analysis, indicating a more voluminous network of HA chains compared to that of mouse samples. Scanning electron micrographs also indicate that NM-R HA chains take on a supercoiled-conformation, characteristic of a “cauliflower” appearance. HA extracted from purified human skin tissue did not have these “supercoiled” structures, rather these samples took on large, flat networks where individual chains could be resolved. In addition to HA isolated from conditioned media of NM-R fibroblast lines, HA was also extracted from NM-R brain tissue. AFM topography demonstrated that isolated NM-R brain HA coincidentally exhibit “brain-like” folds, resembling the gyri and sulci characteristic of a whole brain. Viscoelastic measurements indicate that these supercoils retain a high degree of hydration and elasticity compared to other species that were compared (Kulaberoglu et al., 2019).

1.4.3. NM-Rs as a unique model for Ischemia

Hypoxia is implicated in a number of pathological conditions, including cerebral ischemia (e.g. stroke), cancer, and neurodegenerative disorders like Alzheimer’s disease (Gao, Tian, Gao, and Xu, 2013; Michiels, 2004; Peers et al., 2009). Ischemic induced cell death primarily occurs due to
oxygen deprivation and subsequent lack of ATP production, as oxygen is required for aerobic respiration. The high energetic requirements of the CNS leaves it particularly vulnerable to hypoxic insults. Most of the ATP used by neurons is used to maintaining the sensitive ionic gradients and membrane potentials during synaptic transmission (Harris, Jolivet, and Attwell, 2012). The rapid decrease in cellular ATP levels during ischemic/hypoxic insults disrupts ion and neurotransmitter homeostasis (Haddad and Jiang, 1993; Rolfe and Brown, 1997), increase in intracellular calcium levels, and results in neuronal cell death (Kristián and Siesjö, 1998; Martin, Lloyd, and Cowan, 1994). The switch to an anaerobic metabolic pathway has its dangers, including accumulation of lactic acid, increased extracellular pH, inducing neurotoxicity through the activation of acid-sensing ion channels (ASICs) (Xiong et al., 2004).

NM-Rs are equipped with several advantageous physiological traits to deal with sustained low-levels of oxygen. For example, NM-R hemoglobin has a higher affinity for oxygen than mice (Johansen, Lykkeboe, Weber, and Maloiy, 1976), and their remarkably low basal metabolic rate ($0.27 - 1 \, mL \cdot O_2/g/h$ within its thermo-neutral range, vs mice which are greater than $1.2 \, mL \cdot O_2/g/h$), can be further suppressed under additional hypoxic stress (Goldman, Goldman, Lanz, Magaurin, and Maurice, 1999; Mink, Blumenschine, and Adams, 1981; Selman, Lumsden, Bünger, Hill, and Speakman, 2001). This physiological adaptation is common among hypoxia-tolerant species, to reduce ATP consumption under a period of oxygen deprivation (Buck and Pamenter, 2006).

NM-Rs have also developed hypoxia-tolerant adaptations at the neuronal level. Electrophysiological studies measuring excitatory postsynaptic field potentials (fEPSP) in NM-R hippocampal slices have demonstrated a robust capacity to withstand hypoxic/anoxic conditions via blunted reduction in evoked potential amplitude, functional recovery of fEPSP amplitude to baseline levels after reoxygenation, and significant increase in time to anoxic depolarization, all of which were compared to mice (Larson and Park, 2009). Additional work in NM-R hippocampal organotypic explant cultures has demonstrated robust cell viability under conditions of oxygen-glucose deprivation (Nathaniel, Saras, Umesiri, and Olajuyigbe, 2009). NM-R hippocampal slices perfused with a hypoxic bathing solution accumulated less intracellular calcium than mouse slices, indicative of an
additional adaptive mechanism to reduce intracellular calcium signaling resulting in neurotoxicity (Peterson, Larson, Buffenstein, Park, and Fall, 2012). Recent work in vivo experiments showing that NM-Rs can survive 18 minutes of anoxia, entering a suspended state, and can recover to normal function upon reoxygenation of experimental chamber. During anoxia, NM-Rs may employ a fructose-driven anaerobic glycolytic pathway to resist anoxia and support viability as suggested by an increased global expression of GLUT5 fructose transporter and increased levels of ketohexokinase during anoxic exposures (Park et al., 2017).

1.5 Summary

As a polymer, HA has remarkable mechanical properties; generally of high-molecular-weight and occupying a large hydrodynamic volume in solution through a random-coil configuration. As the concentration of HA in solution increases, HA molecular chains began to entangle and form porous, layered networks. This particular arrangement confers unique visco-elastic properties that are shear-dependent. High-molecular-weight HA in dilute saline solutions occupy a large domain in which the mass the HA itself is 0.1% and the remaining volume is occupied by the solvent. As a biomolecule, HA can bind to several types of proteins and interact with cell surface receptors, such as CD44 and receptor for hyaluronic-acid mediated motility (RHAMM). Thus, signaling pathways that involve CD44 and RHAMM signaling utilize HA as a signaling molecule. Notably, HMW-HA, on the account of its sheer size, is immunosuppressive and anti-angiogenic; possibly by preventing ligand access to cell-surface receptors and cell-cell interactions (Toole, 2000, 2004).

For a simple molecule, HA potentially has profound consequences on mediating the interaction between the intracellular and extracellular compartments of the brain microenvironment, both by influencing cell behavior and the ECS microstructure. However, these two characteristics are not mutually exclusive. The brain ECS, which constitutes the brain external microenvironment, consists of interconnected narrow channels which house extracellular fluid closely resembling cerebrospinal fluid. It serves a conduit of the transport of molecules essential for brain health and function, provides a reservoir of ions essential for neuronal electrical activity, and forms an intercellular communication
network for chemical transmission. Interaction of ECM molecules with the ECS not only influences its microstructure, but also the dynamic process of diffusion. The ECS can be characterized by two fundamental parameters: extracellular volume fraction ($\alpha$), representative of what volume of the tissue is occupied by the ECS; and tortuosity ($\lambda$), representative of how well substances can diffuse through the ECS relative to an obstacle-free medium. Both the geometry of extracellular space and interaction with ECM modulate the diffusion of molecules in the brain. The ECS, depending on its interactions with the ECM, has the potential to differentially regulate the diffusion of various groups of macromolecules and direct them to specific targets. This targeted approach ultimately influences cellular behavior (Kamali-Zare and Nicholson, 2013; Nicholson and Hrabětová, 2017).

NM-Rs have become an exemplary model of longevity with their maximum life-span extending over 30 years, approximately ten times greater than other rodent species of comparable body mass (Buffenstein, 2005). A buffered fossorial habitat coupled with social cooperativity has facilitated several evolutionary adaptations to extend their longevity in their hypoxic environment including lowered basal metabolic rate, exploitation of a fructose-driven glycolytic pathway during oxygen deprivation, a low core body temperature driven by ambient temperature (e.g. poikilothermy), and hemoglobin with increased oxygen affinity, among others (Larson, Drew, Folkow, Milton, and Park, 2014; Park et al., 2017). NM-Rs, which harbor a unique HMM-HA variant in the ECM of a wide spectrum of tissue types, including the brain, may serve as a novel animal model to explore the interplay between ECM composition, ECS micro-structure and cellular resilience to insults such as ischemia. This particular interaction highlights the instructive nature of the ECM, in modulating the ECS geometry and dynamics, and influencing cell behavior.
**Figure 1.1:** Hyaluronan structure and role as an extracellular matrix scaffold.

A) Hyaluronan (HA) is a large unbranched, nonsulfated glycosaminoglycan composed of repeating disaccharide units of N-glucuronic acid and N-acetylglucosamine. Due to the distribution of charges contributed by the amino, carboxyl, and hydroxyl groups on the polymer, HA molecules form transient hydrogen bonds with water molecules, forming water bridges, on adjacent groups of neighboring saccharides (image from Almond, 2005). B) It is hypothesized that HA polymers serve as a molecular scaffold for perineuronal nets (PNNs). Hyaladherins, such as chondroitin sulfate (CS), bind to HA by via the N-terminus of the G1 domain and stabilized by link proteins (image from Galtrey and Fawcett, 2007).
Figure 1.2: The brain extracellular space (ECS)

The ECS consists of individual channels within interconnected narrow spaces, separated by cellular membranes. These spaces span 30-60 nm and are filled with ionic solution and ECM macromolecules, including glycosaminoglycans and proteoglycans. The ECS serves as the conduit for transport of neurotransmitters, metabolites, neuromodulators, nutrients and therapeutic compounds, all of which influence the brain microenvironment. These substances travel through the ECS primarily by diffusion since there is no dedicated active transport. The cartoon depicts the contribution of extracellular matrix components in modulating the space, in particular, the ease with which a molecule diffuses. The micrograph inset highlights the extracellular space (in blue) of a mouse neocortical section (image from Korogod et al., 2015).
Figure 1.3

The volume fraction is the proportion of tissue volume occupied by the ECS relative to the total tissue volume. The volume fraction is a dynamic quantity, primarily due to re-distribution of water across intracellular and extracellular compartments. Typically, the ECS occupies 20% of the brain tissue volume, and this value can drop to 5% during anoxia (values obtained for rat cortex from Syková and Nicolson, 2007).
The tortuosity is the relative hindrance that a substance encounters when diffusing through the ECS as compared to a medium with no obstructions. The presence of extracellular matrix molecules potentially increases the interstitial viscosity of the ECS so that it no longer approximates a free medium. Additional charge interactions between the diffusing molecule and extracellular matrix can provide an additional hindrance. A healthy rat cortex (in vitro) has a tortuosity value of 1.70 (Nicholson, 1993) and when simulating a focal ischemic attack (in vitro), that value increases to 3.29. (Hrabětová et al., 2003)
CHAPTER 2

Research Aims

The African naked mole-rat (NM-R) harbors a unique variant of hyaluronan (HA) known as high-molecular-mass hyaluronan (HMM-HA), which is significantly larger than variants measured in species. This heavy variant is expressed across multiple tissues, including the NM-R brain (Tian et al., 2013), and has been attributed to a potential cancer resistance mechanism; however, very little is known about how the presence of this HMM-HA influences the brain physiology of the NM-R, and in particular, the extracellular space (ECS). Additional work has demonstrated the NM-R’s robust ability to withstand extreme hypoxic conditions through several physiological adaptations, including blunted intracellular calcium accumulation, hemoglobin with increased oxygen affinity, and an alternative glycolytic pathway driven by fructose metabolism (Johansen et al., 1976; Park et al., 2017; Peterson et al., 2012).

The relatively unassuming HA molecule has profound mechanical and biomolecular effects, which are size-dependent. Notably, high-molecular-weight hyaluronan (HMW-HA) in a dilute saline solution occupies a large space where the mass of hyaluronan itself is approximate 0.1% of the volume, and the solvent occupies the remaining space. Additionally, HMW-HA, on the account of its sheer size, is immunosuppressive and anti-angiogenic; possibly by preventing ligand access to cell-surface receptors and cell-cell interactions (Toole, 2004). The purpose of this work is the bridge the biophysical and biomolecular consequences of HA to demonstrate the instructive nature of the ECS on cell behavior. By utilizing the NM-R as a novel animal model, the following two aims will provide additional insight into how external environmental conditions influence brain physiology as it interfaces with the brain microstructure.
Aim 1

**Determine the relative degree of hindrance to diffusion of macromolecules under normal and ischemic states.**

A series of experiments using the Integrative Optical Imaging (IOI) technique is performed in order to characterize how the presence of an HMM-HA variant in the ECM of the NM-R affects the ECS parameter of tortuosity (hindrance to diffusion). This will include an inter-species comparison (with mice). Further characterization of the NM-R ECS will be performed using larger macromolecules to determine the extent of this hindrance. A series of digestion experiments, using a bacterial sourced hyaluronidase (HYase), are performed to determine how the tortuosity changes upon cleavage of NM-R HA. To explore how the NM-R ECS responds to a focal ischemic insult, a thick slice preparation will be used to determine how the tortuosity changes under conditions of hypoxic stress. Finally, to dissociate the effects of cellular swelling from the metabolic shift which occurs under oxygen deprivation, a series of hypo-osmotic stress experiments are performed to determine how tortuosity changes with cellular swelling.

