ELECTRO-STIMULATION, A PROMISING THERAPEUTIC TREATMENT MODALITY FOR TISSUE REPAIR: Emerging roles of sulphated glycosaminoglycans as electro-regulatory mediators of intrinsic repair processes.

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Abbreviations used

AD	Alzheimer's diseas
AkT	a serine/threonine-specific protein kinase, protein kinase B
ALS	Amyotrophic lateral sclerosis
AT	antithrombin
BMP	bone morphogenetic protein
CCL2	C-C Motif Chemokine Ligand 2
CNS/PNS	central nervous system/peripheral nervous system
Cs	chitosan
CS	chondroitin sulphate
DS	dermatan sulphate
ECM	Extracellular matrix
ERM	condensed term based on the first initial of a family of three highly related cytoskeletal proteins, **E**zrin, **R**adixin, ***M***oeisin,
FGF	fibroblast growth factor
GAG	Glycosaminoglycan
HA	hyaluronic acid, hyaluronan
HS	heparan sulphate
IHH	indian hedgehog
KS	keratan sulphate
mAb	monoclonal antibody
Mef2c	MADS box transcription enhancer factor 2. MADS is a conserved motif in a family of MADS box transcription factors. MADS is an acronym derived from the first initial from four founding members of this group, **M**CM1 from *Saccharomyces cerevisiae*, **A**GAMOUS from the thale cress *Arabidopsis thaliana,* **D**EFICIENS from the snapdragon *Antirrhinum majus and* **S**RF from *Homo sapiens*.
NHERF-1	Na^+/H^+ exchanger regulatory factor 1
PDGF	platelet derived growth factor
PDZ	a term derived from the first initials of post-synaptic density protein (PSD95), Drosophila disc large tumour suppressor protein (Dig1), and zona occludens-1 protein.
PEDOT /PSS	poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)
PG	proteoglycan
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
VEGF	vascular endothelial cell growth factor
Sox	SRY-related high mobility group (HMG)-box genes
Wnt	a condensation term derived from the terms for the *Winged* and *Int* genes

Abstract

Glycosaminoglycan's (GAGs) are a family of diverse biomolecules that decorate proteoglycans (PGs) in the glycocalyx and extracellular matrix (ECM) of all cells. They exist as linear polysaccharide chains consisting of repeating disaccharide units that can be variably sulphated and carboxylated along their GAG chain length. These ionisable carboxyl and sulphate groups on GAGs create charged interactive motifs that convey cell regulatory properties important in tissue homeostasis and the maintenance of optimal tissue function. GAGs participate in a number of essential physiological processes including coagulation-fibrinolysis, matrix assembly and stabilization, immune regulation and the complement system. The high fixed charge density and the counter-ions of GAGs is central to their role in the hydration of various connective tissues within the body. Charge transfer properties of GAGs makes them amenable to electro-stimulation and offers a potential mechanism for promoting or enhancing cellular tissue repair processes. This review was undertaken to illustrate these properties and to gain a better understanding of how these processes might be manipulated through electro-stimulation to help improve tissue repair and the recovery of normal function in traumatized tissues. Weight-bearing and tension-bearing, collagen-rich, avascular tissues have intrinsically poor repair properties and represent difficult clinical challenges. Electro-stimulation represents a novel approach with significant potential in the stimulation of repair in these most intransigent of tissues.

1. Introduction.

Glycosaminoglycans (GAGs) are a diverse and ancient family of charged biopolymers that are sulphated at various positions on their sugar rings and these groups are presented in a range of planar spatial orientations [1, 2]. These sulphate groups are important functional determinants in GAGs and they have variable distributions and densities in tissues conveying a range of functional attributes including an ability to act as an electrical conduit from the extracellular matrix (ECM) to the cell during electro-stimulatory applications. Sulphate groups are relatively bulky space-filling groups on GAGs so it was important for all spatial orientations to be explored during natural evolutionary selection in order to optimise GAG interactive potential and to cover all co-operative permutations. Natural evolutionary selection forces thus explored many possibilities in order to uncover structures with optimal cellular mediator capability. Sulphate groups convey interactive molecular recognition and information transfer properties to GAGs, facilitating extracellular interactions with growth factors, receptors, morphogens, extracellular matrix components, proteases and protease inhibitory proteins that regulate cell signaling and many important physiological processes in tissue morphogenesis and skeletogenesis [1, 3, 4]. Knockout GAG-deficient mice have been used to examine the roles of GAGs in human genetic disorders and elucidate their roles in health and disease [5, 6]. Molecular bioinformatics studies have confirmed the important inter-relationship between GAG structures as biomarkers of a number of degenerative processes confirming their functional status in these diseases [4, 5]. The inherent charge transfer and storage properties of GAGs is in reality a bioinformatics "glyco-code" network and a directive language which cells can interpret. This code equips GAGs with sophisticated cell mediatory properties which, in combination with their stereoselective 3D interactive properties at the chemical level, makes them important regulatory entities on cell surface and ECM proteoglycans (PGs) with key roles in the maintenance of optimal tissue function and matrix homeostasis.

1.1 Glycosaminoglycans as in-situ bioinformatics regulatory modules

GAGs have evolved under positive evolutionary selection pressure over several hundred million years of evolution as cellular mediators with molecular recognition and information transfer properties [2]. The fact that GAGs are still employed in essential life processes in a relatively unchanged form in the present day in the regulation of a wide variety of cellular behaviours during tissue development, remodeling and repair testifies to their enormous functional capability [7] and the perfection in their structural form which has evolved by natural selection forces over the millennia [2]. The course of natural selection for such interactive modules was not a facile process and had rigorous inclusion criteria, HS and CS for example, require the co-ordinated activity of 24 or 12 GAG biosynthetic enzymes respectively. That cells should invest so heavily in such sophisticated genetic machinery with the accompanying metabolic energy demands this entails underlies the functional significance of these molecules. Advances in computational algorithms, carbohydrate analytic software and discriminative techniques including neutron scattering and electron detachment dissociation and online analytic tools have reached a level of sophistication that now provide important predictive capability in the elucidation of the atomistic contributions, effects of variations in conformational structure and the protein recognition and interactive properties of specific GAG sequences [8].

1.2 Glycosaminoglycan biodiversity

Five classes of GAGs have been identified on the basis of their repeat disaccharide and linkage structures to PG core proteins and their glycosidic structures overall. Details of the structural organization of these GAGs are shown in Figures 1-4 emphasizing the distributions of the charged groups distributed throughout these structures. Hyaluronan (HA), a copolymer of GlcNAc and GlcA is the simplest GAG and is unsulphated however contains ionisable carboxyl groups on its glucuronic acid components (Figure 1a, Figure 2d).

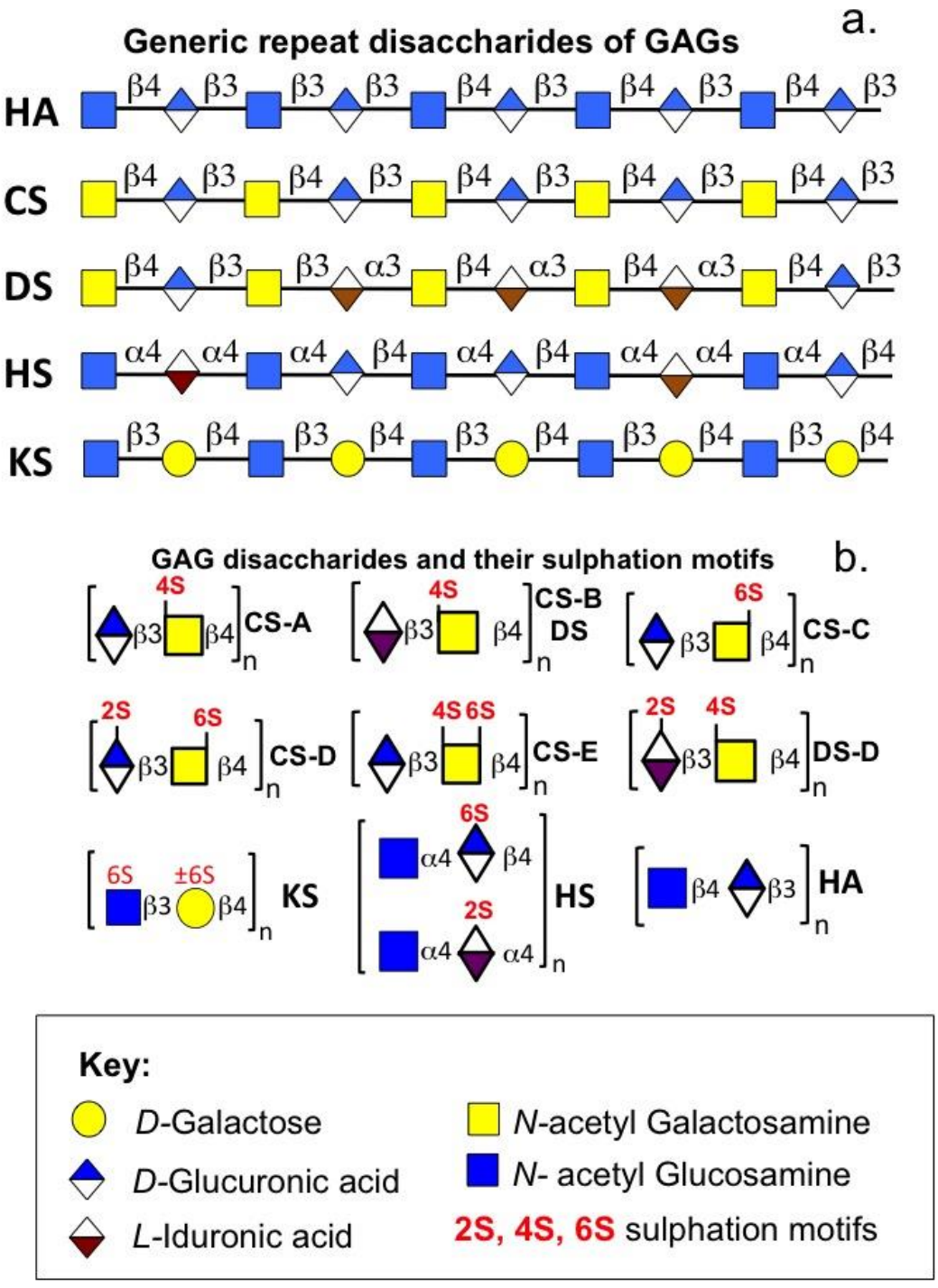

Figure 1. The five classes of glycosaminoglycans (GAGs), Hyaluronan (HA), Chondroitin sulphate (CS), Dermatan sulphate (DS), Heparan sulphate (HS), and Keratan Sulphate (KS) and their constituent glycosidically linked monosaccharides (a) and the characteristic repeat disaccharides of each GAG type and of the CS isomers and their sulphate positions (b).

Heparan sulphate (HS) is composed of the same repeat disaccharides as HA but these residues are modified by sulphation and acetylation like in heparin (Figure 1a, Figure 2a). After heparin, HS is the most heterogeneous and highly charged GAG [9]. HS has a complex

structure containing regions of high sulphation (S domains), high acetylation (A domains) and less modified regions along the HS chain (Figure 3a, b). Around 50% of the disaccharides in HS are GlcA linked to GlcNAc while in heparin, IdoA(2S)-GlcNS(6S) disaccharides make up 75-85% of its total disaccharides (Figure 3a, b).