Aim 2

**Determine if HA size contributes to enhanced NM-R cell viability under conditions of pH stress.**

Here we investigate the potential for the brain ECM to contribute to the environmental tolerance of NM-R neurons. Histological analysis of HA content in NM-R and mouse brain tissue was performed to determine if HA expression levels and distribution differed between the two species. A series of titration experiments involving HA of differing molecular weights (ultra-low, medium, and high) in solution (both pure $\text{H}_2\text{O}$, and artificial cerebrospinal fluid), to determine the buffering capacity of HA. A series of pH-hypoxic stress experiments on NM-R cortical slices and culture were conducted to determine if acute manipulation of extracellular pH stimulates the production of HA. Finally, a set of pH-stress experiments were performed using primary NM-R brain tissue culture to determine if exogenously added HA of various molecular weights influences cell viability.
This body of work posits that the composition of the extracellular matrix is instructive. Specifically, as a structural cue, HA modulates diffusion within the brain extracellular space. As a molecular cue, HA modulates cellular metabolism. The two seemingly disparate, yet intimately involved, cues are attributed to the resilient physiology of the NM-R, which resides in extreme hypoxic environmental conditions. The role of a size-dependent environmental buffer has not been previously explored prior to this collection of work. Future work in this area should further explore the potential for HMM-HA to protect the brain against environmental insults, including stroke and ischemia.
CHAPTER 3

Brain Extracellular Space of the Naked Mole-rat Harboring High-Molecular-Mass Hyaluronan

Introduction

The contribution of the brain extracellular matrix in modulating the brain microenvironment cannot be understated. It plays a critical role in coordinating the development of the central nervous system (CNS) and is involved in remodeling the adult CNS after injury. The presence and alteration of brain extracellular matrix (ECM) can potentially change the diffusion parameters of the extracellular space (ECS). Factors which could influence these parameters include, increased ECS viscosity, charge discrimination capabilities due to fixed charge densities present on select ECM components, and molecular interactions with various embedded receptors (Syková and Nicholson, 2008). However, the extent to which the ECM contributions to these factors remains to be determined. This becomes even more relevant in diseased states, where the ECS is particularly reactive to such pathophysiological changes. For example, the reduction of HA content in the hippocampus through the use of a HAS3 knockout paradigm resulted in an increased propensity for seizures, as well as a 40% reduction in extracellular volume fraction (Arranz et al., 2014). Other pathophysiological states such as ischemia and its subsequent effect on the diffusion parameters in the ECS have been explored (Hrabětová and Nicholson, 2000; Syková and Nicholson, 2008; Syková et al., 1994; Voříšek and Syková, 1997); this body of work will further expand on how the ECM composition may play a role in mediating these parameters.

Focal ischemia initiates an intricate biochemical cascade of events, both metabolic and ionic, resulting in neuronal cell death and tissue injury. Neural tissue is far more sensitive to changes in oxygen than glucose. Thus, this biochemical cascade is driven by severe hypoxia at the site of infarction, supplemented with a relatively preserved influx of glucose. As a result, there is an increase in anaerobic metabolism in response to the reduced oxygen and maintained glucose source. This
metabolic shift, aside from the consequential lactic acid accumulation leading to extracellular acidification, depletes ATP, initiates production of free radicals by mitochondrial electron transport chain, intracellular Na+ accumulation, excess extracellular K+, an influx of excess Ca++, and release of excitatory neurotransmitters such as glutamate. These transmembrane ionic shifts are accompanied by a redistribution of water from extracellular to intracellular compartments, resulting in neuronal and glial swelling (Larson et al., 2014; Lipton, 1999). These profound metabolic and ionic shifts detrimentally impact the brain microenvironment.

The brain ECS, as a vehicle of modulating change in the brain microenvironment, thus is a critical component in orchestrating the changes that lead to eventual cell death in ischemic-hypoxic brain tissue. Notably, there is a precipitous decrease in the ECS volume fraction, $\alpha$, from 20% to as low as 5% as well as a dramatic increase in tortuosity, $\lambda$, from 1.5 to 2.1 in anoxic/ischemic cortical tissue in vivo (Syková et al., 1994; Voříšek and Syková, 1997). In rescue experiments where blood was re-injected or a norepinephrine dose was administered, a full recovery to normoxic ECS diffusion parameters was obtained. It is worth noting a rebound effect was observed, where $\alpha$ increased to 25-30%, but $\lambda$ remained unchanged (Syková et al., 1994). Similar decreases in $\alpha$ and increases in $\lambda$ were obtained by the application of high concentrations of NMDA, glutamate or 50-80 mM K+ to brain slices (Vargová, Jendelová, Chvátal, and Syková, 2001). Rat striatal slices perfused with normal artificial cerebrospinal fluid, gassed with 95% N2-5% CO2 for 10-30 minutes resulted in a substantial decrease of $\alpha$ to 13%, while $\lambda$ remained unchanged (Rice and Nicholson, 1991). Additional work in the stratum pyramidale of the CA1 and CA3 regions of the hippocampus that using a similar approach as Rice & Nicholson, however removing glucose from the media, evoke a further decrease in $\alpha$ to 5%. The CA3 region exhibited a significant change in $\lambda$, increasing to 1.75 from 1.62 (Pérez-Pinzón, Tao, and Nicholson, 1995).

The use of thick slices, cut at 1000 um rather than the normal thickness of 400 um, provided a ready model global ischemia in vitro. RTI measures obtained from thick slice rat cortical samples demonstrated an increase in tortuosity from the normoxic value of 1.66 to 1.99; however, this value dropped to 1.54 upon the addition of 70k dextran to the submersion media. Although the $\lambda$ value
returned to normoxic levels, $\alpha$ was consistently reduced to approximately 10% in all thick slices, regardless of the presence of 70k dextran (Hrabětová and Nicholson, 2000). This finding led to the novel hypothesis that the increase in $\lambda$ in the thick slice model of ischemia may be attributed to newly-formed dead space microdomains in the ECS. These dead-spaces trap diffusing molecules, extending their dwell time, and thus netting a lower speed of diffusion. The addition of high-molecular-weight dextrans might restore $\lambda$ values to normoxic levels by filling up and eliminating these dead-space microdomains (Hrabětová and Nicholson, 2000). Tortuosity measurements from IOI experiments using 3K dextran in thick slice model have yielded increased $\lambda$ values of 3.29, which dropped down to 2.44 upon the addition of dex70k to the perfusion bath (Hrabětová et al., 2003).

A novel animal model, the African naked mole-rat (Heterocephalus glaber; NM-R) provides a unique opportunity to explore the interactions of the brain ECM and ECS during states of health and disease, in particular, focal ischemia. A buffered fossorial habitat coupled with social cooperativeness has facilitated several evolutionary adaptations to extend their longevity in their hypoxic-hypercapnic environment including lowered basal metabolic rate, acid, blunted sensitivity to acid-pain, high oxygen affinity hemoglobin, a low core body temperature (~32°C) that is subject to changing with ambient temperature (e.g. poikilothermy) (Buffenstein and Yahav, 1991; Johansen et al., 1976; O’Connor, Lee, Jarvis, and Buffenstein, 2002; Smith et al., 2011). Electrophysiological studies measuring excitatory postsynaptic field potentials (fEPSP) in NM-R hippocampus slices have demonstrated a robust capacity to withstand hypoxic/anoxic conditions via blunted reduction in evoked potential amplitude, functional recovery of fEPSP amplitude to baseline levels after reoxygenation, and significant increase in time to anoxic depolarization, all of which were compared to mice (Larson and Park, 2009). Recent work in vivo experiments showing that NM-Rs can survive 18 minutes of anoxia, entering a suspended state, and can recover to normal function upon reoxygenation of experimental chamber. During anoxia, NM-Rs may employ a fructose-driven anaerobic glycolytic pathway to resist anoxia and support viability (Park et al., 2017). Studies exploring hyaluronan (HA) production by naked mole-rat fibroblasts have demonstrated that naked mole-rats produce a high molecular mass hyaluronan (HMM-HA) variant which is significantly higher (6-12
MDa) than samples from mouse and guinea-pig tissues (0.5-3 MDa). Additionally, naked mole-rats are able to maintain relatively high levels of HA in tissue due to a combination of a mutated HAS2 variant that constitutively synthesizes HA at a high rate and a reduced rate of hyaluronidase digestion of HA (Tian et al., 2013).

To understand the capacity for this ECM enhancement in modulating the ECS, the following set of experiments in this chapter specifically address the potential of an HMM-HA enhanced ECM influencing the cortical ECS tortuosity in healthy and ischemic states. Specifically, the presence of HMM-HA enhanced brain ECM increases the tortuosity of ECS as measured by a 3000 Da MW fluorescent-dextran (dex3). Additionally, preliminary experiments demonstrate that increasing the size of the macromolecular probe to 70,000 Da MW (Ficoll70) does not increase the ECS tortuosity to the same extent as previously reported values in rat cortical slices (Nicholson and Tao, 1993). To determine the role of HMM-HA in modulating the ECS, digestion experiments using hyaluronidase demonstrated that HA cleavage significantly increases the tortuosity as measured by dex3. In order to determine how the NM-R ECS responds to a simulated focal ischemic insult, experiments using thick slice preparation demonstrated that there was no significant change in tortuosity when compared to normal-thickness cortical slices. Finally, hypo-osmotic stress experiments in normal-thickness NM-R cortical slices indicate that the ECS tortuosity is susceptible to significant increase when inducing cellular swelling via exposure to a hypo-osmolar bathing solution. The primary purpose of this series of experiments is to highlight the ECM’s instructive role in influencing the ECS microstructure.

**Materials and Methods**

**Naked mole-rat brain slices**

Naked mole-rat brain slices were prepared from both male and female mole-rats (age > 6 months) anesthetized with tribromoethanol (Avertin, 125 mg kg, i.p.) and transcardially perfused with ~4°C sucrose-based artificial cerebrospinal fluid (sucrose-ACSF, in mM: 252 sucrose, 5.0 KCl, 2.0 CaCl2, 2.0 MgSO4, 1.25 NaH3PO4, 26 NaHCO3, 10 d-glucose; pH = 7.4). The brain was re-
moved and embedded in low melting point agarose. Coronal cortical sections (400µm & 1000µm) were cut using a Compressstome (Precisionary Instruments), and transferred to a double-walled vacuum sealed tumbler (Contigo) filled with 4°C sucrose-ACSF saturated with 95% O₂/5% CO₂ gas prior to transport. Transportation time of slices between the College of Staten Island and SUNY Downstate was approximately 45 minutes. Upon arrival, slices were transferred to an incubation chamber containing ACSF of a similar formulation as described above but with 126 mM NaCl substituted for sucrose (NaCl-ACSF), maintained at room temperature and oxygenated (95% O₂/5% CO₂). Slices were incubated for 90 minutes before the start of the experiment. All methods involving animals were approved by the Institutional Animal Care and Use Committee at the College of Staten Island in the City University of New York (and SUNY Downstate) and are in accordance with regulations required by the United States Department of Agriculture.

**Integrative optical imaging (IOI) method**

In the IOI method, fluorophore-labeled dextran molecules are pressure-injected from a single barrel glass micropipette into brain tissue or dilute agarose gel and a sequence of images is captured using a charged-coupled device (CCD) camera attached to an epifluorescence compound microscope. The distribution of the fluorescence signal is fitted to the diffusion equation to obtain an estimate of the effective diffusion coefficient ($D^*$, cm² s⁻¹) and the free diffusion coefficient ($D$, cm² s⁻¹) in the brain tissue and dilute agarose gel preparation, respectively. The hindrance imposed on the diffusing molecule by the extracellular microenvironment of the brain, extracellular tortuosity, is calculated as $\lambda = \sqrt{(D/D^*)}$.

The present study measured the diffusion of a Texas Red-labeled dextran (MW 3000) (dex3, 1 mM in 150 mM NaCl; catalog No. D-3329, Life Technologies, Carlsbad, CA) for the inter-species comparison, hyaluronidase digestion, and thick-slice experiments. Preliminary studies to further characterize the extent of tissue hindrance in naked mole-rat cortical slices utilized a FITC-labeled polysucrose polymer (MW 70000) (Ficoll70, 0.1 mM in 150 mM NaCl; catalog No. 51731, Millipore Sigma, Darmstadt, Germany). Each fluorescent molecule was dissolved in a 150 mM NaCl
solution, to reach a final concentration of 1 mM for dex3 and 0.1 mM for ficoll70. Using an electronic micromanipulator (MP 285; Sutter Instruments, Novato, CA), the micropipette was advanced into tissue (or agarose gel) at an angle of 31° from the horizon. The tip of micropipette (2–4 mm in diameter) was positioned 200 µm below the surface of the coronal surface of the cortical slice or 500 µm below the surface of the dilute agarose gel (0.3% in 150 mM NaCl, NuSieve GTG Agarose, FMC Bio-Products, Rockland ME). A nano-volume of the solution of fluorescent molecules was released from a glass micropipettes (single barrel, thin-wall glass tube; catalog No. 6170, A-M System Carlsborg, WA) by a brief pulse of compressed nitrogen (10-200 ms, 10-20 psi) controlled by an electronic value (PicoSpritzerIII; Parker Hannifin, Pine Brook, NJ). The diameter of the injection was typically 20–40 mm. The sample chamber was maintained at 34°C via thermistor feedback (TC-344C Dual Channel Temperature Controller; Warner Instruments; Hamden, CT). The ACSF solution perfusion system was controlled via a peristaltic pump maintained at a flow rate of 2 mL per min.

A 75 W xenon source mounted onto the light-source port on the epifluorescence compound microscope (BX61WI; Olympus America, Melville, NY) provided the necessary excitation wavelengths which were directed to the sample chamber using an electronically-triggered dichroic filter wheel fitted with Texas Red or fluorescein (FIT-C) filter sets. The microscope was equipped with water immersion objectives (UM PlanFl 10x, NA 0.3 and LUM PlanFl 40x, NA 0.8; both, Olympus America). A timed sequence of images capturing the diffusion of fluorescent molecules was taken with a cooled charged-coupled device camera (QuantEM 512SC; Photometrics, Tucson, AZ) attached to the microscope. The image sequence acquisition parameters (interval and duration) depended on the molecular weight of the diffusing molecule and diffusion medium. For dex3, 40 images were captured over 80 seconds; for ficoll70, 40 images were captured over 320 seconds. To prevent photobleaching, a shutter (model Uniblitz VS35) in the excitation light path was triggered to close in between image captures. The acquisition instrumentation was interfaced with an 8-channel stimulator (Master-8; A.M.P.I; Jerusalem, Israel) and controlled using custom-built MATLAB-based acquisition software.

Analysis of image sequences was analyzed using custom-built MATLAB-based analysis soft-
ware. Briefly, a series of background images captured before the injection which are used to subtract the background from the set of images post-injection. The intensity profiles were extracted along the major, minor, and two diagonal axes of the diffusion cloud in each image. These intensity profiles were fitted with a Gaussian curve using a non-linear least-squares curve-fitting algorithm. The values of $D^*$ (or $D$) was obtained by using linear regression on Gaussian-fitted intensity profiles.

**Digestion of Hyaluronan Using Hyaluronidase**

A series of hyaluronidase digestion experiments were conducted to determine how digestion of the unique HMM-HA in the NM-R brain affected the degree of hindrance to diffusion in normoxic cortical slices. A digestion chamber was fabricated using a 3D printer (Ultimaker 2+ 3D printer; Ultimaker, Utrecht, Netherlands) to incubate a single slice submerged in 1 mL of digest solution. The digest solution consisted of 75 units of bacterial hyaluronidase dissolved in normal ACSF oxygenated with 95% O$_2$/5% CO$_2$. Candidate slice(s) for digestion were randomly chosen once the slices were transferred from the transport vessel to the incubation chamber for the 90 minute acclimatization period. For a given experiment, a series of IOI measurements using dex3 as the diffusing fluorophore was used to measure tortuosity in undigested normoxic NM-R cortical slices. After a two-hour digestion period, a series of IOI measurements using dex3 was used to measure the tortuosity in the hyaluronan-digested normoxic NM-R cortical slice.

**Osmolarity of ACSF and Osmotic Stress Experiments**

The osmolarity of ACSF solutions was measured using a freezing-point osmometer (5002 OSMETTE Automatic High Sensitivity Osmometer; Precision Instruments; Natick, Ma). Normal ACSF solutions for incubation of slices and non-osmotic stress experiments were 300-310 mOsm. For osmotic stress experiments, NM-R cortical slices were exposed to the following sequence of ACSF perfusions: normo-osmolar ACSF (300-310 mOsm) for baseline IOI measurements, hypo-osmolar ACSF(145-155 mOsm) for a hypo-osmotic stress response, and normo-osmolar (300-310 mOsm) for a recovery response. When switching between these three phases, a 20 minute acclimation period
was given for the tissue to equilibrate with the new ACSF formulation, prior to making measurements.

**Immunohistological analysis**

Undigested and digested slices (n = 3 slices per treatment group) were placed into separate scintillation vials with approximately 10 mL of 4% paraformaldehyde. After a 24 hour fixation period (vial were maintained at 4 deg celsius), slices were transferred to a 30% sucrose solution for another 24 hours before embedding in 2% agarose for sectioning. Slice-embedded agarose blocks were sectioned at 50µm using a vibratome while being submerged in 1X phosphate-buffered saline (PBS) solution. Sections were transferred to appropriately labeled microscope slides where they were sectioned off using a grease pencil to establish wells for microliter volumes of treatment solutions. Sections were treated with a 3% blocking solution (normal goat serum in PBS), followed by a series of PBS washes. After the PBS washes, the slices were treated with a Hyaluronic-Acid Binding Protein primary antibody (HABP, 1:200 dilution with 3% goat serum in PBS; catalog No. 385911-50UG, Millipore Sigma, Burlington, MA). After a 3-hour incubation period, sections underwent a series of PBS washes, after which they were treated with 500 uL of 3,3′-Diaminobenzidine (DAB) substrate buffer solution. DAB signal intensity was measured using wide-view microscopy. Acquired images were separated into corresponding channels of the DAB-HABP signal using the color deconvolution plugin in ImageJ. Pixel intensities were measured from randomly selected regions of interest (n=100) using a custom macro within a manually selected region of flat and unfolded tissue. Intensity values were converted to an optical density metric using the following formula:

$$O.D. = -\log \left( \frac{255}{Mean\ Pixel\ Intensity} \right).$$

Average O.D. was calculated for each slice from, and a two-tailed heteroscedastic t-test was used (after determining equality of variance using F-test) to determine statistical differences in HA expression between undigested and digested sections.
Statistical Analysis

For tortuosity and immunohistochemistry data, statistics were computed on mean values for each slice (i.e. mean $\lambda$ value per slice and mean optical density value per slice). All data represented in graphs depict (Mean ± SEM). For the comparison of means between two groups, an F-test of equality of variances was computed to test for the null hypothesis that two normally-distributed populations have the same variance. Subsequent 2-tailed unpaired t-tests (Student’s t-test) were then computed to compare the means of the two groups of interest. Each group consists of randomly selected samples collected from two different populations (or treatments), and thus are independent of one another; this serves as the basis for utilizing the Student’s t-test. Significance was accepted when $p < 0.05$. Since statistical comparisons were made between 3 treatments on the same sample for a given independent experimental trial, One-way analysis of variance (ANOVA; R version 3.5.3) was computed followed by pair-wise multiple comparisons between groups using post-hoc Tukey-HSD test for parametric ANOVA for osmotic stress experiments. Significance was accepted when $p < 0.001$ for ANOVA. Parametric method(s) were utilized with the underlying assumption that the data is normally distributed and consistent with statistical approaches used previously for the same measures.

Results

Normoxic naked mole-rat (NM-R) cortical slices exhibit a greater degree of hindrance to diffusion compared to mouse cortical slices.

Normoxic naked mole-rat (NM-R) cortical slices exhibit a greater degree of hindrance to diffusion compared to mouse cortical slices. The behavior of dex3 in normoxic mouse cortical slices ($n = 7$ slices) was first examined to establish a control group. Briefly, fluorescently tagged dex3 was pressured injected $200\mu m$ below the surface of the slice, and its subsequent movement was observed using IOI. The diffusion fluorescently-tagged dex3 volume was imaged every 4 seconds for 160 seconds. A complementary set of similar experiments were performed on normoxic NM-R cortical
slices for comparative purposes. An additional set of normoxic mouse cortical slices (n = 2 slices) underwent the transport protocol to ensure the process of transporting tissue between the 2 campuses did not significantly compromise the parameters of diffusion. Figure 3.1A depicts an inter-species comparison of tortuosity values, which represents the degree to which diffusion is hindered, denoted by \( \lambda \). For the mouse cortical slices, \( \lambda = 1.82 \pm 0.26 \) (mean ± SD, n = 9 slices); whereas, for NM-R cortical slices, \( \lambda = 2.10 \pm 0.14 \) (mean ± SD, n = 17 slices). Thus, there was a statistically significant difference between the ECS tortuosities of both species, as measured by dex3 (\( p = 0.015 \), unpaired t-test, unequal variance, 2-tailed). NM-R cortical slices exhibited a 14.28 % increase in tortuosity compared to mouse cortical slices for a macromolecule of the same size. Figure 3.1B depicts the comparison of tortuosity values measured from mouse cortical sections sourced from CSI and Downstate. There was no statistically significant difference in \( \lambda \) values between the two groups (Downstate: \( \lambda = 1.84 \pm 0.29 \); CSI: \( \lambda = 1.75 \pm 0.006 \) (mean ± SD; \( p = 0.537 \), unpaired t-test, unequal variance, 2-tailed).

Dex3 is used to approximate the tortuosity of the brain ECS typically experienced by the diffusing small molecules and ions. As the size of the molecule increases, there is a proportionate increase in the hindrance experienced by that molecule as it diffuses through the ECS. To determine how larger molecules behave in an ECS which is supported by a high-molecular-mass variant of hyaluronan, a series of preliminary experiments were performed to determine the extent of hindrance to diffusion that a larger molecule undergoes within in the brain ECS of the NM-R cortex in vitro. The experimental paradigm is as follows, for a given normoxic NM-R cortical slice, an initial set of IOI measurements were taken with dex3 as the diffusing molecule. A subsequent set of IOI measurements are taken with ficoll70 as the diffusing molecule. Figure 3.1C depicts the comparison of average \( \lambda \) values for each of the two different molecular weights of fluorophores used. For the dex3 injections, \( \lambda = 2.11 \pm 0.005 \) (mean ± SD, n = 2); whereas, for the ficoll70 injections, \( \lambda = 2.45 \pm 0.004 \) (mean ± SD, n = 2). An additional slice underwent IOI measurements using just ficoll70; the average \( \lambda = 2.32 \pm 0.386 \) (mean ± SD, for 7 injections).
Digestion of Hyaluronan increases hindrance of diffusion of Dextran-3K in NM-R cortical slices

HA-digested experiments cited from prior literature used a 1 hour digestion period. The increased mass, as well as the robust nature of HMM-HA as described by Tian et. al, was the primary motivation for increasing the digestion time to 2 hours. An alternating treated-slice paradigm was utilized in which IOI measurements were taken in an undigested slice, followed by a digested slice, and finally in another undigested slice. As depicted in Figure 3.2, the undigested slices, $\lambda = 2.07 \pm 0.11$ (mean $\pm$ SD, n = 11 slices) and of the 5 slices that were digested, 4 slices underwent a 2-hour digestion period and 1 slice underwent a 3-hour digestion period. For slices under the 2-hour digestion regime, $\lambda = 2.47 \pm 0.12$ (mean $\pm$ SD, n = 4 slices). The 3-hour digested slice had a $\lambda = 2.23$; although this value deviates the 2-hour digestion average, it should be noted that the condition of the slice was structurally-weak and several compromised areas indicated that the slice viability was rapidly diminishing. Thus, this series of digestion experiments demonstrate that there was a statistically significant increase in tortuosity in the digested slices compared to the undigested control slices (for all digested slices, p = 0.0066, n = 5 slices); for the set of 2-hour digested slices, p = 0.0056 (n = 4 slices); both p-values were computed using an unpaired t-test, unequal variance, 2-tailed). Immunohistochemical analysis of undigested-digested pairs of slices was performed to determine the extent of digestion, depicted in Figure 3.2 B-C. There was no significant difference in DAB-HABP signal (undigested: O.D = 0.688 $\pm$ 0.052 (n = 3 slices); digested: O.D. = 0.748 $\pm$ 0.025 (n = 3 slices); p = 0.1724; unpaired t-test, unequal variance, 2-tailed).

Diffusion parameters of ischemic thick NM-R cortical slices

To model the effects of an ischemic insult, a series of experiments using the thick-slice model were conducted to determine how an ischemic brain microenvironment influences the diffusion in the ECS supported by an HMM-HA based extracellular matrix. The experimental paradigm used is as follows; over the course of five experiments, IOI measurements using dex3 as the diffusing molecule was used to measure tortuosity in 11 thick slices. For two of the experiments, IOI measurements in normoxic slices (n=2), were taken for comparative purposes. Figure 3.3A depicts these
comparative measures; for thick slices, $\lambda = 2.10 \pm 0.356$ (mean $\pm$ SD, n = 11 slices). For the normoxic slices, $\lambda = 2.15 \pm 0.072$ (mean $\pm$ SD, n = 2 slices). There was no statistically significant difference between the normoxic and thick slices from this series of measurements ($p = 0.731$, Unpaired t-test, unequal variance, 2-tailed). For an additional comparison, a running average of tortuosity for all normoxic slices measured up until this point was computed $\lambda = 2.11 \pm 0.136$ (mean $\pm$ SD, n = 32 slices). There was no statistically significant difference between the running average of normoxic slices and thick slices from this series of measurements ($p = 0.925$, Unpaired t-test, unequal variance, 2-tailed).