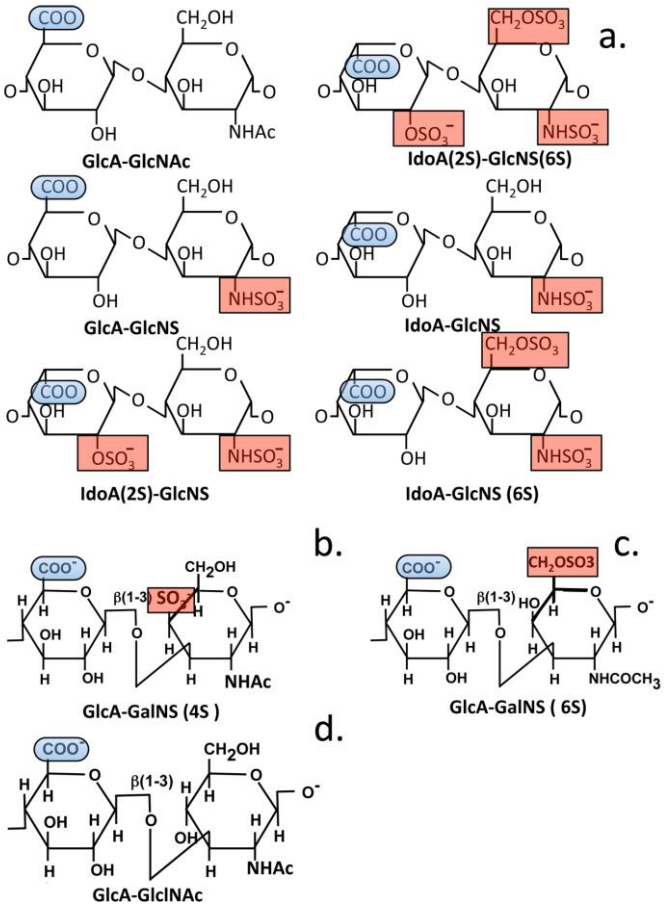

Figure 2. Haworth projections of the major disaccharides of HS, CS and HA. Of these GAGs, HS is the most heterogeneous and most highly charged, the rare 3-O sulphated Glucosamine (GlcNS(3S, 6S) disaccharides of HS or those containing the free amine group (GlcNH3+) are not shown (a). The repeat disaccharides of Chondroitin-4 sulphate (b), Chondroitin-6-sulphate (c) and hyaluronan are also shown (d). The generic GlcA-GlcNAc disaccharide can be modified with sulphation, indicated by pink shading in the structures shown, HA is non-sulphated (d). Glucuronic acid carboxylate groups are indicated in blue shading. Acetylation and epimerization of the GlcA to IdoA to form DS can also occur, these are not shown. Brief perusal of these structures clearly shows the broad distribution of ionisable groups they carry, these are potential motifs that can carry electrical charge to cells.

Keratan sulphate (KS) is also a heterogeneous GAG and has been categorised into four sub-types on the basis of its linkage region to PG core proteins and internal structural organisation [10] (Figure 3c-f). Corneal KS-I is found in many cartilaginous and non-cartilaginous tissues (Figure 3c), KS-II, skeletal KS is found in tensional and weight bearing tissues (Figure 3d, e) and KS-III is a form of KS found in the PNS/CNS [11] (Figure 3f). KS assembly occurs by

additions of GlcNAc and Gal to the PG linkage region acceptor module by specific glycosyl transferases producing sequential additions of GlcNAc and Gal in a polylactosamine chain which undergoes sulphation at C6 by a family of sulphotransferases [10, 11]. This assembly process by glycosyl transferases and sulphotransferases is undertaken in a co-ordinated manner to form monosubstituted regions in the nascent KS chain, disulphated regions where Gal and GlcNAc are both substituted at C6 also occur towards the non-reducing terminus of the KS chain. These mono-and disulphated regions vary in length and are separated by non-sulphated regions (Figure 3 c-f). The non-reducing terminus of KS-I is end capped by sialic acid, Gal, GlcNAc or GalNAc [10, 11] (Figure 3c). Skeletal KS-II has a similar overall structure to KS-I but its disulphated, monosulphated and non-sulphated regions are more variable and end-cap modifications less extensive (Figure 3d, e).

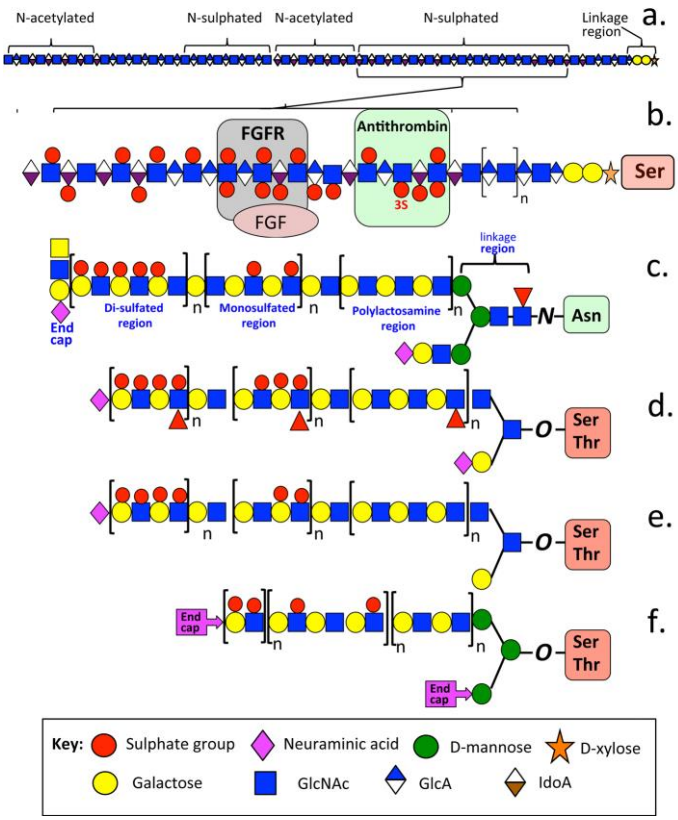

Figure 3. Glycan organisation and sulphate distributions in representative HS and KS structures. The distribution of potential N-Acetylated, N-sulphation and minimally modified HS regions of HS and the linkage region of HS to core proteins is shown (a). Higher power image showing potential sulphation patterning along a HS chain and the structure of the Antithromin-III binding HS pentassacharide, and FGF and FGFR binding sequences (b), Structural organization of N (c)- and O- linked KS (d-f) in the GAG side chains attached to KSPG core proteins. Representative GAG structures are depicted however these are highly heterogeneous structures both in terms of chain length and charge localisation. HS can

be further edited in the tissue by the sulphate editing enzymes Sulf 1, Sulf 2 and heparanase and CS by the CS hydrolase HYAL4. Each of the GAG chains shown have different linkage regions which act as acceptor groups for the sequential addition of sugars and sulphate groups by a series of glycosyl and sulphotransferase enzymes during GAG biosynthesis. The distribution of sulphate groups is nonuniformin HS which is the most highly charged and heterogeneous GAG (a, b). Several areas of differing sulphate substitution have been noted along the KS-I chain, a disulphated region of high charge density close to the non-reducing terminus, an internal monosulphated region mainly on GlcNAc, and a non-sulphated polylactosamine region (b). GlcNAc in the linkage region may be substituted with Fucose residues. The non-reducing terminus is end capped with a number of residues including GlcNAc, GalNAc, Gal, or sialic acid. The minor branched chain of KS-I is prematurely truncated (c). Skeletal KS-IIA from weight bearing cartilage has a similar overall structure to KS-I but is O-linked to PG core proteins through Serine or Threonine residues it is also more heavily fucosylated but less extensively end-capped (d). The KS-IIB from non-weight bearing tissues such as trachea and epiglottis does not contain fucosylation (e). A further form of KS is found in brain, KS-III has a mannose containing O-linkage through Serine residues to PG core proteins, its end capping structures also differ but have not received detailed characterization (f).

Chondroitin sulphate is assembled from the GAG repeat disaccharide glucuronic acid β 1-3 glycosidically linked to D-galactosamine. The D-galactosamine can be sulphated at C4 or C6 in the main chain, however a proportion of these are di-sulphated when they occur at the non-reducing terminus [12]. HYAL4 cleaves at the non-reducing terminus generating the 3-B-3(-) epitope which is characteristic of human foetal developmental cartilage and occurs predominately in transitional areas of tissue morphogenesis. Chondroitin-4-sulphate is a common component of foetal cartilages, however it is largely replaced in mature cartilage by the chondroitin-6-sulphate glyco-form. A number of monoclonal antibodies have been developed with identify the 3-B-3(-) non-reducing terminal epitope and native 4-C-3 and 7-D-4 and 6-C-3 CS sulphation motifs in the CS chains (Figure 4a). These epitopes are produced by stem/progenitor cells located in niches in the surface regions of foetal articular cartilages and perichondrium in developmental diarthrodial joints (Figure 5d-f, Figure 6a, b) [13]. Cell clusters have been observed in normal ovine IVDs (Figure 6c-e) [14] and in regions of the annulus fibrosus undergoing reparative remodeling in an ovine model of experimental disc degeneration (Figure 6f). These appear to represent nests of stem cells, surrounded by HA which has regulatory properties over stem cell recycling and survival (Figure 6h).

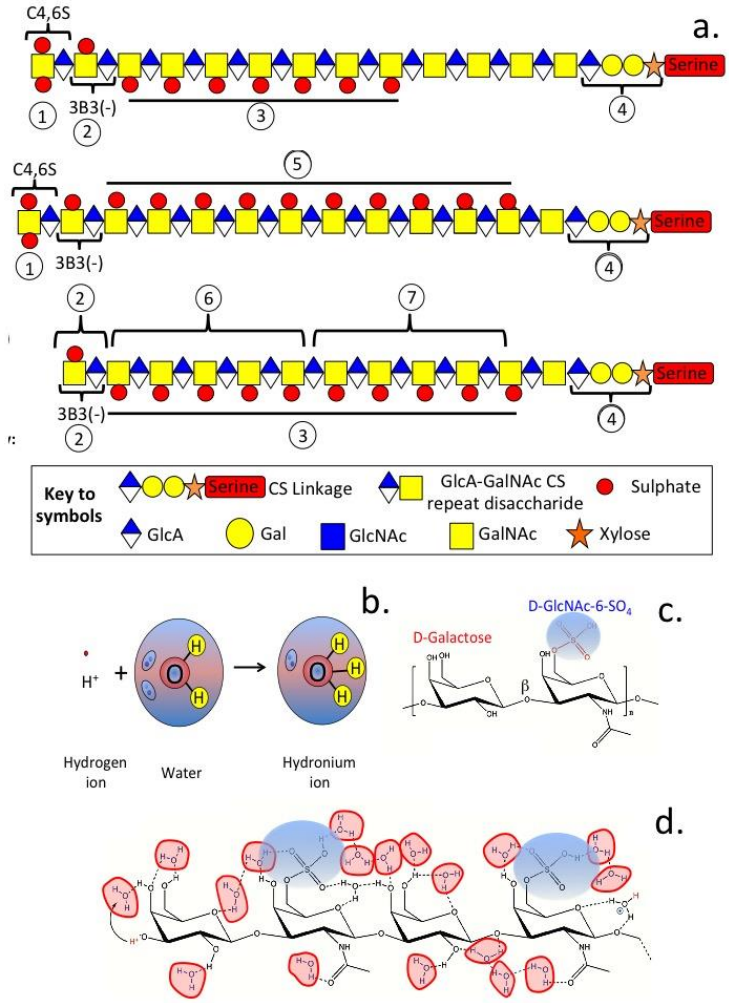

Figure 4. (a) Generic organization of the possible structure of CS chains showing areas of sulphation, organization of non-reducing terminal structures and regions of the CS chain identified by some CS selective monoclonal antibodies. 1. Non-reducing terminal sulphated CS epitope. 2.Non-reducing terminus generated by HYAL4 generating the 3-B-3(-) CS sulphation motif. 3. Chondroitin-4 sulphate epitopes which predominate in foetal tissues. 4. CS linkage attachment region to Serine residues of proteoglycan core proteins. 5.Chondroitin-6-sulphate epitopes predominating in adult weight bearing tissues. 6. CS chain region detected by MAb 4-C-3. 7. CS chain region detected by MAb 7-D-4. The 4-C-3 and 7-D-4 CS sulphation motifs are expressed in a spatiotemporal manner during cartilage and joint development and are also expressed by foetal stem cells in niches located in the surface region of cartilage (see Figure 5). which predominate in foetal Jssues. (b-d) Diagram of the hydronium ion and the proton interactive network KS assembles through cooperative hydrogen bonding. Generation of the hydronium ion by reaction of a proton with water (b). The structure of the KS disaccharide (c) and the KS hydrodium ion co-operative network cloud formed through hydrogen bonding (d). Water has the ability to attract H+ ions (protons) since it is a polar molecule. However hydrogen ions do not actually exist as such in aqueous solution but take the form of the hydronium ion. The bound proton does not remain with a single water molecule but cycles rapidly between many water molecules many times per second ensuring the proton remains entrapped in the KS hydrogen bonding cloud and also generates hydronium ions which are the reactive species for attracting further protons. Figure modified from [15] under Open Access using the terms of the Creative Commons Attribution License.