**Osmotic stress is not the primary driver of ischemic insult resiliency in NM-R cortical slices**

An ischemic insult triggers a cascade of ionic and metabolic events which activate multiple signal transduction pathways leading to neuronal cell death. The brain ECS undergoes a dramatic reduction in volume fraction (approximately 50%), with cell swelling being the primary cause of this precipitous decrease. A series of experiments were conducted to determine if the resiliency of NM-R cortical thick-slices are due to withstanding cell swelling or neuronal cell death. To dissociate these two factors, an osmotic stress experimental paradigm was employed in normoxic NM-R cortical slices. The purpose of this particular exposure was to determine if reducing the osmolarity of the ACSF, and thereby induce cellular swelling, results in the same degree of resiliency as demonstrated in the NM-R thick-slice model. Briefly, two sets of ACSF solutions were prepared: normo-osmolar (300-310 mOsm), and hypo-osmolar (145-155 mOsm). IOI measurements were taken in normoxic NM-R cortical slices using dex3 as the diffusing molecule. The experiment was designed to compare the tortuosity of the ECS under normo-osmolar conditions, hypo-osmolar conditions, as well as during a recovery period in which normo-osmolar ACSF was used after a prior hypo-osmolar exposure (~90 minutes) for each slice. Figure 3.3B describes the obtained results. During the normo-osmolar exposure (baseline), $\lambda = 2.11 \pm 0.172$ (mean $\pm$ SD, n = 7 slices). After a 20 minute acclimatization period, hypo-osmolar exposure resulted in an increase in tortuosity; $\lambda = 3.04 \pm 0.377$ (mean $\pm$ SD, n = 7 slices). For the recovery phase, again after a 20 minute acclimatization period, the tortuosity
was reduced, $\lambda = 2.02 \pm 0.180$ (mean $\pm$ SD, $n = 7$ slices). One-way ANOVA was performed (p-value < 0.0001), and adjusted p-values were computed using post-hoc Tukey’s HSD test for pairwise comparisons. The increase in tortuosity induced by reducing the osmolarity of the ACSF was significantly higher than the baseline measurements taken before the exposure (p adjusted < 0.00001). There was not a statistically significant difference between the tortuosity values taken during the baseline normo-osmolar exposure and the recovery exposure following a hypo-osmolar exposure.

**Discussion**

**NM-R ECS hinders diffusion of large macromolecules**

The presence of a very high molecular weight variant of HA in the NM-R ECS provided an opportunity to explore its contribution to the ECS microstructure. In particular, it provides an experimental model to determine how ECM components modulate the ECS parameter of tortuosity (hindrance to diffusion). Transport of NM-R tissue between two locations posed a unique challenge, thus it is worth noting that by using a cold (0-4 deg c) sucrose-based ACSF solution for transcardial perfusion, slicing, and transport of tissue, preserved its viability. Prior work has demonstrated that replacing sodium content with sucrose during the initial phases of slice recovery drastically reduces the majority of neuronal swelling (Ting, Daigle, Chen, and Feng, 2014), and maintaining slices in ice-cold conditions curtails ischemia-induced excitotoxicity (Campos et al., 2012).

Mean tortuosity measurements previously recorded in rat cortical slices using 3-kDa dextran was 1.70 (Nicholson and Tao, 1993). Here we report a mean transported mouse tortuosity value of 1.76. Increasing the size of the molecule to 70-kDa (Ficoll), increased the tortuosity to 2.45. It has been previously reported that a molecule that size increased the tortuosity measure to 2.26 in rat cortical sections (Nicholson and Tao, 1993). This is a 28.28 % increase in hindrance compared to a 14.91 % increase recorded in NM-R samples. Taken together, the presence of an HMM-HA based ECM in the NM-R confers a greater degree of a hindrance to the diffusion of large molecules. Interestingly, when increasing the size of the diffusion molecule, this hindrance does not increase similarly as it does with mice.
With previous work describing the substantially more viscous conditioned media of NM-R fibroblast culture producing HMM-HA (Tian et al., 2013), it possible that a similar enhancement of ECS viscosity is seen here. This provides additional information regarding the NM-R ECS microstructure; however, further details of the ECS geometry remain open-ended. From recent scanning electron microscopy micrographs of HA extracted from NM-R brain tissue have demonstrated a voluminous, cloud-like appearance. This was vastly different from HA extracted from mice, which occupied less volume, had a flattened network configuration (Kulaberoglu et al., 2019). Granted, these samples underwent a chemical extraction process using a series of alcohols. Nevertheless, if NM-R HA takes on such a unique configuration within the ECS, it would potentially alter its geometry.

The observed increase in NM-R cortical ECS tortuosity highlights the role with which ECM components dictate ECS diffusion parameters. The highly viscous nature of HMM-HA, in addition to its sheer size, is most likely the source of the increased $\lambda$ values seen in NM-R cortical slices. However, preliminary results indicate that increasing the size of the diffusing molecule does not increase the subsequent $\lambda$ value to the same extent as observed with other species (cortex). These findings highlight a potential functional role of HMM-HA in the NM-R brain, notably as a structural scaffold to the ECS, which influences the diffusion of critical molecules for growth, development, and survival.

Work by (Margolis et al., 1975) demonstrated that in 7-day old rat pups, 90% the HA in the brain is extractable by water alone, as compared to only 15% in adult animals, and this large amount of soluble hyaluronic acid in young rat brain is relatively inactive metabolically. It is conjectured that the purpose of the highly soluble HA content in the developing brain facilitates the diffusion of critical molecules, such as growth factors, during maturation. Incidentally, (Penz et al., 2015) characterized several anatomical and physiological hallmarks that demonstrate an extremely protracted period of brain maturation. These hallmarks include prolonged retention of expression of PSA-NCAM, critical for neurite outgrowth, and uncoupling of neuronal morphology and excitability in CA1 region of the NM-R hippocampus.
HA molecules of heavier mass entangle to form continuous, porous networks which are conferred with a high hydration capacity, retention of flow, and resistance to gel-formation (Cowman et al., 2015; Krause et al., 2001). These properties of heavier HA polymers are perhaps contributing factors in the highly soluble nature of early postnatal HA, as seen by (Margolis et al., 1975). Thus, the two following correlates arise: The HA contents of the developing postnatal brain may be of a heavier molecular mass, and that the retention of such trait in the NM-R may also contribute to its neotenuous phenotype. Also, the blunted $\lambda$ measure of ficoll70 injections allude to a narrower range of tortuosity values exhibited in the NM-R cortex, allowing for facilitated diffusion of larger molecules.

**HA Digestion further increase ECS tortuosity**

Using a bacterial hyaluronidase, cleaving HA resulted in a significant increase in ECS tortuosity as measured by dex3. A 2-hour digestion window resulted in an 18.75% increase in tortuosity, from 2.08 to 2.47. For a 3-hour digestion window, the average tortuosity value was 2.22; however the quality the slice was notably compromised. HA digestion on this time scale although visibly compromised the integrity of the tissue, the increase in tortuosity was not as pronounced as with the 2-hour digestion window. This was a rather surprising finding, assuming that a heavy HA variant is more viscous and adds to the hindrance of diffusion, digestion of HA seems that it reduces tortuosity, further facilitating diffusion. In light of this novel finding, it is worth considering the HA’s role as a scaffolding component of the brain ECM. Hyaladherins, such as brevican, neurocan, versican, and aggrecan, phosphacan, chondroitin sulfate, along with tenascin-R and other various link proteins use HA as a molecular scaffold to fold extracellular structures such perineuronal nets (Deepa et al., 2006; Yamaguchi, 2000). Upon cleavage of HA, it could conceivably cause these interconnected ECM components to collapse onto one other resulting in an ECS “log-jam”. The obstruction of the narrow, connected channels of the ECS would increase the hindrance to diffusion of large macromolecules such as dex3. It is also worth considering if the increase in HA polymer length also leads to changes in expression levels of other proteoglycan components. The extent of this “log-jam” effect may be
dependent on the size of the HA-chain backbone, i.e. the heavier the chain, the more hyaladherins present, and thus more material to obstruct the ECS.

For immunohistological analysis of HA-digested vs undigested samples, there was no statistically significant difference in HABP (hyaluronic acid-binding protein antibody) signal between the two groups. Although there was a substantial increase in tortuosity upon digestion, it is possible that HABP still binds to digested HA components. The 2-hour digestion may not have cleaved HMM-HA chains sufficiently enough, resulting in the linkage proteins in the HABP antibody to still bind with the digested product. This particular finding highlights the role of HA size in HA turnover; Tian and colleagues (Tian et al., 2013) noted that HMM-HA turnover was relatively lower compared to other species. The sheer size of HMM-HA may preclude a hyaluronidase from effectively digesting the substrate; possibly since the conformation of an HMM-HA molecule obstructs a sufficient number of glycosidic bonds on which the active site of the enzyme acts on. The cleaved product may also be conducive to readily re-form glycosidic bonds, to larger HA molecules, thus reducing the efficacy of the hyaluronidase reaction.

**NM-R ECS is resilient to focal ischemic insult**

NM-Rs are among the most hypoxic-hypercapnic tolerant mammals, sharing an underground nest with upwards of 100 colony members. Several physiological adaptations, in addition to being eusocial, has equipped the species to thrive in their fossorial habitat. In order to explore how the NM-R ECS responds to such a hypoxic insult, we used a thick-slice (1000 um) preparation to simulate a focal ischemic event. NM-R thick cortical slices were remarkably tolerant to hypoxic-stress, showing no statistically significant difference in tortuosity values compared to normal-thickness NM-R cortical slices. There was however much variability in tortuosity records obtained for each thick-slice, suggesting some regions were more susceptible to the injury than others.

Previous work done in rat cortical thick-slices shows that the tortuosity values, as measured by 3-kDa dextran, reaches 3.66. Interestingly, the addition of a 70-kDa dextran to the bath medium reduces this precipitous increase in tortuosity, yielding a value of 2.37 (Hrabětová et al., 2003). Exper-
periments using RTI showed a similar result, increased from a normoxic value of 1.66 to 1.99 in thick-slices; however that value dropped to 1.54 when 70-kDa dextran was added to bathing medium. The measure of volume fraction yielded a curious finding. The volume fraction for normoxic slices was 0.24, which reduced to 0.13 in thick-slices. By adding the dextran the bath, the volume fraction of thick-slice decreased from 0.12 to 0.10 (Hrabětová and Nicholson, 2000).

The increase in tortuosity during ischemia is was previously attributed to the decrease in volume fraction; however, work by Hrabětová & Nicholson showed that tortuosity and volume fraction can change independently in ischemic tissue with the addition of dextran to the bathing media (Hrabětová and Nicholson, 2000). These works collectively suggest that with the ischemia-induced cellular swelling, there is a drastic decrease in volume fraction (~50%). This reduction in the volume fraction will also reduce the width of interstitial channels in the ECS, potentially forming dead-space microdomains. These dead-spaces increase the tortuosity by transiently trapping diffusing molecules, prolonging the time of travel. Thus, the addition of dextran molecules to the bath could block these dead-spaces, which would marginally reduce the volume fraction, but vastly improve the ease with which molecules move throughout the ECS (Hrabětová et al., 2003; Hrabětová and Nicholson, 2000; Patlak, Hospod, Trowbridge, and Newman, 1998).

Prior work in tumor tissue demonstrated hypoxia-induced changes in HA content, notably an increase in HA production as well as hyaluronidase activity. This upregulation in hyaluronidase activity subsequently leads to an increase in LMW-HA content, which has been shown to promote angiogenesis. This angiogenic effect is perhaps a compensation mechanism for hypoxic insults in tumors (Gao et al., 2005). Relatively little is known about hyaluronidase activity in NM-R brain tissue, particularly under pathophysiological conditions. An increase in native hyaluronidase activity during periods of ischemia could conceivably cleave the HMM-HA to a size which would block the dead-space microdomains, thereby providing pockets of viable tissue.

Cellular swelling is the primary factor in the ischemic-driven reduction of ECS volume fraction. Oxygen deprivation reduces cellular ATP stores, leading to substantial shifts in the concentration gradients of several ions. These shifts move water from the ECS into intracellular compartments,
resulting in cellular swelling and subsequent drop in volume fraction, and consequential increase in tortuosity (Hrabětová and Nicholson, 2000; Lipton, 1999). To determine how the NM-R ECS responds to cellular swelling, an osmotic stress paradigm was used. By exposing normoxic tissue to a hypo-osmotic ACSF solution, simulates the cellular swelling event that occurs during an ischemic insult, without triggering the cascade of signaling pathways which compromise metabolic function resulting in cell death. Upon exposure and acclimatization to a hypotonic ACSF solution (150 mOsm), the tortuosity increased by 42%, from 2.10 to 3.00. Reversing the osmotic gradient back to normo-osmolarity, restored the tortuosity to normoxic levels, 1.99.