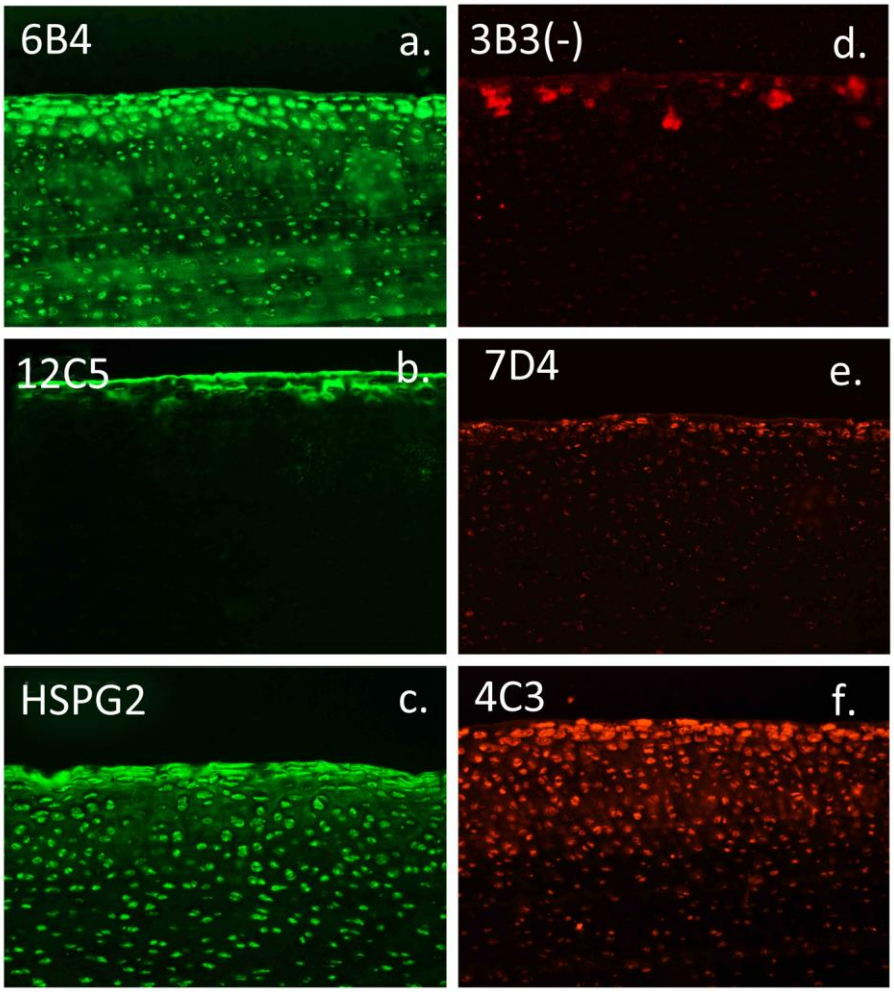

Figure 5. Fluorescent confocal immunolocalisation of aggrecan using MAb 6B4 (a), versican using MAb 12C5 (b) and perlecan (HSPG2) (c) compared to the distribution of the CS sulphation motifs 3-B-3(-)(d), 7-D-4 (e), and 4-C-3 (f) in young bovine knee tibial articular cartilage. The secondary antibodies used in a-c were FITC labeled and those used in d-f were Alexa 488 labelled. Aggrecan has a widespread localization through the full depth of the cartilage (a) and has a similar distribution to perlecan (c) which is also a chondrogenic marker while versican is primarily localized to the surface regions of cartilage (b). Small pockets of cells in the articular surface regions express the 3-B-3(-) and 7-D-4 CS sulphation motifs. These apparently are niche stem cells. MAb 4-C-3 has a broad distribution pattern throughout the cartilage (including the surface regions) similar to the distribution patterns for aggrecan and perlecan. Figure kindly supplied courtesy of Prof B. Caterson, University of Cardiff, Wales, UK.

Significantly the 3-B-3(-) and 7-D-4 CS sulphation motifs are also expressed by such cell clusters in inner AF regions undergoing reparative changes [16] , this is consistent with the synthesis of these CS sulphation motifs by stem cell populations [17] and the cell directive properties proposed for these CS glyco-forms [18]. Administered stem cells exhibit a strong reparative response in experimental disc degeneration, producing a 95% recovery in disc height and recovery of a normal tissue composition and biomechanical material properties [19]. Cell clusters have also been observed in grade IV degenerate human IVDs (Figure 6i)

suggesting that a reparative, but incomplete, response had been mounted in such degenerate human IVDs [14].

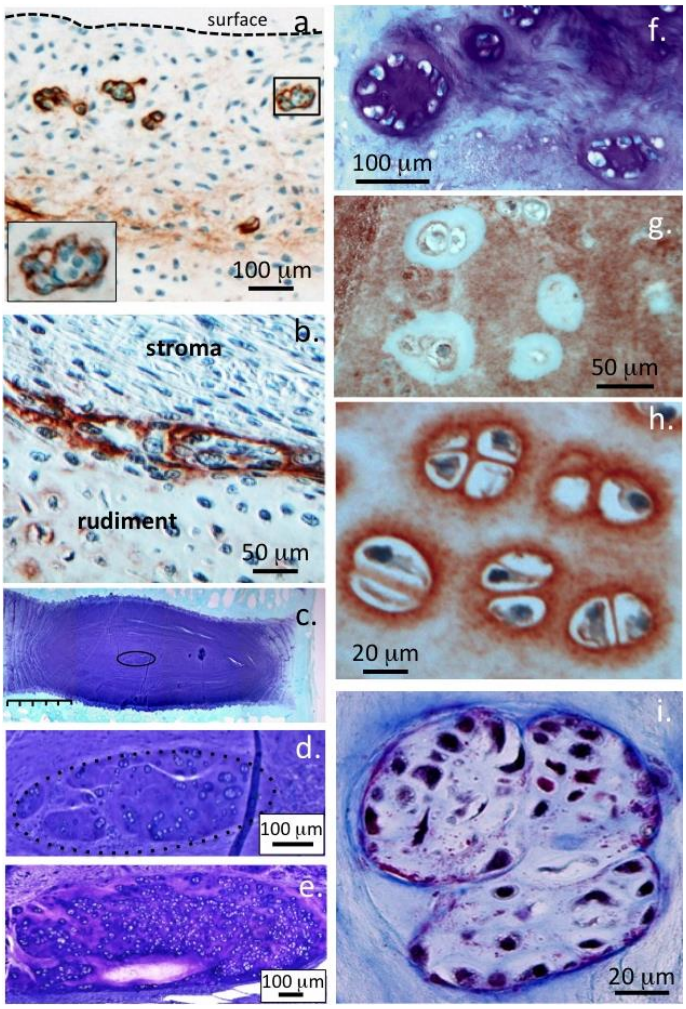

Figure 6. Strategic positioning of glycosaminoglycans (GAGs) in the ECM and pericellular matrix conveying hydration, weight bearing properties and mechanosensory cell directive properties in the pericellular matrix at the cell-matrix interface. Immunolocalisation of perlecan around progenitor stem cell nests in foetal human hip (a) and knee joint rudiments (b) in the surface regions of the rudiment cartilages. The boxed area in (a) is also shown as an inset. Low power image of a toluidine blue stained 12 month old sheep IVD showing an indistinct central progenitor stem cell nest in the nucleus pulposus (circled) (c). The cell nest in (c) shown at higher magnification (d). A cell nest in a 4 year old sheep IVD is also shown for comparison (e). Cell clusters in the annulus fibrosus of a remodeling sheep IVD in which degeneration had been induced in a model of IVD degeneration (f). Immunolocalisation of aggrecan in a 2 year old sheep nucleus pulposus (g). Small stem cell nests are evident as areas devoid of aggrecan in this image. Hyaluronan was localized in the same pericellular regions using biotinylated aggrecan G1 domain affinity probe and avidin conjugated with alkaline phosphatase using NovaRED chromogen (h). Cell clusters in a grade IV degenerate human IVD (i). Figure modified from [14] with permission.

CS sulphation motifs have also been observed in human foetal knee and elbow cartilages in developing joints [13, 20] (Figure 7). Chondroitin sulphate has been employed in composite bioscaffolds in repair biology to act as a director of stem cell differentiation and proliferation

[21]. Cells expressing the 4-C-3 and 7-D-4 CS sulphation motifs in developmental human foetal elbows identify the surface region of the articular cartilage and the perichondrium of the humerus as stem cell niche regions (Figure 7a-d) [20].

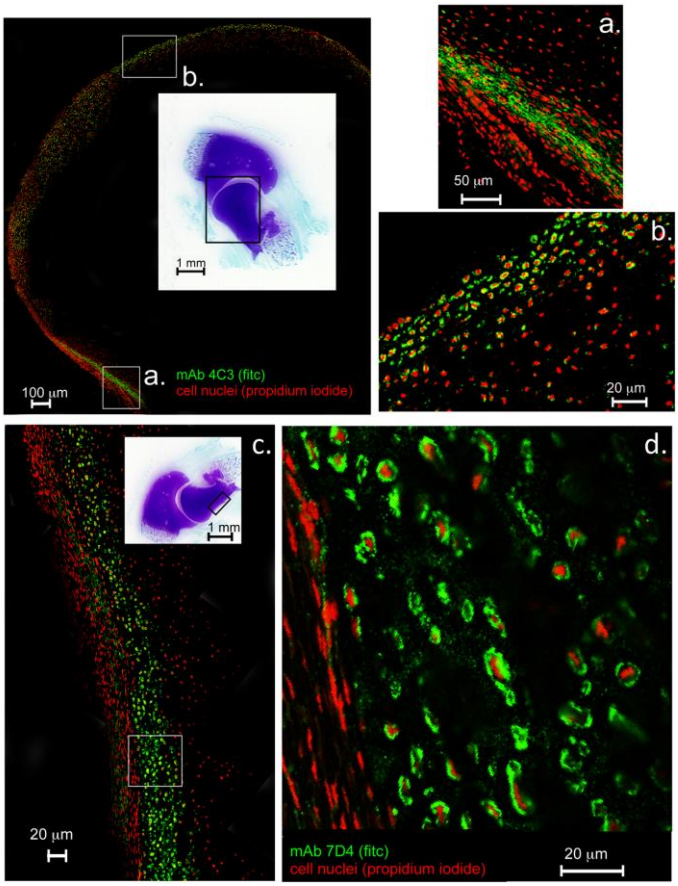

Figure 7. Confocal images of a human foetal elbow joint (12 weeks gestational age) depicting fluorescent immunolocalisation of the 4C3 (a, b) and 7D4 CS sulphation motifs (c, d) on discrete progenitor cell populations in the surface cartilage (b) and perichondrium (c, d). Images reproduced from [20]with permission.