Kume-Kick and colleagues (Kume-Kicket al., 2002) demonstrated how the volume fraction and tortuosity are altered in normoxic rat cortical slices under various osmotic conditions. Under normo-osmolar exposure (305 mOsm), the volume fraction was 0.24 and tortuosity was 1.69. Reducing the osmolarity to 150 mOsm, decreased the volume fraction to 0.12, and increased the tortuosity to 1.86. Interestingly, increasing the osmolarity to 350 mOsm reduced the tortuosity to 1.67, where it remained constant as the osmolarity was further increased to 500 mOsm. The volume fraction, however, continued to increase, reaching 0.42 at the highest osmolarity exposure. A similar study was performed by Tao (Tao, 1999) using IOI and 3-kDa dextran to measure tortuosity showed similar trends as demonstrated by the diffusion of TMA+ ions. Although the data collected by Tao were measures of effective diffusion coefficients, tortuosity values can be calculated using $\lambda = \sqrt{\frac{D}{D^*}}$, where the free diffusion coefficient (D) was sourced from tortuosity measure in agarose as described by (Nicholson and Tao, 1993). In rat cortical slices, there was a 33.5% increase in tortuosity with hypo-osmotic treatment (150 mOsm), from 1.85 to 2.47. Not only did NM-R cortical slices mirror the same effect previously seen in other species, but it also seemed that it was more susceptible to hypo-osmotic stress with a 42% increase in tortuosity. Given that the ECS tortuosity in the NM-R cortex is not susceptible to a drastic increase during a simulated insult as seen with other species, but is just as sensitive to changes in osmolarity, alludes to a potential metabolic-control mechanism which resists hypoxic cell death. Park and colleagues (Park et al., 2017) recently described a fructose-driven glycolytic mechanism which supports anoxia resistance in the NM-R.
Perhaps a shift to this metabolic pathway allows for the production of sufficient ATP molecules to stave off cellular swelling for a longer period compared to other species.

It is worth considering, then, the incubation times employed for such osmotic stress experiments. In this present study, the slices were given 20 minutes to equilibrate with the new ACSF solution differing in osmolarity. Tao showed that the effective diffusion coefficient began to change after ~ 5 minutes after changing the solution, and reached a steady value within 10 minutes (Tao, 1999). Complementary work by Hrabětová and colleagues (Hrabětová, Chen, Masri, and Nicholson, 2002), showed normoxic slices accumulated water within the first 30 minutes of incubation post-slicing. Thick-slices (1000 um), however, continued to gain water through the course of incubation. Notably, the largest water redistribution occurred at the onset of ischemic insult in thick-slices, likely due to the large shifts in ion movement across compromised concentration gradients. Perhaps the NM-R ECS, supplemented with an HMM-HA based ECM exaggeratedly compartmentalizes due to the high hydration capacity of HA.

Taken together these novel findings characterizing the NM-R ECS parameter of tortuosity highlights the interplay between ECM composition, ECS microstructure, and geometry. ECM components may add to the viscosity of ECS, and it is conceivable that a heavy variant of HA would do. However, the presence of such a component may also directly influence the organization and geometry of the ECS. In order to gain a full appreciation of how the unique composition of the NM-R ECM influences the ECS, additional work to determine the native volume fraction would be needed. It would provide further insight into how ECS dynamics are altered in the NM-R brain under various physiological and pathological conditions, with and without the presence of HMM-HA.
Figure 3.1: NM-R ECM confers more hindrance to diffusion compared to that of mice

A) Inter-species comparison of tortuosity values from NM-R and mouse cortical slices. For mouse cortical slices, $\lambda = 1.82 \pm 0.26$ (mean $\pm$ SD, $n=9$ slices); for NM-R cortical slices, $\lambda = 2.10 \pm 0.14$ (mean $\pm$ SD, $n=17$ slices, (*) $p<0.05$, t-test). B) Determining whether the transport protocol compromises tortuosity measures. Downstate: $\lambda = 1.84 \pm 0.29$; CSI: $\lambda = 1.75 \pm 0.006$ (mean $\pm$ SD; $p = 0.537$, unpaired t-test, unequal variance, 2-tailed). C) Comparison of tortuosity values obtained from 2 different sizes of fluoroscent probes. Magenta-dotted markers indicate values previously reported in rat cortical slices by (Nicholson & Tao, 1993). For dex3, $\lambda = 2.11 \pm 0.005$ (mean $\pm$ SD, $n=2$ slices); whereas, for ficoll70, $\lambda = 2.45 \pm 0.004$. 
Figure 3.2: Digestion of HA increases hindrance to diffusion

A) Digestion of HA on tortuosity in NM-R cortical slices. Data from 2 Hour digestion period depicted here. For the undigested slices, $\lambda = 2.07 \pm 0.11$ (mean ± SD, n = 11). For the digested (2-hr) slices, $\lambda = 2.47 \pm 0.12$ (mean ± SD, n = 4 slices). (**) $p = 0.0056$; unpaired t-test, unequal variance, 2-tailed. B) Immunohistological analysis of HA digestion. DAB-HABP signal is depicted as optical density (O.D.). O.D. describes the degree to which a refractive medium retards the transmission of light; lower O.D. equates to increased HA signal. Undigested: O.D. = 0.688 ± 0.052 (n = 3 slices); digested: O.D. = 0.748 ± 0.025 (n = 3 slices); $p = 0.1724$; unpaired t-test, unequal variance, 2-tailed. C) Images of DAB-HABP labeled sections from digestion experiments are shown here. Images underwent color deconvolution to isolate the DAB signal. Mean pixel intensities were pulled randomly selected regions (3x3 pixels) sampled color deconvoluted images. D) Diffusion cloud from point source injection of 3K-Dextran in NM-R cortical slice. Top three panels demonstrate the change in intensity of point source over time. Bottom panel shows intensity profiles across the time series for the prescribed axis.
Figure 3.3: NM-R thick slices are resilient to simulated ischemic insult; however not to osmotic stress

A) NM-R slice thickness on tortuosity. For the normoxic slices (400 μm), $\lambda = 2.15 \pm 0.072$ (mean ± SD, n = 2 slices). For thick slices (1000 μm), $\lambda = 2.10 \pm 0.356$ (mean ± SD, n = 11 slices). There was no statistically significant difference between the normoxic and thick slices from this series of measurements (p = 0.731, Unpaired t-test, unequal variance, 2-tailed). Dotted markers depict historical values for similar experiments performed in rat cortical slices. Normal (400 μm) SD-rat cortical slices [magenta]: $\lambda = 1.70$ (Nicholson and Tao, 1993); Thick (1000 μm) SD-rat cortical slices [green]: $\lambda = 3.66$ (Hrabětová et al., 2003). B) Hypo-osmotic stress on tortuosity. During the normo-osmolar exposure (baseline), $\lambda = 2.11 \pm 0.172$ (mean ± SD, n = 7 slices). After a 20 minute acclimation period, hypo-osmolar exposure resulted in an increase in tortuosity; $\lambda = 3.04 \pm 0.377$ (mean ± SD, n = 7 slices). For the recovery phase, again after a 20 minute acclimizaation period, the tortuosity was reduced, $\lambda = 2.02 \pm 0.180$ (mean ± SD, n = 7 slices). One- way ANOVA was performed p-value < 0.0001), and adjusted p-values were computed using post-hoc Tukey’s HSD test for pairwise comparisons; (****) adj. p-value < 0.0001. Bronze-dotted markers depict historical values for similar experiments performed in rat cortical slices (Tao, 1999). Although the data collected by Tao were measures of effective diffusion coefficients, tortuosity values were computed using free diffusion coefficient (D) was sourced from tortuosity measure in agarose as described by (Nicholson & Tao, 1993). Hypo-osmolar exposure: $\lambda = 2.48$; Normo-osmolar exposure: $\lambda = 1.85$; Recovery (exposure to normo-osmolar solution, after hypo-osmolar exposure): $\lambda = 1.73$. 
**Figure 3.4**

<table>
<thead>
<tr>
<th>Undigested</th>
<th>HA Digested</th>
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<tbody>
<tr>
<td>Hyaluronan</td>
<td></td>
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<tr>
<td>Tenascin-R</td>
<td></td>
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<tr>
<td>Chondroitin Sulfate</td>
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<td>Various</td>
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<tr>
<td>Proteoglycans</td>
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**Figure 3.4:** Log-jam of extracellular matrix components obstructing the ECS.

Upon digestion of the extracellular matrix hyaluronan-based scaffolding, the hyaladherins collapse on top on one another within the narrow channels of the ECS. The collapsed material obstructs the diffusing molecule (red), thereby requiring more time for it to traverse the channel. This increase in time is recapitulated by an increase in ECS tortuosity.
CHAPTER 4

Metabolic Stimulation of Naked Mole-rat Neurons with Exogenous Hyaluronan

Introduction

African naked mole-rats (Heterocephalus glaber; NM-R) require the capability to tolerate wide excursions in environmental conditions, from the low oxygen/high CO2 conditions they likely encounter in the burrow nest, to the normoxia and normal atmospheric CO2 concentrations they encounter near the burrow surface. Understanding the mechanisms which allow for this wide tolerance may provide insight into the prevention of neuronal cell death during stroke-induced hypoxia (Larson et al., 2014; Lewis et al., 2016; Xiao et al., 2017). Hypoxia leads to extracellular acidification and tissue death through several mechanisms involving ion exchangers including NHE1 (Detweiler, Vigil, Resta, Walker, and Jernigan, 2018; Dhaka et al., 2009; Sedmak et al., 2016; Svatová et al., 2004; Yingjun and Xun, 2013; Yu, Quinn, Garg, and Hales, 2008). pH regulation is an essential process for all mammalian cells, but it is of particular necessity in excitable cells where ion balance across the membrane plays a crucial role in establishing and maintaining membrane polarity. pH deregulation in neuronal tissue has been associated with ischemia (Xiong et al., 2004) and seizure (reviewed in (Obara, Szeliga, and Albrecht, 2008)), as well as neurodegenerative diseases such as AD, Parkinson’s disease, and Multiple Sclerosis (Demetrius, Magistretti, and Pellerin, 2014). While systemic factors, such as blood (Johansen et al., 1976), are modified in NM-R consistent with increased hypoxia tolerance, a number of studies demonstrate that the tolerance for hypoxia and hypercapnia-induced acidosis exists ex vivo (Larson and Park, 2009), even in isolated neurons (Husson and Smith, 2018).

Here we investigate the potential for the brain extracellular matrix (ECM), a system which is dynamically regulated by environmental conditions, to contribute to the environmental tolerance of NM-R neurons. Hypoxia is linked to increased ECM deposition and altered composition in fibrotic
breast tumors (reviewed in (Gilkes, Semenza, and Wirtz, 2014)) and osteoblasts (Markway, Cho, and Johnstone, 2013) through mechanisms involving the activation of HIF1α target genes. Work in human osteoblasts shows that, in addition to the increased expression of several genes associated with ECM components, hypoxia increases the expression of HAS2 (Markway et al., 2013). HAS2 is responsible for both the production of high molecular weight hyaluronic acid and its release into the extracellular space (ECS) (Toole, 2004).

The ECS is a highly complex environment with dozens if not hundreds of molecules that provide mechanical and chemical signals to parenchymal cells. The ECS volume fraction can rapidly change in response to hypoxia (Rice and Nicholson, 1991; Syková et al., 1994). Mice deficient for HA production are prone to epileptic seizure owing to decreased ECS volume and increased neuronal packing (Arranz et al., 2014). HA in the ECS has a number of roles including regulatory T-cell recruitment during the inflammatory response (reviewed in (Bollyky, Bogdani, Bollyky, Hull, and Wight, 2012)), angiogenesis (Ghose, Biswas, Datta, and Tyagi, 2018), and wound repair (Aytekin, Haserodt, Comhair, and Dweik, 2009; Jiang, Liang, and Noble, 2007). Interestingly, the molecular weight of HA in the ECS has a discrete impact on the effect of this molecule on parenchyma. Low molecular weight HA (LMW HA) promotes angiogenesis and acts as a pro-inflammatory signaling molecule that classically activates macrophages (Rayahin, Buhrman, Zhang, Koh, and Gemeinhart, 2015). Whereas high molecular weight HA (HMW-HA) provides increased viscosity in the ECS (summarized in (Cowman et al., 2015)), alternative macrophage activation (Rayahin et al., 2015) which can antagonize inflammation, increased clustering of the HA receptor CD44 (Yang et al., 2012), anti-angiogenic signals, and impedes differentiation in multiple tissues (Kujawa, Pechak, Fiszman, and Caplan, 1986; Passi et al., 2004).

HAS2 is a target gene of HIF1α. As such, an enhanced role for HA in the ECM of the NM-R is supported by previous work which has demonstrated high levels of active HIF1α in the NM-R compared to mice (Xiao et al., 2017), a mutated form of HAS2 that results in the constitutive production of a uniquely high molar mass species of HA (HMM-HA) (Tian et al., 2013), and high levels of HA compared with other rodents in a variety of tissues. Though there have been a number of groups to
suggest that high molecular weight HA is responsible for the observed cancer resistance in these animals, no one has yet to associate HMM-HA with neuroprotection.

To understand the capacity for ECM enhancement in controlling the extracellular environment, we investigated how HMW influences NM-R neuronal viability, as well as the dynamic regulation of HA in response to manipulation of extracellular pH by in vitro and ex vivo methods. We report that NM-Rs do not have an altered deposition pattern of HA in the brain when compared with mice as determined by alcian blue staining. Also, there is no significant difference in HA expression levels in formalin-fixed paraffin-embedded brain tissue (FFPE). We also report that HMM-HA alters buffering dynamics of artificial cerebrospinal fluid (ACSF). Acute manipulation of extracellular pH in slice culture or primary neuronal culture did not stimulate neurons to produce more HA in the time frame investigated here, however, we did find that addition of exogenous HA improved cell viability in vitro as determined by WST assay at neutral pH. Taken together, these data suggest that HA is not dynamically produced in NM-R neuronal tissue in response to an acute insult by pH manipulation, but that constitutively HMM-HA provides a stimulatory effect that likely sustains neurons through a wide range of insults.

Materials and Methods

Titration Experiment

Stock solutions of lyophilized Hyaluronan (R&D Systems) of different molecular weights were prepared using millipore-Q water as a solvent. Three molecular weights of HA were used to prepare stock solutions: ultra-low MW (4.8 kDa) at 1 mM, medium MW (289 kDa) at 10 µM, and HMW (1.35 MDa) at 1 µM. Stock solutions were stored at 4°C. Strong acid and strong base solutions were prepared at 1 N for Hydrochloric acid and Sodium Hydroxide respectively. For titration experiments using HA-H2O solutions, stock solutions for each respective MW of HA were diluted to 100 nM using millipore-Q water. The titrant was added 10 µl at a time until a total added volume of 500 µl was reached. The pH was measured (Hanna Instruments pH 210 pH meter) with each 10 µl addition to the solution which was in constant motion on a stir plate with a magnetic stir bar. HA-
ACSF solutions for the ultra-low and HMW forms of HA were prepared in similarly as the HA-H2O solutions, with artificial cerebrospinal fluid (126 mM \(NaCl\), 5 mM \(KCl\), 1.25 mM \(Na_3PO_4\), 2 mM \(MgSO_4\), 26 mM \(NaHCO_3\), 20 mM HEPES, 2 mM \(CaCl_2\), 10 mM D-Glucose; pH = 7.4) used as the solvent. For the titration experiments using HA-ACSF solutions, HA-ACSF solutions were bubbled with 95% Oxygen-5% CO2 as 25 µl titrant was added at a time. Titrant was added until each respective HA-ACSF solution shifted by at least 1 pH unit.

**pH slice experiment**

Adult naked mole-rats (\(N = 2\) animals; \(n = 18\) slices) were sacrificed using 1.25mg/kg Avertin according to IACUC approved handling practices. The brain was then removed and embedded in 5% agar ACSF. Coronal sections (400 µm) were made using a compressstome. Each section was then floated in a well of a 24 well plate containing 2 mL of ACSF as above at pH 4, 7, or 10. Slices were incubated for 60 minutes before removal for either Alcian blue staining or immunocytochemistry. Each pH condition was performed in triplicate (\(n = 9\) slices per animal).

**HA ELISA**

HA in conditioned ACSF was measured by ELISA as first described by Martins et. al. (Martins et al., 2003). Briefly, high binding 96 well plates (Fisher Scientific: 07-000-627) were coated with hyaluronic acid binding protein (HABP, 100uL, 20 ug/mL, Fisher Scientific: 38-591-0100UG) overnight. Conditioned ASCF (100 uL, collected from the pH slice experiment at the time points indicated) was incubated for 2 hours, followed by incubation with biotinylated HABP (100 uL, 1mg/mL, Fisher Scientific NC1087781). Streptavidin-conjugated 3,3’,5,5’-Tetramethylbenzidine (TMB) was then incubated for 1 hour, followed by TMB substrate. Enzymatic conversion of TMB substrate was allowed to continue for 30 minutes before quenching in quench buffer (10% acetic acid in phosphate-buffered saline, PBS). Absorbance was recorded at 450 nm.
Alcian blue staining

Staining was performed on brain tissue from mice (N = 3 animals) and naked mole-rats (N = 3 animals) following complete anesthesia with 1.25mg/kg Avertin and transcardial perfusion with 4% paraformaldehyde in PBS pH 7.4, except for the pH slice exposure studies. Some of the perfusion fixed brains were paraffin-embedded, sectioned coronally, mounted on lysine-coated slides, deparaffinized, rehydrated, and incubated with either PBS or hyaluronidase (2 units/mL; Fisher Scientific: ICN15127082) in PBS before staining. Tissue was stained with Alcian blue solution (1% Alcian blue 8GX in 3% acetic acid, pH 2.5) for 30 minutes at room temperature, as has been described (Tian et al., 2013). Sections (n = 6 for each mouse and NM-R undigested sections, n = 3 for each mouse and NM-R HYase-digested sections) were then washed running water for two minutes and then rinsed in distilled water. Sections were then dehydrated in an ethanol series, mounted in Permount mounting media with a coverslip.

Image Collection and Quantification of Alcian blue Staining

Alcian blue-stained tissue was imaged using a Leica Aperio slide scanning microscope. Color images were captured sequentially using identical lighting conditions and exposure times. For quantification of Alcian Blue staining of tissue exposed to a range of pH, the captured images were separated into corresponding channels of the Alcian blue stain using the color deconvolution plugin in ImageJ. Pixel intensities were measured from randomly selected regions of interest (n = 100 regions) using a custom macro within a manually selected region of flat and unfolded tissue. Intensity values were converted to an optical density metric using the following formula:

$$O.D. = -\log\left(\frac{255}{Mean\ Pixel\ Intensity}\right) .$$

Average O.D. was calculated for each slice from, and a two-tailed heteroscedastic t-test was used (after determining equality of variance using F-test) to determine statistical differences in HA expression between species. A similar analysis was performed between the digested and undigested samples for each species.

High-resolution imaging of Alcian Blue staining was achieved by measuring reflectance at 633 nm using Leica SP2 scanning confocal microscope (Glykys and Staley, 2015). The 633 nm channel
of the image was used to quantify HA patterning of the tissue using Fourier analysis. A randomly selected region of interest was selected from an area devoid of cells as identified using NeuroTrace. Candidate images were obtained by averaging the images within a stack for a given slice (n = 4 slices per species, with 3 candidate z-plane sections averaged together per slice). A 2D fast Fourier transform was applied to the averaged image (per slice). The resulting 2D Fourier transformed image was then adjusted so that low spatial frequencies were centered within the image before the 2D power spectrum. The azimuthally averaged 1D power spectrum was computed from the 2D power spectrum. Azimuthally averaged 1D power spectrums of the 4 slices (per species) were averaged together to represent a particular species. Image processing and manipulations were performed using the Fiji distribution of ImageJ2. Fourier analysis of confocal images was performed using custom-written Python scripts.

**Primary Cell Culture**

NM-Rs (N = 4 animals) ranging in age from 4-6 months were sacrificed according to IACUC approved standards. Whole brains were then removed, gently minced with 2 standard razor blades, and placed into a digestion buffer composed of Hibernate A (catalog no. A1247501, ThermoFisher Scientific), supplemented with GlutaMax™ (catalog no. 35050079, ThermoFisher Scientific), Penicillin-Streptomycin (Pen/Strep, catalog no. 15240062, ThermoFisher Scientific), and Papain (2 mg/mL, catalog no. NC0056304, Worthington Biochemical). Brain tissue was digested by shaking at 33°C for 30 minutes in 5 mL of digestion mix in a 15 ml Falcon tube. After shaking, tissue was further dissociated via pipette trituration. Visible chunks of undigested material were removed to a separate vial. The cell suspension was washed 2 times by pelleting at 200g for 5 min and full volume replacement with growth media (Hibernate A with Glutamax and Pen/Strep). Cells were then counted and seeded on poly-D-Lysine coated 96 well plates in growth media at a density of 7,000 - 10,000 cells/well. Cells were allowed to adhere for at least 24 hours in an incubator maintained at 5% CO₂ and 37°C before experimentation.
Flow cytometry

Adherent primary neuron culture of NM-R brain tissue was stained with NeuroTrace™ (NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain, catalog no. N21479, ThermoFisher Scientific), fixable viability dye (Fisher Scientific: 50-112-8846). Adherent cells were then fixed and permeabilized before being incubated with biotinylated HABP. Biotinylated HABP was visualized with Alexa 633-Streptavidin (Streptavidin, Alexa Fluor™ 633 conjugate, catalog no. S21375, ThermoFisher Scientific). Cells were then removed from culture using 0.5% trypsin solution, filtered, and analyzed using flow cytometry (BD Accuri C6 Flow Cytometer, BD Biosciences). Only cells positive for NeuroTrace were used for analysis. A minimum of 10,000 NeuroTrace positive cells was analyzed from each condition. Figures are displayed with identical axes depicting NeuroTrace staining on the x-axis and HABP staining on the Y-axis.

WST-1 Assay

WST-1 colorimetric assay reagent (CytoScan™ WST-1 Cell Cytotoxicity Assay, Cat. # 786-212, 786-857) was used to assess the metabolic activity of NM-R primary tissue culture treated with HAs of varying MW and/or pH-treated ACSF conditions. Briefly, the assay quantifies metabolic activity via the cleavage of tetrazolium salt, MTS, by mitochondrial dehydrogenases to form formazan in viable cells. The greater the number of viable, metabolically active cells, the greater the amount of formazan product produced following the addition of WST-1. After NM-R primary tissue cultures were given at least 24 hours for the cells to adhere in wells of 96-well plates, the culture media was exchanged for fresh pH-treated ACSF solution (ph 7 for the dose-response experiments, or appropriate pH-treated ACSF solutions ranging from pH 5 to pH 10). Exogenous HA of various MWs [ultra-low HA (4.8 kDa), medium (289 kDa), or high molecular weight HA (1.35 MDa)] were added from stock solutions of lyophilized HA commercially available from (vendor). Cultures were incubated with respective HA treatments for 6 hours prior to the exchanging the media with fresh pH 7-treated ACSF, and subsequent addition of the WST-1 reagent (10 uL per well, prepared as described in the reagent kit). WST-1 reagent was also added to wells contained just ACSF solution without cells as a
The reagent was incubated for 30 minutes prior to taking measurements on the microplate reader (Tecan Spark Multimode, Tecan Life Sciences). Absorbance measurements were taken at 450 nm, with a reference wavelength of 610 nm to adjust for background emission of the signal. Relative absorbance measures were calculated by normalizing experimental groups to control groups. For dose-response experiments, the experimental groups were individual concentrations of varying MW of HA and the control group(s) were the untreated (no HA) samples. For the metabolic activity measurements, the control groups were defined as the sample which did not receive HA treatment(s) in that particular pH experimental groups (i.e. for the samples incubated in the pH 5 ACSF solution, the control group consisted of the wells with no HA added and the ultra-low, medium, high MW treated groups were compared to this control group). For each 96-well plate, each treatment was run in at least triplicate. Multiple two-tailed, heteroscedastic, t-test comparisons were performed to compare metabolic pro-/anti- stimulatory effects of HA treatments for the dose-response experiments. The relative percentages of dose-responses (WST absorbance) were compared to the untreated samples (normalized to 100 %). For the pH-treated experiments, the average normalized metabolic activity percentage was computed for each ph-treatment of each HA group for each experiment. A one-way ANOVA with posthoc Tukey HSD test was calculated across all HA treatments (ultra-low, medium, and high) for each pH group to determine statistical differences between HA treatments on metabolic activity percentage.

Results

Interspecies comparison of brain HA expression

Previous work has demonstrated that the NM-R has high levels of HA compared with other vertebrates such as rat and guinea pig, in a variety of tissues (Tian et al., 2013). Additionally, Tian and colleagues also described a uniquely high molecular weight species of HA ranging in size from 2 to 6 MDa in the skin and ~0.8-1.3 MDa in the brain. Given our focus on the potential link between HA and neuronal cell viability during pH deregulation, we focused our attention on HA levels in the
brain of NM-Rs compared with mice, as depicted in Figure 4.1A. We found comparable levels of total HA between these two vertebrate models in all brain regions examined as determined by Alcian blue staining of thin sections from formalin-fixed paraffin-embedded (FFPE) brain tissue (mice: n = 4 sections, NM-R: n = 4 sections).

Alcian blue quantification was performed using the color deconvolution ImageJ plugin to separate histological signals into respective channels, followed by converting the mean intensity into an optical density measure. Optical density (O.D.) was computed using the following equation: $O.D. = -\log\left(\frac{255}{\text{Mean Pixel Intensity}}\right)$, where MaxIntensity for an 8-bit grayscale image is 255. Thus, the lower the mean intensity of the signal of interest, the higher the optical density (i.e. the higher the absorbance of light through the sample). There was no significant difference between the O.D. measures for mouse and NM-R sections (n = 4 each), depicted in Figure 4.1B (left); however, mice samples (n = 3 sections for each species) digested with hyaluronidase (ph20, MP Biomedicals™ Hyaluronidase, Bovine Testes, catalog no.ICN10074080, Fisher Scientific) resulted in a significant decrease in O.D. whereas the digested NM-R showed no significant difference, depicted in Fig 4.1B (right). Though this result was somewhat unexpected, we hypothesized that the high molecular mass of the NM-R HA, due to the constitutively active HAS2 mutation in the NM-R (Faulkes, Davies, Rossiter, and Bennett, 2015), maybe more important than the total amount of HA produced when considering the hypoxia adaptation of these animals. Also, perhaps the HMM-HA variant expressed in NM-R tissue is far more robust and able to withstand degradation compared to lower-molecular HA variants. To this end, Fourier analysis of confocal images of reflected Alcian blue staining was performed to determine if the patterning of HA differed between the two species, depicted by Figure 4.1C, D. There are no clear spatial oscillatory components that differ between the power spectrums of the two species across all images measured.