1.3 The glyco-code impacts on cellular regulation, tissue development and repair processes

As stated previously, the GAG glyco-code evolved over several hundred million years of evolution as a cell regulatory mechanism involved in tissue development, physiology and repair [1, 10, 12, 18, 22, 23, 24, 25]. The longevity of GAGs over this protracted evolutionary period points to the essential roles they played in essential cellular survival processes [1]. The sulphation patterns on GAGs direct these cellular molecular recognition activities [10, 18, 23, 24, 25]. Deciphering of the glyco-code using sophisticated techniques [26] demonstrates the significant cell regulatory properties and biodiversity of these molecules [1, 27] and the recognition of

diverse roles for GAGs in cellular regulatory processes in health and disease [22, 28]. This has led to the development of bioscaffolds containing bio-directive GAGs to guide cellular activities in an effort to promote tissue repair and regeneration [12, 29] GAGs are also electro-conductive molecules and conductive bioscaffolds have been developed to stimulate repair processes and optimized for specific cell types such as hepatocytes and neural cells.

1.4 GAGs and the importance of their sulphation motifs in tissue morphogenesis

Several years ago [30] it was noted that monoclonal antibodies (mAbs) 3-B-3(-) and 7-D-4 raised against unique sulphation motif epitopes, identified chondrocyte "cell-clusters" in pathological (osteoarthritic) canine and human articular cartilage and at that time these cell clusters were considered a classical feature of the onset of late stage degenerative joint disease. In these early publications knowledge of stem/progenitor cells in cartilage was not known and expression of PGs (aggrecan) with CS GAG chains recognized by mAbs 3-B-3 (-) and 7-D-4 were interpreted to indicate a failed, late-stage, attempt to repair and replace PGs in an ECM that had been extensively degraded by matrix proteases. An alternative hypothesis however has now emerged, these 'chondrocyte clusters' are evidence of adult stem/progenitor cell niches in these tissues [14]. An important feature of the stem/progenitor cell niche is the sulphation of the PG GAG side chains. Variable expression levels of GAG sulpho-transferases and glycosyl-transferases in the stem/progenitor cell niche environment supports such an interpretation [31]. Studies of animal and human intervertebral disc (IVD) have identified similar chondrocyte clusters within the ageing nucleus pulposus that show distinct distributions for different CS sulphation motif epitopes i.e. 3-B-3(-), 4-C-3, 6-C-3 and 7-D-4 (Figure 4) [14, 32]. Cell clusters express biomarkers that are synonymous with the stem cell niche eg Notch 1 and CD166 [33]. Studies on the chondroprogenitor stromal cells from bone marrow have also shown that these cells elaborate PGs decorated with the 4-C-3, 7-D-4 and 3-B-3 (-) CS sulphation motifs [17]. These motifs have specific localization patterns in tissue morphogenesis

consistent with the roles of chondroprogenitor cells in early tissue development [18, 34]. The current understanding is that these CS sulphation motifs have specific cell directive properties in transitional tissues in development and in tissue repair [17, 18] which however, await full characterisation [13].

The perichondrium is a dense layer of connective tissue that surrounds the cartilage rudiments of developing bones, the cells of its innermost layer produce chondroblasts and chondrocytes whereas those in its outer layer produce cells of fibroblastic phenotype. Once vascularised the perichondrium gives rise to the periosteum, a fibrous layer surrounding the nascent bone. This tissue thus represents an important transitional tissue and signaling centre containing bone and cartilage progenitor cells important in foetal bone development. Figure 5 depicts the specific immunolocalisation of the 4C3 and 7D4 CS sulphation motifs on PGs elaborated by discrete progenitor cell populations in the perichondrium and surface cartilage regions of a developmental human foetal elbow [20]. The pericellular matrix compartment represents a strategic location in these developmental tissues and has important roles to play in cell-matrix communication that is consistent with the cell directive properties of these CS sulphation motifs [13, 17, 18, 20]. FGF-18 has been shown to promote chondrogenic differentiation of bone marrow derived progenitor cells *in vitro* in micro-mass pellet cultures initially; with maturation there is a down regulation of type II collagen production and a switch to the induction of osteogenic differentiation and calcium deposition [35]. Significantly, the 4-C-3 and 7-D-4 CS sulphation motifs are also upregulated in these regions of calcium deposition. This is consistent with the immunolocalisation of FGF-18 in the columnar hypertrophic chondrocytes of the tibial and femoral growth plates during skeletogenesis and indicates that FGF-18 together with these CS sulphation motifs play important roles directing growth plate chondrocyte maturation during the endochondral ossification process leading ultimately to bone formation and extension of the axial skeleton. Perlecan also promotes chondrogenesis

and angiogenic processes which facilitate tissue development [20] and these are functions conveyed by its GAG side chains, thus modulation of GAG fine-structure in variable tissue environments during tissue development may also modulate perlecan's functional properties [36, 37]. Identification of specific GAG sequences and sulphation motif presentations in the HS side chains interactive with growth factors (FGF family, PDGF, VEGF, midkine, pleiotropin), morphogenetic proteins (BMP-2, 4) and transcription factors (*IHH, Sox 9, 10, Mef2c, Wnt*) further reinforces the central roles that sulphation motifs play in molecular recognition and cellular regulation required to direct tissue development [37]. Moreover, specific GAG sequences and sulphation patterns in HS interactive with biomolecules such as AT, lipoprotein lipase and FGF-2 further reinforces the directive properties of GAGs have on specific physiological processes [18]. The electro-conductive properties of HS and its sulphation motifs are of importance in these cell guidance directional properties.

Macrophages are capable of synthesizing HS and CS/DS GAGs, which decorate PGs on their surface and play important roles in cell signaling and guidance via chemokine gradients. Recent data has shown macrophage polarization, caused by alterations in their ECM microenvironment leads to altered expression profiles of numerous HS and CS/DS sulpho-transferases and the emergence of M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage populations. These populations exhibit surface PGs with distinctly altered structural features and hence functional properties. GAGs on PGs synthesised by M2 (anti-inflammatory) macrophages have been shown to be more efficient at presenting FGF-2 in cell signalling in an assay of tumour cell proliferation indicating that the changes in GAG structure contribute to the altered functional properties of polarized macrophages [38]. Such observations suggests that electro-stimulation may have the potential to alter the structural and functional properties of the GAGs expressed on macrophage PGs that are more conducive towards tissue repair. Studies to confirm this proposal have yet to be undertaken however the approach may

represent a potential mechanism of action whereby electro-stimulation exerts its beneficial effects as discussed further below.

2. Electro-conductive effects in tissues regulate cellular behavior

2.1 Ion channels and electrostimulation

The movement of calcium ion (Ca2+) in tissues not only generates an electrical current as it moves, but also directly influences the plasma membrane's permeability to other ions, leading to additional transcellular events [39]. Ionic currents are integral components of the morphogenetic mechanism [40]. Embryonic currents have been measured in a wide range of animal cellular systems in development [41, 42], wound repair [43] and in tissue regeneration [42, 44]. The neuron is a well-known electrosensitive cell type which develops into extensive communication networks throughout the body that direct the activity of other cell types in multisystem components [45]. Ion channels facilitate the development of the nervous system and are controlled by various gating mechanisms during developmental stages and the maturation of neural and related cell types. Variation in ion channels provides distinct signalling mechanisms in neural cell development supporting neural cell proliferation and neuronal differentiation, critical to developmental events which drive the earliest stages of the morphogenesis of the neural tube through to the establishment of diverse neuronal systems throughout the embryo and formation of the spinal cord, brain stem and brain [45]. Something that also needs to be considered in these developmental tissue processes is the role of proteoglycan GAG side chains which carry counterions and their potential roles as ion reservoirs in specific developmental niches [46, 47]. KS is a widely distributed GAG in sensory tissues and displays variable sulphation levels which may facilitate fine levels of control over the counterions they make available for the generation of ion channel generated events in specific developmental contexts [10, 24, 25, 48].

2.2 KS polymer roles in proton detection

Proton (H^+) conductivity is important in many natural cellular phenomena [49] including oxidative phosphorylation in mitochondria and energy production [50], uncoupling of membrane potentials during membrane polarization and neural potentiation [51] and in the priming of cells for proliferative events, apoptosis or cell migration [52]. Electro-chemical reactions control cell and tissue polarity and regulate cell behaviour [53]. An analysis of the proton conductivity of GAGs shows some surprising findings. KS is the best proton detection GAG generating the hydroxonium or hydronium ion [54] through co-operative events involving inter and intramolecular hydrogen bonding with the detected proton (Figure 4b-d).

2.3 KS controls cellular behavior

The conduction of protons occurs through interactions along chains of hydrogen bonds between water and hydrophilic residues on the KS chain and is important in many natural cellular phenomena [15]. CS, DS, HS and HA are also electro-conductive GAGs but to a far lesser extent than KS [55]. In nature, protons (H(+)) can mediate metabolic processes through enzymatic reactions. A KS substituted PG-mucin-like glycoconjugate gel isolated from the Ampullae of Lorenzini, a sensory pore-like system present on the surface of the skin of elasmobranch fish species (sharks, rays, skates), is the best proton detection polymer known in Nature [56] (Figure 8). KS in such mucinous deposits equips coupled neurosensory networks in the fish skin with the ability to detect electric fields generated by the muscular activity of preyfish species, a process known as electro-location [47] and is used to hunt prey fish under turbid water conditions of poor visibility. A similar process is used by some freshwater fish species such as the Mormyridae gymnotiform Elephant fish (genus *Gnathonemus*) of the Belgian Congo or the Sternopygidae Glassknife fish (*Eigenmannia* sp) of the Amazon in a process known as electro-communication [57] (Figure 9a, b). In this case, the electrical signals emitted by these fish identify individuals and the amplitude and frequency of the emissions

can be used to determine their status within the hierarchy of their social group or even the level of sexual maturity of individuals within the group [58]. Thus, in this context, these electrical emissions serve as a social communication system [59].

The electric eel uses controlled high intensity electrical discharges to immobilise prey species but also uses low intensity discharges for navigational purposes in turbid water conditions (Figure 9c). The electric eel (*Electrophorus electricus*), was first described 250 years ago, inhabiting the Amazon and Orinoco rivers in South America. Charles Darwin also identified the electrical eel in his historical explorations conducted on HMS Beagle. Until 2019, The electrical eel was classified as a single species in its genus. However recent work has identified several species of electric eel and, despite its name, it is not an eel, but rather it is a member of the knifefish family [60]. A new species of electric eel *Electrophorus voltai* can deliver 860 volt discharges and has even been reported to fly from rivers in pursuit of land-based prey, *Electrophorus electricus* can deliver up to 600 Volts [61]. Electric eels use high intensity electrical discharge to immobilise prey items, however they also emit low intensity electrical emissions which allow social communication and navigation by electro-location to identify other fish species and predators such as Cayman crocodiles under turbid water conditions of low visibility [61, 62].