**Altered pH dynamics**

Hyaluronic acid, a weak acid, has an intrinsic pKa of approximately 3.0 (Brown and Jones, 2005). As such, we hypothesized that increasing the length of this polymer would affect pH buffer-
ing dynamics in the extracellular space. To investigate this possibility, we performed acid and base titrations in 100 nM solutions of HA at three molecular weights: ultra-low (4.8 kDa), medium MW (289 kDa) or high MW (1.34 MDa). Titrations were performed in either water (Figure 4.2A left and right) or artificial cerebrospinal fluid (ACSF) (Figure 4.2B left and right). We found that the molecular weight of HA had little effect on buffering at 100 nM in water. However, both ultra-low and high MW species of HA solutions resisted acidification by HCl addition in ACSF at (Figure 4.2B (left)), but sped alkalization by NaOH (Figure 4.2B (right)). At larger volumes of NaOH titrant, HMW HA species of HA resisted further alkalization hydroxide saturated solutions. It should be noted that ACSF contains sodium bicarbonate, and was constantly bubbled with 95% $O_2$ and 5% $CO_2$. These results indicate that the presence of HA in the extracellular space may help resist acidification resulting from $H^+$ ion accumulation, but this effect is likely not directly due to the molecular weight of the molecule, rather it may be attributed to the negative charge density present on HA.

**No observed increase in HA production in response to the pH manipulation**

We observed that HA could mitigate acidification in ACSF during acid titration. We, therefore, hypothesized that altering pH, especially in the acidic direction, would induce increased production of HA in neuronal tissue as a protective response. To investigate this possibility, we incubated acute slices (400 um) of NM-R brain in ACSF at either pH 4, 7, or 10 maintained at room temperature. The slices were incubated in their respective treatments without being oxygenated to induce a hypoxic stress event. We looked for HA in the conditioned ACSF by ELISA after 20, 40, or 60 minutes of incubation as previously described (Martins et al. 2003) (Figure 4.3A). HA levels could be reliably detected under all experimental conditions. We did not detect, however, any significant difference in HA release between the pHs investigated. We further investigated HA levels in the slice itself by Alcian blue staining (Figure 4.3D, E) and found that HA was no significant difference in optical density across all pH conditions. Mechanical dissociation and immunocytochemistry revealed reduced HA in both acidic and alkaline pH conditions and increased cell death in tissue incubated with acidic ACSF (Figure 4.3B). It has recently been reported in that rat neurons express HAS1-3
and can produce HA directly (Fowke et al. 2017). To determine if NM-R neurons increase HA production in response to altered extracellular pH, we cultured primary NM-R neurons for 24 hours before incubating them in either acidic (pH 5), neutral (pH 7), or alkaline (pH 9) ACSF for 90 minutes. Neurons were labeled with NeuroTrace and a viability dye before being lifted from culture, permeabilized, and stained for HA. We analyzed 10,000 NeuroTrace positive cells in each condition by flow cytometry and found no significant increase in cells positive for HA staining as a result of incubation in either acidic or alkaline ACSF (Figure 4.3C).

HA induces enhanced metabolic output in primary neuronal culture as determined by WST assay.

Having determined that altered pH does not increase HA production in neurons or neuronal tissue within 60 minutes of exposure, we hypothesized that the MW of HA could have variable effects on the metabolic output of primary NM-R neurons. Others have reported that varying the length of HA polymer alters the extent to which the HA receptor CD44 clusters (Yang et al., 2012) and cell behavior (Fuchs et al., 2013; Saad, Maria, and David, 2016). To quantify the metabolic output of NM-R primary brain tissue cultures, a WST-1 assay was used and absorbance values were normalized to the control groups to yield a relative percentage value. When comparing the metabolic activity of NM-R cultured cells between different pH conditions ranging from a pH 5 to 10 (Figure 4.4A), there was no significant difference between the relative absorbance values (as compared to pH 7 control group).

We then tested various concentrations of either ultra-low HA (4.8kDa), medium (289kDa), or high molecular weight HA (1.35MDa) on primary neuronal viability. We chose to use a molecular weight for our HMM-HA that was in the range of the high MW HA collected from brain tissue as demonstrated by Tian et al. (Tian et al., 2013). Initially, we found that adding exogenous HA to NM-R primary neurons significantly increased metabolic activity (relative to samples not treated with HA) at higher concentrations (50 nM-100 nM), regardless of HA MW, as determined by WST assay in a dose-dependent manner (Figure 4.4 B-E). However, the high MW-HA dose-response curve did
not plateau at the concentrations used, thus an extended dose-response (Figure 4.4E) experiment was conducted to determine at which concentration does the stimulatory effect plateau. Interestingly, the extended dose-response yielded confounding results. The ultra-low MW HA treatment significantly reduced the metabolic activity of the cultured samples across all concentrations tested. There were no significant changes in the metabolic activity of the samples treated with medium MW HA. Each concentration of high MW-HA used significantly increased the metabolic activity; however, the effect plateaued at 1000 nM.

To determine if HA confers a metabolic stimulatory effect under pH stress, thereby improving cell viability, we used a working concentration of 100 nM for each of HA MWs used (ultra-low, medium, and high) which was added to the pH-treated ACSF culture media. Relative absorbance values were calculated by comparing experimental treatment with control samples where no HA was added under the same pH condition. There was no difference in metabolic output, as determined by the WST-1 assay, across all pH groups tested and MWs of HA used (Figure 4.4F).

**Discussion**

The goal of the present study was to determine whether high molecular weight HA, previously identified in the NM-R brain, has neuroprotective properties. Here we measure neuroprotection as improved cell viability in acidic/basic pH conditions, compared to neutral pH conditions. Prior work by Tian and colleagues demonstrated that not only was the NM-R HMM-HA variant was significantly larger compared to other species, but it was also present in several tissue types including the brain (Tian et al., 2013). Here, we expand on this finding by performing immunohistochemical analysis of HA expression between mice and NM-R brain tissue. The overall HA expression levels and distribution did not seem to vary between mice and NM-R brain tissue. Also, Fourier analysis of the HA expression spatial frequency did yield any clear differences in oscillatory components between the two species. Although, a qualitative analysis indicated that there were differences in HA expression between various NM-R cortical layers, and hippocampal regions. Further quantitative analysis is required to reliably characterize these differences.
Given that there appears to be a ubiquitous expression of HA in both mouse and NM-R brain tissue, and that the NM-R variant is significantly larger, it is plausible that not all the candidate sites of HA molecules are being labeled by Alcian blue. The efficacy of Alcian blue staining of HA requires titrating the dye-solution to a pH of ~2.5. This allows for the polyvalent dye to bind to the negatively charged domains present on HA polymer. Recent work by (Kulaberoglu et al., 2019) utilized scanning electron microscopy to study the ultrastructure of the HMM-HA extracted from NM-R brain tissue. These micrographs indicate that these HMM-HA molecules have a voluminous, cloud-like appearance. This was vastly different from HA extracted from mice, which occupied less volume, had a flattened network configuration. Due to this unique conformation, it is conceivable that the polyvalent ions of the Alcian blue are not able to access the deeper portions of an HMM-HA chain. Conversely, this would also explain how “lower” molecular weight HA would be labeled readily by Alcian blue since a greater portion of the negatively-charged domains is exposed.

Also, our analysis demonstrated that NM-R HMM-HA was significantly more robust, showing no significant decrease in Alcian blue signal after hyaluronidase digestion. This finding was also seen with immunohistochemical analysis of bacterial hyaluronidase digestion of normoxic NM-R cortical slices (acute slices were post-fixed with 4% paraformaldehyde) using HABP as the HA marker. The digested product HMM-HA may be sufficiently large enough to be labeled by the HABP and Alcian blue, i.e. these markers do not discriminate between the resulting size of HA, after digestion.

The fixed negative charge density on HA molecules posed as an attractive potential mechanism for buffering against pH shifts in aqueous solutions. The addition of the HA to bicarbonate-based ACSF resisted acidification; however, did not show any improvements in buffering against alkalinization at a physiologically-relevant pH range. The blunted acidification response can be attributed to the bicarbonate-$CO_2$ buffering component present in the ACSF; however, it should be noted that the addition of HA (of any size), slightly alkalized the solution,$H^+$ “sponge”. Upon the addition of larger volumes of a strong base, ACSF supplemented with a high molecular weight HA solution showed a remarkable resistance to further alkalinization, outside a physiologically-relevant
pH range (above 8.2). Biochemical characterization of HA by Maleki and colleagues, demonstrates that the degradation of HA occurs at pH<4.0 and pH>11, and within this range, HA molecules are quite robust showing no polymer disruption (Maleki, Kjøniksen, and Nyström, 2008). There may be an additional charge interaction component between the negatively charged domains of the HA molecule and the other ions present in the ACSF. A one-off experimental paradigm, where a similar titration experiment could be performed with one component of the ACSF formulation removed to determine which ions, in particular, facilitate the aforementioned charged interaction.

Acute manipulation of extracellular pH in slice culture or primary neuronal culture did not stimulate neurons to produce more HA in the time frame investigated here. The addition of exogenous HA increased the net metabolic output as determined by the WST assay in a dose-dependent fashion. However, there was no discernible improvement in net metabolic output between the sizes of HA and pH conditions tested. Work by Solis and colleagues showed that Human placenta-derived mesenchymal stem cells (PDMSC) cultured on HMW-HA-coated surfaces promoted mitochondrial biogenesis which led to a hyaluronan dose-dependent increase in ATP content, oxygen consumption rate, and mitochondrial membrane potential (Solis et al., 2016). Also, there was a significant reduction in reactive oxygen species. Thus, HMM-HA might serve as an additional source of energy production, supplementing the proposed fructose-driven glycolytic pathway described by Park and colleagues for the NM-R brain (Park et al., 2017). This highlights HA’s role as a signaling molecule, specifically differentially activating CD44/RHAMM receptors based on variations in HA size. HA, however, seems to have dual-functions in signaling mechanisms involved with inflammation. For example, the presence of CD44 is involved in the initiation of arthritis, while its absence (via genetic deletion) in an arthritis mouse model increases rather than decreases disease severity. Similar dual functions of CD44 exist in the initiation and progression of cancer (Misra et al., 2003).

It is also conceivable that rather than exerting its influence as an extracellular ligand, HA may be internalized and subsequently interact with intracellular signaling mechanisms. Previous work by (Hua, Knudson, and Knudson, 1993) shows evidence of HA internalization mediated by receptor-mediated endocytosis, specifically by CD44-HA interactions, in chondrocytes. Intracellular accumu-
lation of HA has been observed in aortic smooth muscle cells during pre-mitotic and mitotic stages (Evanko, Angello, and Wight, 1999; Hascall et al., 2004). This supports earlier work showing an elevated synthesis of HA in G2/M stages of the cell cycle in fibroblasts (Brecht, Mayer, Schlosser, and Prehm, 1986). Moreover, living smooth muscle cells treated with hyaluronidase to remove pericellular HA, prevented internalization of HA. This abolished most of the intracellular HA. This suggests that a portion of intracellular HA may be derived from its uptake and translocation into intracellular compartments (Hascall et al., 2004). Taken together, we suggest that, in addition to this cell-intrinsic adaptation of using a fructose-driven glycolytic pathway during anoxia, the NM-R potentially possesses a cell-extrinsic component in the form of HMM-HA that directly promotes a metabolic stimulatory effect, which improves cell viability across wide excursions in extracellular pH.
Figure 4.1: HA staining in mouse and NM-R FFPE brain sections indicates no difference in HA content and similar staining pattern compared to the NM-R.