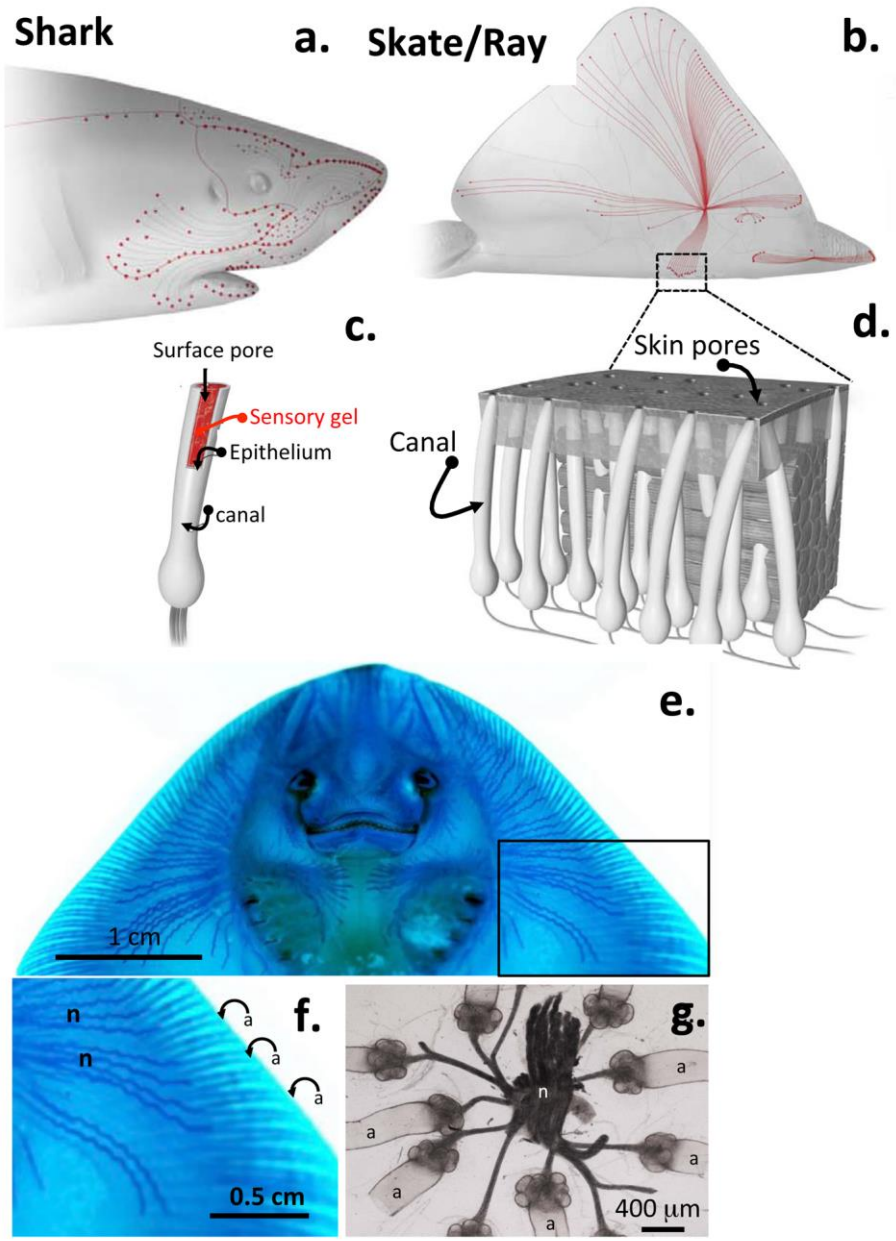

Figure 8. Electrolocation in Sharks, Skates and Rays employs a network of sensory pores containing a sensory glycosaminoglycan gel which detects proton gradients generated by the muscular activity of prey fish species. Schematic of a shark head (a), ray and skate (b) with the sensory networks indicated in red. Diagram of a single pore and its interconnected canal filled with a sensory gel matrix (c) and a collection these pores emerging at the skin surface (d). Macroscopic images of the underside of a ray stained with alcian blue to depict the sensory pores termed ampullae of lorenzini (a) in the skin interconnecting with underlying sensory neurons via canals filled with sensory gel which stain positively with alcian blue (e). The inset in (e) is shown at higher magnification (f). Electron microscopic view of a skin segment showing the ampullae (a) connecting with a collection of neurons (n). Images a-e reproduced from and e-g reproduced from with permission. Image a-d modified from [47] under open access with permission of Advanced Biosystems. Images e-g reproduced from [63] under licence and permission of Springer-Nature publishing (licence number 4657210716625).

Figure 9. The glassknife fish Eigenmaneia viescens (a), Elephant fish *Gnathonemus petersii* (b) which use electrolocation as a means of social communication and the electric eel *Electrophorus electricus* which is also a member of the glassknife fish family. The electric eel uses low intensity electrosensory discharges for navigational purposes and high intensity electrical discharges to immobilize prey species. E. electricus can produce 600V discharges and E. voltai 860V emissions capable of immobilising a horse. a, b are Alamy stock images reproduced under licence. Image c reproduced from Encyclopedia of life under licence cc-by-nc-2.0.

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Mucinous deposits have also been observed surrounding sensory cilia on neurosensory cells in mammals and these are decorated with sialic acid and KS which have roles in the conduction of electro-sensory signals to interconnected neurons in a number of sensory tissues [25, 46]. The conductive properties of KS substituted mucin glycopolymers synthesised by tumour cells has also been exploited in the development of electro-chemical biosensors which detect the conductive polymers produced by the tumour cells in secretions and biological fluids and can be used prognostically to assess disease status [64].

KS-PGs are the most sensitive proton detection molecules known in nature facilitating the process of electro-location in the elasmobranch sharks, skates and rays (Figure 8) and in two terrestrial animals, the duck billed platypus (Figure 10) and long and short nosed echidna of Australia (Figure 11), as well as electro-communication in a number of fish species [55, 56, 65]. The predominance of KS substituted molecules in neural tissues also testifies to the unique functional properties conveyed by this GAG in sensory processes [25, 46, 47, 55, 56]. In weakly electric fish species which employ electro-location or electro-communication, a characteristic electric oscillatory discharge is generated and emitted by a pacemaker organ. This electrical discharge can be modulated in terms of its amplitude, frequency and the waveform of the signal elicited and other fish can interpret this signal which thereby forms a sophisticated means of social communication. Any distortions in this emitted electric field due to the presence of obstacles, predators or other prey fish species are also detected and this

information provides the fish with spatial awareness and aids in navigation, facilitates escape from predators and the capture of food species even in poor light or in turbid conditions of poor visibility.

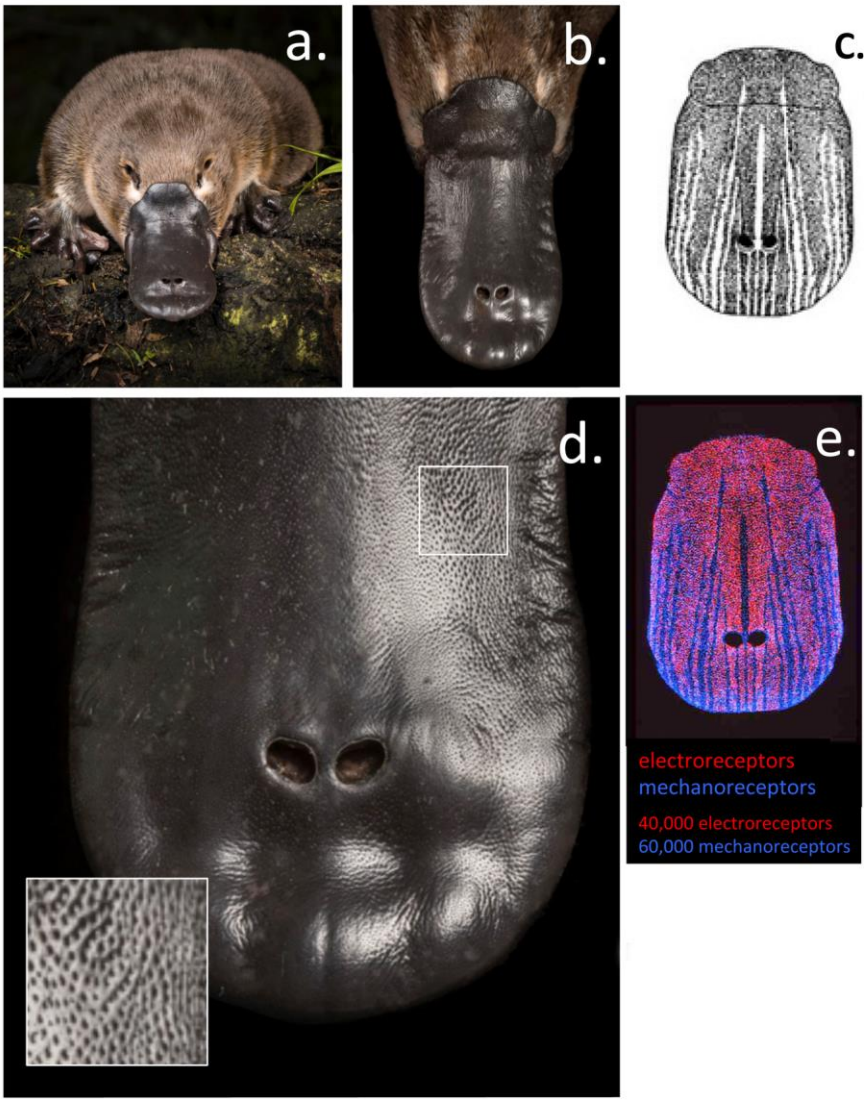

Figure 10 The duck bill platypus uses electrosensory receptors in its bill to detect its prey species when hunting. Sketches of the electrosensory receptors in the platypus bill (c) and mapped with mechano-receptors also mapped in the bill (e). The inset in (d) shows an enlargement of the platypus bill pores. Images a, b, d kindly supplied by Douglas Gimesy, Wildlife photographer, © Gimesy 2012. The sketch in (c) and image in (e) reproduced from [66] with permission.

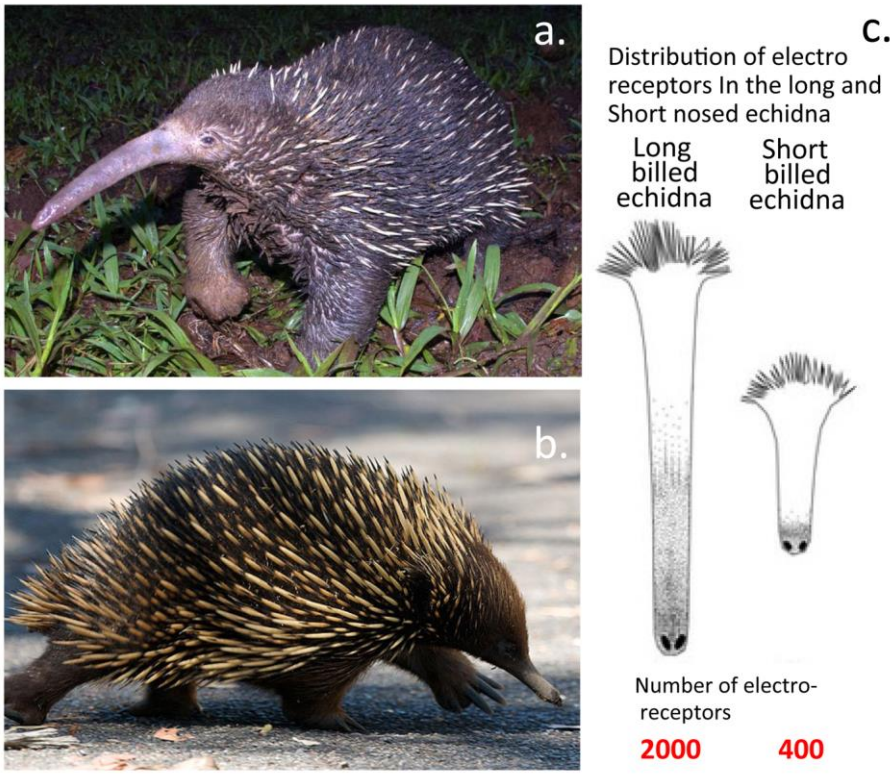

Figure 11. The long (a) and short nose echidna (b) and sketches of the distribution of electroreceptors in the bill (c). The Echidna images were kindly supplied courtesy of Douglas Gimesy, Wildlife photographer, © Gimesy 2012. The sketch in (c) was reproduced from [66] with permission.