A) Alcian blue staining of FFPE brain sections from mouse and NM-R. B) (Top) Alcian blue signal between undigested samples of each species as determined by optical density. No significant differences were found between the two species (unpaired t-test; p > 0.05). (Bottom) Alcian blue signal comparison between digested (Hyase) and undigested (Ctrl) samples for both species. Digested mouse sections have a significantly lower O.D. measure, compared to undigested control (Mean ± SEM; unpaired t-test; p = 0.000186807). C) (Left) Confocal microscopy of FFPE brain sections from mouse and NM-R dentate gyrus. Images were captured by reflecting a laser at 633nm off of Alcian Blue stained brain tissue. (Right, top) Sample region of interest used in Fourier analysis. (Right, bottom) The 2D power spectrum of the transformed image with low spatial frequencies centered. D) 1D power spectrum of averaged sections (n = 4 per species) depicting the decomposition of discrete spatial frequencies extracted from the candidate image.
Figure 4.2: HA molecular weight affects pH buffering capability

A) 100 nM solutions of low molecular weight (4.8kDa) medium (289kDa) and high (1.35MDa) HA were prepared in water. (Left) Each solution was titrated with 1N HCL in 10 μL increments until 500 μL HCl was added. (Right) Solutions as in left panel were titrated with 1N NaOH in 10 μL increments until 500 μL HCl was added. B) ACSF was prepared containing 100 nM of either low molecular weight (4.8kDa) or high (1.35MDa) HA. (Left) ACSF-HA solutions were then titrated with 1N HCL in 25 μL increments until 5 mL total volume was added. (Right) Titration was performed as in left panel, but with the addition of 1N NaOH.
Figure 4.3: Manipulating extracellular pH does not increase HA production

A) ELISA for detecting HA in conditioned ACSF under the time points indicated following addition to brain slices (n = 3 per pH treatment). There were no significant differences between detectable HA content from conditioned media. Markers indicate Mean ± SEM; repeated measure, one-way ANOVA with Geisser-Greenhouse correction and Tukey HSD posthoc comparisons. B) IHC staining of mechanically dissociated brain tissue from panel A after 60 minutes of incubation with ACSF at the pHs indicated. Mechanically dissociated tissue was labeled with Streptavidin-conjugated HABP for Hyaluronan (red), viability dye (blue if dead), and NeuroTrace (green). C) (Left) Flow cytometry of cultured brain tissue from panel A. At least 10,000 NeuroTrace positive cells were analyzed for HA production. Cultured cells were incubated with ACSF at the pHs indicated for 90 minutes. Turquoise box gates area of interest counted cells which most likely express HABP and NeuroTrace markers. (Right) IHC staining of primary tissue culture; samples were labeled with viability dye (red; dead cells) and NeuroTrace (green). D) Alcian blue staining of brain slices from panel A collected 60 minutes after the addition of ACSF at the pHs indicated. E) Alcian blue signal of slices from Panel (D) (n = 3 for pH 5 and ph 10 treatments; n = 5 for ph 7 treatment). Markers indicate Mean ± SEM; One-way ANOVA and Tukey HSD posthoc comparisons were performed; no significant differences were found.
**Figure 4.4:** Exogenous HA alters metabolic output off brain tissue in culture.

All graph markers quantify Mean ± SEM. Metabolic activity of primary brain tissue cultures was determined by WST assay. Primary NM-R neuronal cell cultures were established for 24hrs before being incubated in HEPES buffered ACSF and/or pH-treated HEPES buffered ACSF. Incubation time was 6 hrs. The media was then replaced with neutral HEPES buffered ACSF without HA along with the WST reagent. Absorbance was recorded 30min after the addition of WST. Panel A: Primary NM-R brain tissue culture was established and incubated in HEPES buffered ACSF at the pHs. WST conversion was normalized to pH 7 control sample. Panel B-C: Primary NM-R brain tissue culture was established and incubated in HEPES buffered ACSF pH 7 to determine dose-response interaction. Panel D: Extended Dose-response interaction represented for all three HA treatments. The effect of High MW HA plateau at 1000 nM before dropping. Unpaired t-tests were used to compare experimental groups (dose) to No HA (control) treatment; * p < 0.05. Panel F: Comparison of relative metabolic stimulatory effects of exogenous HA in different pH conditions. WST conversion was normalized to No HA treatment groups of each respective pH. No statistical difference between percentages (when compared to No HA group control for a given pH). One-way ANOVA with Tukey HSD posthoc test was used to compare each HA treatment within each pH group.
Figure 4.5: Extended dose-response curves

All graph markers quantify Mean ± SEM. Metabolic activity of primary brain tissue cultures was determined by WST assay. Primary NM-R neuronal cell cultures were established for 24 hrs before being incubated in pH-treated HEPES buffered ACSF. Incubation time was 6 hrs. The media was then replaced with neutral HEPES buffered ACSF without HA along with the WST reagent. Absorbance was recorded 30 min after the addition of WST. Panel A: The effect of High MW HA plateau at 1000 nM before dropping. Panel B: No significant difference between Medium MW HA doses when compared to No HA group. Panel C: All ultra-low MW HA doses resulted in a reduction of metabolic activity when compared to the untreated control group. Unpaired t-tests were used to compare experimental groups (dose) to No HA (control) treatment; * p < 0.05; ** p < 0.005; *** p < 0.0005.
CHAPTER 5

General Discussion and Concluding Remarks

The intracellular and extracellular compartment of the brain microenvironment responds to critical cues provided by the external environment, such as oxygen content, in order to establish brain homeostasis. The brain ECS serves as the conduit for the transmission of information regarding the status of the microenvironment. Thus, the ECS orchestrates communication between the intracellular and extracellular compartments. The brain ECM serves to support the ECS by influencing the parameters of diffusion, which can either facilitate or hinder the homeostatic function of the ECS. This body of research demonstrates the instructive nature of the brain ECM, in particular how HA can influence both the extracellular and intracellular compartments of the microenvironment. The fossorial NM-R utilizes a number of remarkable physiological adaptations which allow this eusocial species to tolerate and thrive in sustained hypoxic-hypercapnic environments. It has been previously demonstrated that a number of tissue types harbor a unique HMM-HA variant, which is substantially larger than that found in other species. Thus, the NM-R serves as a unique animal model with which to explore the functional implications of an HMM-HA based brain ECM on the diffusion of large macromolecules and cell viability.

The body of research demonstrates that the ECM specifically modulates the ease with which molecules diffuse through the ECS. Specifically, the NM-R ECS, which harbors HMM-HA, increases the tortuosity of molecular diffusion. The digestion of HA further increases the tortuosity. HA molecules form porous, layered, networks that possess a high hydration capacity. Notably, high-molecular-weight hyaluronan (HMW-HA) in a dilute saline solution occupies a large space where the mass of hyaluronan itself is approximate 0.1% of the volume, and the solvent occupies the remaining space. This confers HMW-HA the ability to occupy large spaces and maintain the structural integrity of the tissue. This set of findings suggest that perhaps NM-R HMM-HA occupies a larger portion
of the ECS, compared to other species, which is accompanied by an increase in tortuosity due to a reduction in the volume fraction. HA also serves as a scaffold for other ECM components, including proteoglycans. The digestion of HA could conceivably compromise this scaffolding, resulting in the collapse of the linked ECM components into the ECS. This would further reduce the available volume fraction, resulting in an additional increase in tortuosity.

Using a thick-slice preparation to simulate a focal ischemic insult, NM-R brain tissue was remarkably resilient to hypoxia as demonstrated by no significant change in tortuosity values compared to normoxic tissue. However, by presenting the NM-R brain with an osmotic stress event to induce cellular swelling, the tortuosity values significantly increased. This suggests the NM-R’s resiliency to hypoxia is independent of the effects of cellular swelling. Oxygen deprivation causes a switch to the anaerobic metabolic pathway(s) which ultimately reduce the net ATP content of the cell. This reduction in ATP disrupts the concentration gradient of ions maintained by active transport processes. This disruption in ionic equilibrium results in a shift of water content from the extracellular to the intracellular domain. This ultimately leads to shrinkage of the ECS, as water is transported into the intracellular compartment, thereby increasing the hindrance to diffusion. The resiliency of the NM-R brain to ischemic insult, but not to hypo-osmotic stress, suggests that there is an additional metabolic mechanism(s) preventing this disruption of ionic concentration gradients.

The remainder of this body of work explored how HA’s size may contribute to this postulated metabolic mechanism. To this end, we stained for HA in mouse and NM-R brain sections, determined the effect of HA on the buffering capacity of ACSF, looked for increased HA production in response to acute pH manipulation in both slice and primary neuronal culture, and measured viability in primary NM-R neuronal cultures with HA at a variety of molecular weights. The naked mole-rat has a known tolerance to hypoxia and has been reported to have constitutively high levels of HIF1α (Xiao et al., 2017). Among other ECM components, HAS2 is a target gene of HIF1α and is becoming increasingly interesting in NM-R biology because of the uniquely high molecular weight HA (HMM-HA) that this enzyme produced in the NM-R. HMM-HA has been suggested to be responsible for this animal’s long life and cancer resistance (Tian et al., 2013). Additionally, HAS expression
has been linked to proliferation in fibroblasts (Brecht et al., 1986) and smooth muscle cells (Evanko et al., 1999). Given the involvement of HA in these various fundamental processes, it is tempting to speculate that the uniquely high molecular weight of the HA found in the NM-R, is causally related to this animal’s special biological properties, i.e. long life, cancer resistance, and hypoxia tolerance.

Here we found that NM-R brain tissue did not appear to differ in HA expression levels compared to mice brain tissue. There was also no apparent differences in the distribution of HA expression between the two species. Given the reported effect of high molecular weight HA to be anti-inflammatory (Rayahin et al., 2015), and highly viscous (thus increasing the volume fraction of the ECS) it became an attractive target of our attention to be investigated as a neuroprotectant and mitigator of the observed hypoxia tolerance of these animals (Larson et al., 2014). Given the observed acidification in glial cells (Sloan and Barres, 2014) and brain tissue as a result of oxygen deprivation (Goldman, Pulsinelli, Clarke, Kraig, and Plum, 1989; Orlowski, Chappell, Park, Grau, and Payne, 2011; Rehncrona, 1985), as well as the reported resistance of NM-R neurons to acid-induced death (Husson and Smith, 2018) we investigated the effect of HA on buffering capacity of ACSF under physiological conditions. We observed that the addition of HA resisted acidification of ACSF, however, this effect was irrespective of molecular weight. Given this observation, we attempted to determine if acute pH manipulation in slice culture, or in primary neuron culture, would induce an increase in HA production. We did not see any such increase in production in either slice culture or primary neuronal culture.

Aside from the role of HA in maintaining a healthy volume fraction in the ECS, other work in both neuronal and non-neuronal systems has demonstrated unique cell responses in culture to HMM-HA (Brecht et al., 1986; Dzwonek and Wileczynski, 2015; Fuchs et al., 2013; Hu et al., 2018; Itano et al., 2002; Nam, Oh, Lee, Yoo, and Shin, 2015; Rayahin et al., 2015; Tian et al., 2013). To directly assess the potential of HMM-HA to exert a neuroprotective effect, we cultured primary NM-R neurons and assessed viability by WST salt conversion. Interestingly, we found the addition of exogenous HA to culture media increased net metabolic output in a dose-dependent manner as measured by the WST-1 assay. In particular, the incremental increase in HMW-HA dose consistently increased
WST-1 assay absorbance signal, indicative in increased metabolic output. However, there was no discernible improvement in net metabolic output between the sizes of HA and pH conditions tested. In fact, NM-R primary brain tissue cultures were remarkably resilient to wide pH, showing no significant differences in net metabolic output across the pH conditions tested.

Taken together, the NM-R provides a unique opportunity to explore the interaction of ECM composition, with the intracellular and extracellular dynamic processes that tend toward maintaining brain microenvironment homeostasis. This homeostatic function not only demonstrates how the external environment exerts its influence on the brain microenvironment, but it also highlights the instructive potential of the ECM in healthy and diseased states.
## APPENDICES

### Commonly Used Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>ACSF</td>
<td>Artificial Cerbrospinal Fluid</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CS</td>
<td>Chondroitin Sulfate</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>ECS</td>
<td>Extracellular Space</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<td>GlcUA</td>
<td>Glucuronic acid</td>
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<tr>
<td>HA</td>
<td>Hyaluronic Acid</td>
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<tr>
<td>HABP</td>
<td>Hyaluronic Acid Binding Protein</td>
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<tr>
<td>HARE</td>
<td>Hyaluronan Receptor for Endocytosis</td>
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<tr>
<td>HMM-HA</td>
<td>High-Molecular-Mass Hyaluronan</td>
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<td>HMW-HA</td>
<td>High-Molecular-Weight Hyaluronan</td>
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<td>HYase</td>
<td>Hyaluronidase</td>
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<tr>
<td>IOI</td>
<td>Integrative Optical Imaging</td>
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<td>LWM-HA</td>
<td>Low-Molecular-Weight Hyaluronan</td>
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<td>MWM-HA</td>
<td>Medium-Molecular-Weight Hyaluronan</td>
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<tr>
<td>NM-R</td>
<td>Naked Mole-rat</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PNN</td>
<td>Perineuronal Nets</td>
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<tr>
<td>RTI</td>
<td>Real-Time Iontophoresis</td>
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<tr>
<td>TMA</td>
<td>Tetramethylammonium</td>
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REFERENCES


References


Lewis, K. N., Soifer, I., Melamud, E., Roy, M., McIsaac, R. S., Hibbs, M., and Buffenstein, R.


neurons, perineuronal nets and experience-dependent plasticity in murine barrel cortex. *European Journal of Neuroscience.*


Saad, K., Maria, F., and David, C. (2016). Effect of glucose levels on high versus low molecular weight hyaluronan and transfection of bone marrow derived human mesenchymal stromal cells. *Frontiers in Bioengineering and Biotechnology*, **4**.


Sherman, L. S., Matsumoto, S., Su, W., Srivastava, T., and Back, S. A. (2015). Hyaluronan synthesis,


Svastová, E., Hulíková, A., Rafajová, M., Zat’ovicová, M., Gibadulinová, A., Casini, A., … Pas-