2.4 KS-PGs and their roles in neuronal electro-physiological processes.

Aggrecan is a large KS-CS-PG (Figure 12 a, b) which forms perineuronal nets (PNNs) around neurons in the brain (Figure 12c-f). PNNs are protective structures that stabilise the matrix of the PNN, promote neuronal and synaptic plasticity and are important for correct neuronal activity. The form of aggrecan found in brain tissues differs from that seen in articular cartilage, containing fewer KS and CS chains (Figure 12a, b). Some of the CS chains of brain aggrecan are replaced by the HNK-1 carbohydrate motif which provides it with additional interactive properties not seen in cartilage aggrecan [25]. HNK-1 is also a component of several structural glycoproteins and PGs [18] which have roles in the myelination of nerves, ensuring they maintain efficient signal conduction velocities important for the efficient functioning of neural networks. (Figure 13a-d). HNK-1 has important roles to play in embryonic development in the guidance of neural crest progenitor cells which lay down neural networks

and associated tissues such as the neural tube, notochord, heart and its associated large and small vessels and other connective tissues and the brain stem (Figure 13e, f).

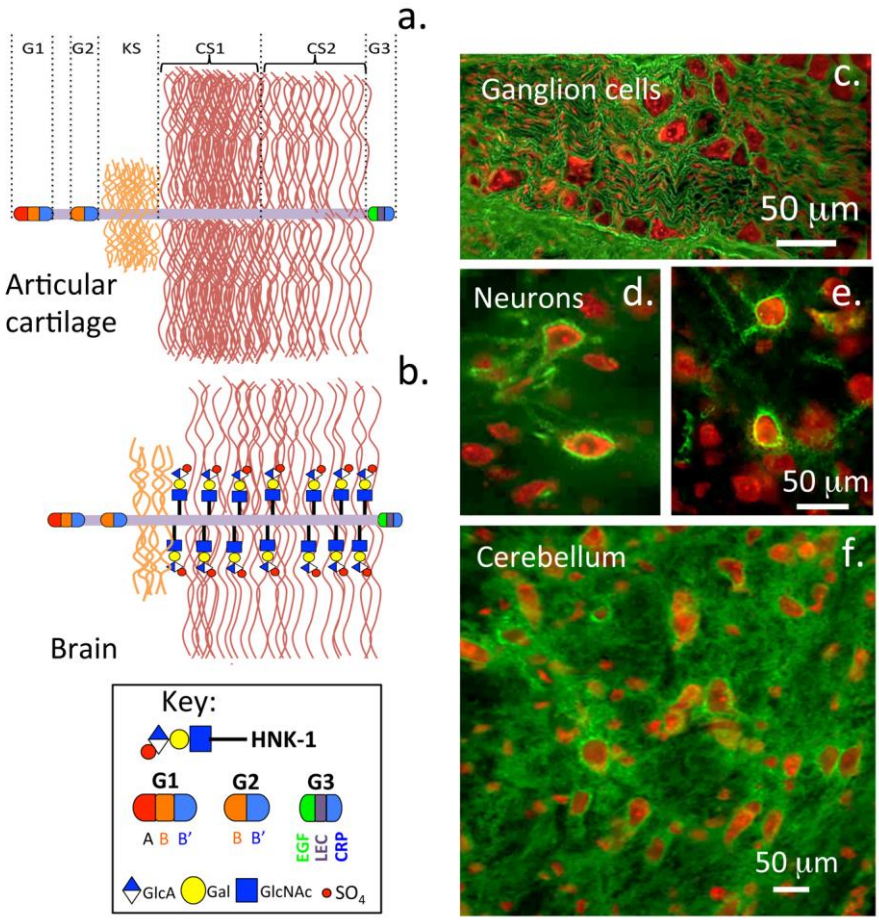

Figure 12. Aggrecan has important roles in perineuronal net formations in brain tissue. Schematic depiction of aggrecan from articular cartilage (a) and from brain tissues (b) showing differences in the GAG glycosylation with the presence of the HNK-1 carbohydrate motif reduced CS and KS content of brain aggrecan compared to cartilage aggrecan. Immunolocalisation of perineuronal nets associated with ganglion cells, neurons and the cerebellum using MAb 1-B-5 which detects proteoglycans displaying the non-sulphated CS glycoform. Aggrecan, versican, neurocan and brevican are all detected using this antibody anf phosphacan decorated with the 1-B-5 epitope would also be detected. Segments c-f modified from [67] under open access.

Several other large brain PGs that are also members of the lectican proteoglycan family include versican (Figure 14b), neurocan (Figure 14c) and brevican (Figure 14d, e); brevican also occurs as a GPI anchored form (Figure 14e). These can also form aggregate structures with HA stabilised by tenascin-R and Bral-1 (Figure 12c-f). Phosphacan is another major brain PG which occurs in several molecular forms and can be variably substituted with CS, KS and HNK-1 trisaccharide (Figure 14 f, g, h) [68]. A truncated non-glycanated form of

phosphacan has also been described which displays neurite outgrowth promoting activity [69](Figure 14i). Phosphacan has also been described as a component of PNNs [70].

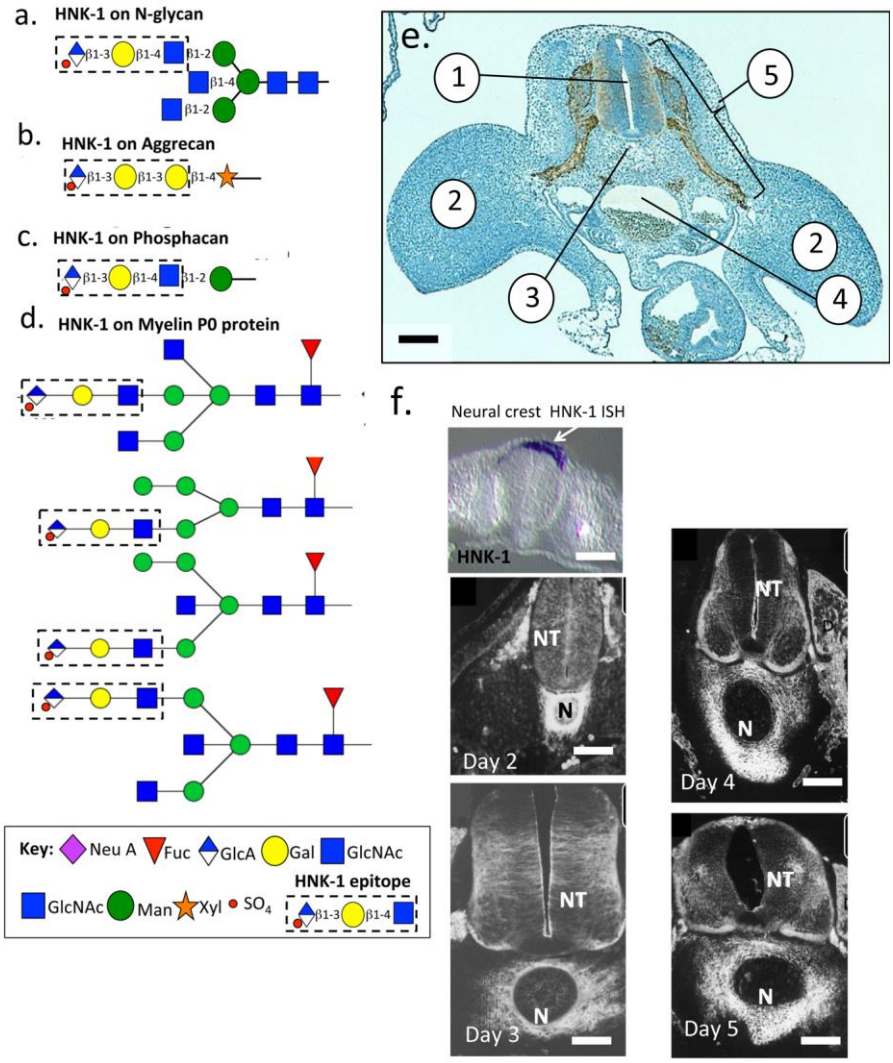

Figure 13. Schematic depiction of HNK-1 structural forms identified in brain tissues on N-glycans (a), aggrecan (b), phosphacan (c) and various structural forms in myelin associated glycoproteins (d). Immunolocalisation of HNK-1 trisaccharide in a section of human chick embryo (e). Labelled features are 1. Neural tube, 2. Wing anlagen, 3. Notochord, 4. Stumoch, 5. Neural network and brain stem, Fluorescent immunolocalisation and in-situ hybridization (ISH) of HNK-1 in 2-5 day old chick trunk sections associated with the neural tube (NT) and notochord (N) development (f). Images in segment f. reproduced from [71] Image e supplied courtesy of Professor Ronald S. Goldstein, Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, ISRAEL.

2.5 GAGs are widely distributed in tissues in the ECM, PCM, at the cell surface and within the cytoplasm

ECM PGs are decorated with HS, CS and KS and these facilitate electro-communication between the cells and their extracellular microenvironments. Cells can

therefore sense changes in their microenvironments through micromechanical and electro-chemical cues and these allow the cell to respond by synthesising ECM components which are detected at deficient levels to maintain a homeostatic balance in the composition of tissues and optimal functional properties. Electro-stimulation would thus be expected to be highly effective in modulating cellular activity to promote tissue repair processes.

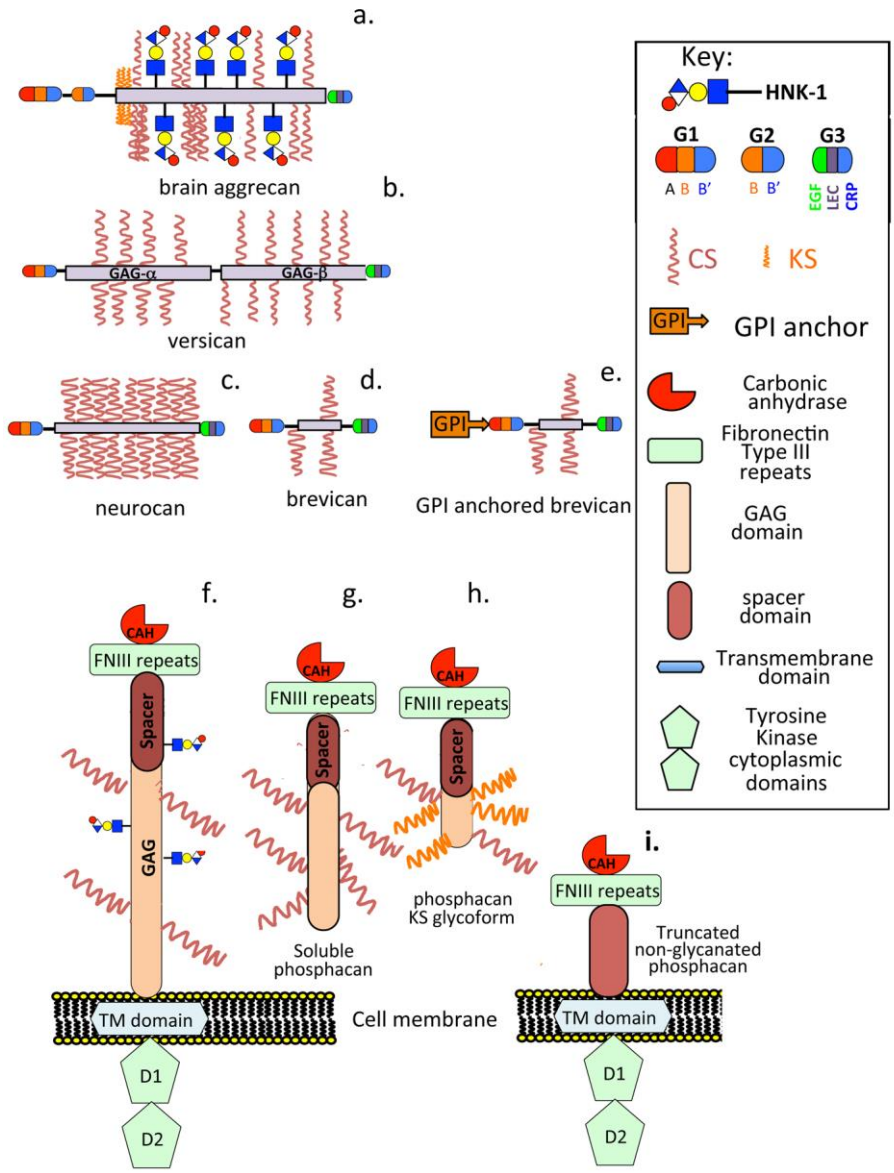

Figure 14. Schematic depiction of the major structural features of brain lecticans (a-e) and the molecular forms of phosphacan identified in brain tissues (f).

3. Neurons are electrically sensitive cell types

Neurons are well known to be an electrically-excitable cell type participating in electro-communication in neural networks. Neurons cultured on peptide-coated gold surfaces [72] and multi-electrode microarrays are innovative technologies that have facilitated the measurement of neuronal membrane action potentials and whole cell ion currents [73]. Thus, intracellular ion-currents and membrane potentials can be measured in NG108-15 cells cultured on gold surfaces [74]. Atomic force microscopy and SEM is also useful for the characterisation of these cultured neurons [75] and facilitates the determination of specific roles for regulatory ion channels [76], regulated by G-coupled receptors [77] and sensory filopodia [78]. The GAG chains of KSPGs and associated counter-ions (Ca2+) serve as intracellular ion-reservoirs or ion-traps and promote these electro-physiological processes. The intracellular SV2 KS-PG, transports neurotransmitters in synaptic vesicles to the synaptic gap where depolarisation of the neuron synaptic membrane facilitates neurotransmitter release in a co-ordinated manner and this results in neural signal transduction. Membrane de-polarization progressively from the soma down to the synaptic gap is an important aspect of the neuro-transductive process resulting in controlled release of neurotransmitters into the synaptic gap. Nanotechnology offers promising therapeutic delivery options for the treatment of neurodegenerative disorders, liposome delivery can modulate neuronal electrical properties promising an amelioration of the cognitive decline evident in AD mice. Cytosolic PSD-95 interactor (cypin), the primary guanine deaminase in brain, modulates neuronal circuits and neuronal survival in traumatic brain injury, however its mechanism of action is largely unknown [79]. *Opuntia ficus indica*, indicaxanthin, a bioactive betalain pigment, has antioxidant and anti-inflammatory properties and can penetrate the blood brain barrier where it can modulate hippocampal neuronal bioelectric activity displaying potential utility in AD therapy [80]. Presenelin 1 and 2 (PS1 and 2) have been suggested as therapeutic targets in the treatment of familial AD [81]. The functional importance of the regulation of Ca2+ signalling by PS1 and

PS2 in AD is impacted by presenilin gene mutation [82], drugs that target PS1 and 2 activity thus show therapeutic potential in the treatment of AD.

4. The cell membrane potential regulates proliferation and cellular attachment in soft tissue repair

The cell membrane is a dynamic structure and membrane potential directly correlates with the cell's proliferative capacity, cellular attachment and cell migratory properties. For example, the high proliferative capacity of liver cells *in vivo* is attributable to their membrane polarization potential, the liver cell is well known as one of the most depolarized of all differentiated cell types [83]. Furthermore, while liver cells are highly proliferative *in vivo* this is not the case *in vitro* and the culture of this cell type has necessitated the development of novel culture conditions. Conductive biopolymers, which increase electrical communication between cells, promote hepatic cell proliferation and attachment. Tissue engineering methodology in hybrid electro-conductive bioscaffolds consisting of blends of HA, poly 3, 4-ethylenedioxythiophene (PEDOT), gelatin, collagen and chitosan promote the proliferation and attachment of hepatic cells in regenerative strategies developed to promote liver repair [83]. PEDOT composite scaffolds have also been useful in neural repair strategies and neural cells are particularly responsive to electro-stimulation [84, 85].

5. Electro-stimulation and the development of conductive bioscaffolds as a therapeutic modality for the treatment of skin wounds

Hydrogels are biocompatible, non-toxic hydrophilic, biodegradable polymers. Electro-conductive hydrogels have proven useful as a smart gel matrix bio-conductor in biosensor and electro-stimulated drug delivery systems in neuron, muscle and skin tissue engineering. Furthermore, injectable, electro-conductive hydrogels based on chitosan and dextran polymers have antibacterial properties against *Escherichia.coli* and *Staphylococcus*

Aureus with *in vitro* kill rates of 90-95% and can also be used to culture mesenchymal stem cells, and C2C12 myoblasts .

Most cell types are responsive to electrical stimulation and this is reflected in their membrane polarization microdynamics *in vivo* [86]. Monocytes and macrophages are particularly responsive to electro-stimulation and voltage-regulated macrophage and monocyte genes involved in cellular proliferation and immune responses have been identified. Monocytes and macrophages express several members of the TRP (transient receptor potential) subfamily of ion channels [87, 88]. These are weak voltage regulated cation channels with roles in immune and inflammatory responses and are regulated by temperature, mechanical force, electrophiles, ligands, membrane composition and pH to control monocyte and macrophage functions such as phagocytosis, production of chemokines and cytokines, cell survival, and macrophage polarization [87]. Identification of environmental stimuli which modulate macrophage phenotype *in situ* and thus control their bioelectric properties represents a promising therapeutic approach to specifically target the macrophage in regenerative medicine [89]. As stated previously, macrophages occur as M1 (pro-inflammatory) or M2 (anti-inflammatory) cell populations; M1 macrophages cause joint erosion and secrete pro-inflammatory cytokines such as TNF-α and IL-1 whereas M2 macrophages stimulate vasculogenesis, tissue remodeling and repair. Thus, it would be desirable to control macrophage activation to minimize tissue damage in chronic conditions such as rheumatoid arthritis [90].

Bioelectric modulation of macrophage polarization has also been undertaken by targeting ATP-sensitive potassium channels using glibenclamide, an ATP channel blocker and pinacidil, an ATP channel opener to modulate macrophage polarization, and phenotype [89]. Modulation of macrophage cell membrane electrical properties using this approach can fine-tune macrophage plasticity, thus glibenclamide decreases the M1 phenotype while pinacidil

augments it, glibenclamide also up-regulates specific features of M2 macrophages. Glibenclamide (glyburide) is a sulphonyl urea drug that was originally developed almost 50 years ago for the treatment of type II diabetes [91] and acts by targeting the ATP sensitive potassium channel sulphonylurea receptor-1 (SUR-1) in pancreatic β-cells [92]. Inhibition of SUR-1 causes membrane depolarization, opens calcium channels and stimulates insulin release in pancreatic β-cells. Pinacidil is a cyanoguanidine drug that opens ATP sensitive potassium channels producing peripheral vasodilation of blood vessels to reduce blood pressure [93]. Glibencalmide also promotes macrophage activation enhancing specific M2 markers during M2 polarization [89]. The use of glibenclamide/pinacidil represents an interesting therapeutic approach for the modulation of M1 and M2 macrophage cell populations and is of potential application in wound repair processes during inflammation and in regenerative medicine [94].

Some tumour suppressor proteins also modify cell membrane components and regulate macrophage cell populations. These include PTEN (phosphatase and tensin homolog) protein and NHERF-1 (Na^+/H^+ exchanger regulatory factor 1). PTEN negatively regulates Akt (protein kinase B [PKB], a serine/threonine specific protein kinase) signalling [95]. PTEN inhibits macrophage polarization and the progression of M1 macrophages to an M2 phenotype [96] through down-regulation of CCL2 (C-C Motif Chemokine Ligand 2) and VEGF-A (vascular endothelial cell growth factor-A) and a synergistic effect with NHERF-1. PTEN, is a tumour suppressor protein with phosphatidylinositol-3,4,5 triphosphate 3-phosphatase activity and is a regulator of macrophage polarization controlling cell division, cell death, cell migration, adhesion and the formation of new blood vessels. NHERF-1, ERM (ezrin, radixin, moesin) binding protein 50 (EBP50) is a scaffold protein which also has tumour suppressor activity. NHERF-1 has two tandem PDZ domains and a C-terminal ezrin-binding module that facilitates its interactions with a range of cytoskeletal proteins and the formation of multi-

protein complexes such as G-protein-coupled receptors and receptor tyrosine kinases with important roles in many disease processes [97].

Macrophage polarization alters the expression and sulphation pattern of cell surface HS and CS/DS [38]. These sulphation motifs mediate interactions with a broad range of ligands and are synthesized by a family of diverse sulphotransferase isoenzymes. Cell-surface GAGs are also electro-conductive molecules but their electro-stimulatory effects on cell populations are rarely considered in wound repair. Macrophage polarization and conversion of M1 into M2 populations also alters the expression of these HS and CS/DS modifying sulphotransferases. Furthermore, M2 macrophage cell surface GAGs are highly interactive with FGF-2, dramatically altering macrophage functional plasticity and proliferation providing a mechanism of how electro-stimulation may facilitate wound repair.

5.1 The application and challenges of the use of electrical stimulation in tissue engineering.

5.1.1 Electricity in animals was first discovered in the eighteenth century

Whole body electrical emissions from humans have been known of since the 1860's when it was first observed that a current of ~1 ⍵A was emitted from small epidermal wounds [98] and this observation was confirmed by Herlitzka in 1910[99]. Emissions of 10-30 ⍵A/cm^2 have also been measured from child amputees [100]. Electric fields are morphogenetic cues which drive embryogenesis, tissue growth and wound-healing. Embryonic electric fields drive cell migration and tissue development by resident cell populations that synthesise matrix components resulting in tissue expansion and network assembly [101]. Mammalian skin and corneal epidermal wounds generate electrical fields lateral to the wound [102]. Disruption of epithelial cell layers at wound sites generates a current out of the wounded area of ~40-200 mV/mm. Many human cell types including macrophages, lymphocytes and keratinocytes detect electric fields of this magnitude and respond by migrating towards the wound site where they promote tissue repair and combat any potential microbial infection.

5.1.2 Electrostimulatory effects on cells can promote tissue repair

Most cell types are responsive to electrical stimulation which effects membrane polarization *in-vivo* [103]. The cell membrane is a dynamic structure and membrane potential directly correlates with the cell's proliferative capacity, attachment and migratory properties. Voltage gated macrophage and monocyte genes regulate cellular proliferation and immune responses through members of the TRP (transient receptor potential) subfamily of ion channels [104, 105]. These regulate immune and inflammatory responses, phagocytosis, chemokine and cytokine production and macrophage polarization [105]. M1 pro-inflammatory or M2 anti-inflammatory macrophage cell populations have tissue destructive and repairative properties respectively thus effective regulation of macrophage phenotype is of importantance in tissue repair and homeostasis. Macrophage polarization alters the expression and sulphation pattern of cell surface HS and CS/DS [106]. Endogenous electric fields occurring around wound sites (30~100 mV) between the epidermis and dermis result in voltage gradients across cell membranes, termed action potentials, these constitute part of the cell signalling and communication machinery that regulates cellular behaviour and effecting tissue repair.

5.1.3 The charge transfer properties of GAGs which promote tissue repair

Transfer and sharing of electrons is the basis of chemical bond formation, the essential life elements N, O, P and S have reactive extra outer shell electrons responsible for the formation of amino, carboxyl, phosphate and sulphate groups as components of Carbon based sugar skeletons. These are functional groups with electroconductive properties at the cellular level. The constructed GAGs containing these functional groups are electroconductive components that transfer electric charge and modulate cellular activity. Certain specialized cell types such as neurons are particularly sensitive to electro-stimulation while in osteocytes and chondrocytes the ECM surrounding these cells has piezoelectric charge generating properties

when the tissue is compressed and these have modulatory effects on the cells embedded in such matrices. The intrinsic piezoelectric properties of collagen are a dynamic feature of densely packed, stressed, weight and tension bearing connective tissue while bone is a microcrystalline collagenous tissue with well-known piezoelectric properties. These electrostimulatory forces have important roles in bone formation, resorption and the ability of connective tissue cells to promote wound repair processes through the laying down of supportive new collagenous tissues spanning the defect site [107].

5.1.4 Use of electrostimulation in tissue engineering protocols to effect tissue repair

Based on the natural electrostimulatory properties that occur in tissue development it is a logical development that electrostimulation should be explored using tissue engineering constructs to assess if this can be harnessed to promote tissue repair using these biomaterials. Organic chemistry has risen to this technical challenge with the development of several electroconductive bioscaffolds with cell directive capability [108].

Some particularly stubborn intransigent tissues in terms of self-repair come to mind such as cartilage, tendon/ligament, IVD, and the myotendinous junctional tissues which might potentially benefit from such procedures. Electrostimulation can potentially be used to stimulate repair processes in resident cell populations in tissues or in cells seeded into specialised electroconductive scaffolds. Electrosimulation of bone marrow progenitor cells has even been explored as a means of modulating stem cell differentiation to produce cells with an ability to promote tissue repair processes in tissue engineering applications [109].

Some examples of this approach have been explored for the repair of nerves, bone, skin and cardiac tissues. Hollow nerve conduits used in the repair of peripheral nerve defects are detrimentally affected by inferior recovery, and nerve extension is hampered by scar tissue adhesion formation during the repair process. Chitosan/oxidized hydroxyethyl cellulose/reduced graphene oxide/asiaticoside liposome based hydrogels have been used to

fill such hollow nerve conduits [110]. The conductive reduced graphene oxide (rGO) of these hydrogels provides an electroconductive microenvironment for peripheral nerve regeneration. The asiaticoside component of this hydrogel is released from the hydrogel during nerve repair and has a significant inhibitory effect on the growth of fibroblasts which populate such constructs and collagen secretion [110]. This eliminates scar formation in regenerated nerves, promoting the functional recovery of defective peripheral nerve bundles. The conductivity of this non-toxic hydrogel was $5.27 \pm 0.42 \times 10^{-4}$S/cm. This hydrogel was suitable for adhesion and proliferation of nerve cells in-vitro and application of electrical stimulation using this hydrogel promoted the differentiation and proliferation of nerve cells, accelerating nerve regeneration [110]. 3D Printing of polycaprolactone-polyaniline scaffolds are electroactive and have been used in bone tissue engineering [111]. These constructs are cytocompatible with adipose stem cells seeded into these scaffolds which displayed appropriate compressive strength and electroconductive properties for applications in bone regeneration. Hybrid bilayer PLA/ nanofibrous chitosan scaffolds containing ZnO, Fe_3O_4, and Au nanoparticles have bioactive properties in skin tissue engineering applications for the treatment of full-thickness skin injuries [112]. This polymer displayed electroconductive properties and minimal cytotoxity and was suitable for the treatment of severe burn patients. Black TiO_2 nanotubes have been investigated as electrodes in electric field-induced stimulation of stem cell growth in osteogenic applications [113]. These show considerable promise in bone regeneration and tissue engineering applications in the repair of bony defects [113]. Electroconductive natural polymer-based hydrogels and their potential biomedical applications have also recently been reviewed in the search for appropriate natural electroconductive materials for tissue reconstructive applications [114]. Electrical stimulation of cardiac adipose tissue-derived progenitor cells caused changes in cell phenotype making them more suitable for cardiac regenerative procedures. Such electrical "pre-conditioning or cell-training" procedures have been suggested as a beneficial pre-treatment step prior to the use of

cardiac progenitors in cardiac engineering procedures [115]. Thus bioartificial myocardium has been developed using electrostimulation of 3D collagen scaffolds seeded with such stem cells [116]. Electrostimulation of cell-seeded collagen matrices changes stem cell morphology, biochemical characteristics and increases expression of cardiac marker proteins. Thus, pre-conditioning using electrostimulation of MSC-derived differentiated cardiac progenitor cells grafted in biological scaffolds is an interesting approach with the potential to generate useful functional tissues for tissue engineering applications aimed at addressing myocardial diseases.

5.2 Use of percutaneous electro-stimulation in the treatment of neurological pain and soft tissue injuries

In an early study in 1972, electro-stimulated amputated rat limbs underwent partial regrowth with new bone, bone marrow, cartilage, nerve, skin, muscle, and epiphyseal growth plate demonstrating the potential of electro-stimulation in repair biology [117]. Earlier studies had shown that an electric current of 1 μA flowed out of a finger cut and currents of 35 and 10~30 μA/cm^2 occurred in amputated fingers of children and guinea pig wounds [118, 119]. Endogenous electric fields occur naturally, around wound sites and a potential difference of 30~100 mV, (the transepithelial potential), has been measured between the epidermis and dermis in normal skin.

Electro-stimulatory devices have been developed for the treatment of neuropathic pain including transcutaneous electrical nerve stimulators, electro-acupuncture or surgical implants which act as peripheral nerve stimulators, spinal cord stimulating devices and dorsal root ganglion stimulators. These devices rely on the inherent excitability of nerve, muscle and connective tissue cells to trigger regenerative processes and minimise tissue inflammation [120]. Electrical stimulation stimulates growth of microcapillary networks which service damaged nerves to stimulate regenerative processes. Accumulation of macrophages at sites of nerve injury may contribute to tissue repair with the scavenger function of M1 macrophages

important, M2 macrophages also have beneficial regenerative properties [120, 121]. Electro-stimulation promotes the recruitment of cell populations to skin wound sites [122] and has been successfully used to treat human crush injuries and, to some degree, rat and canine models of severe nerve transection [122]. Not only are glia and neurons affected at the defect site but macrophage recruitment aids in the ingestion of cellular and tissue debris. Accumulation of M1 macrophages can result in neuroinflammation which inhibits tissue repair and which resolves when M2 macrophages predominate at the wound site. Electrical potentials of between 10-60 mV have been measured at various points in the human body and a so-called epidermal battery identified [123]. Injuries result in the generation of a focal electric field at a wound site which guides cell migration for tissue repair [119].

At the individual cell level, voltage gradients occur across cell membranes. These so called action potentials form part of the cell signalling and communication machinery, however the precise mechanism by which electro-stimulation exerts its effects on tissue repair is still poorly understood. In tissues with a high crystalline component, e.g. bone , an electro-chemical 3D environment around osteoblasts and osteoclasts and piezoelectric electro-stimulatory effects exert stimulatory effects. Stem cells are also responsive to compressive loading and are probably also affected by piezoelectric stimulatory forces [124]. Piezoelectric bioscaffolds generate electrical activity due to mechanical deformation without the need for an external power source, emulating forces bone cells encounter *in vivo* [125]. In articular cartilage, chondrocytes are also surrounded by an electro-conductive ECM with cartilage aggrecan acting as electro-conductive media due to charge mediated ionisable carboxyl and sulphate groups on its GAG side chains [126]. Chondrocytes are sensitive to electro-stimulation [126, 127] and interstitial fluid and its ions act in mechanical signal transduction and are important in cartilage homeostasis [128]. Electro-stimulation increases the expression of collagen type II

mRNA and aggrecan mRNA in human chondrocytes and mesenchymal stem cells, further supporting a role in tissue repair [129].

6. Organic Bioelectronics

The development of electro-conductive scaffolds has allowed applications to be explored in repair biology in combination with electro-stimulation [130].

6.1 The artificial synapse

Synapses are essential for the transmission of nervous electrical signals in the CNS/PNS. Synaptic plasticity allows changes in synaptic responses to be made, this flexibility allows the brain to learn and adapt its responses based on past stimulatory history. The field of organic bio-electronics has designed artificial synapses that attempt to emulate these dynamic properties, a complex and technically demanding exercise [131], and innovative procedures and synaptic devices have been developed to attempt to build artificial sensory systems. Polypyrrole substrate-doped CS conjugated to type I collagen forms a 3-D fibrillar matrix which can be assembled at the conducting polymer interface in artificial synapses [132]. This emulates the anchorage of acetyl cholinesterase in the synapse of the NMJ by interactions involving its C-terminal collagenous tail and heparin binding domains [133].

6.2 Utilization of the piezoelectric properties of therapeutic polymers in combination with electro-stimulation to promote tissue regeneration and repair

Electro-stimulation has been examined as a treatment modality for skin wound repair for over three decades and shows many beneficial properties in terms of cellular recruitment for tissue repair [134]. More recently, electro-conductive graphene-based nanocomposite non-toxic hydrogels with rubber-like elastomeric properties, high mechanical strength, and tunable responsive drug delivery properties have been developed and these show considerable promise as new-age viscoelastic smart biomaterials for skin repair procedures [135]. Electrically-

conductive HA hydrogels containing single-walled carbon nanotubes and/or polypyrrole also provide dynamic bioscaffolds and 3D environments conducive to the proliferation and differentiation of human neural stem/progenitor cells. Electro-active nanofibrous scaffolds containing shell/core poly (3,4-ethylenedioxythiophene) (PEDOT)/chitosan nanofibres provide 3D nanofibrillar electrical environments conducive to tissue repair by promoting cellular adhesion, proliferation, and tissue growth under optimal external electrical stimulation[84, 136, 137] . These scaffolds can also promote the regeneration of ligaments and nerves [84, 136, 138]. GAGs and PEDOT-type derivatives provide a compatible electrically-conductive, hydrophilic neural interface mimicking the intrinsic electro-conductive hydrophilic properties of KS-PGs in neural and sensory tissues [10, 24, 25, 46, 47, 137, 139]. PEDOT:PSS is an electro-conductive polymer consisting of the semi-conductor PEDOT and the dopant PSS which raises the room temperature electrical conductivity of the PEDOT:PSS composite to high levels (100 S/cm, even up to 1,000 S/cm with proper optimization).

The intrinsic piezoelectric properties of collagen are a dynamic feature of stressed weight bearing and tensional connective tissues. These properties play important roles in bone formation and resorption and also the repair responsiveness of connective tissue cells to promote wound healing [140, 141]. The piezoelectric properties of collagen are due to highly aligned fibrillar type I and II collagen fibre packing and the cross-linked structures that are evident in bone and cartilaginous tissues [140, 142]. Composite PEDOT-HA/CS scaffolds emulate the properties of collagen *in situ* and represent smart tissue regenerative scaffolds [143].

6.3 Piezoelectric effects and electro-stimulation of IVD tissues

Piezoresponse Force Microscopy (PFM) has recently been used to measure deformation of the IVD and induction of piezoelectric effects, for the first time, by physical deformation of this structure [144]. Piezoelectric effects were greater in the AF than in the NP,

owing to its organised collagenous lamellar networks. Voltage generated longitudinal piezoelectric effects *in vivo* of ~1 nV were measured. This may represent an intricate electro-mechanically coupled system that could have distinct physiological roles to play in the AF and NP. This parameter awaits detailed characterization but may represent a new functional dimension in the IVD which can modulate the biology of resident IVD cell populations and could also contribute to nerve stimulation and pain generation, a feature that has not previously been considered in the pathobiology of the IVD. Low back pain of discal origin is now recognized as the number one musculoskeletal condition affecting 80% of the general population some time in their lifetime [145]. It is estimated that 9–12% of the global general population (632 million) have low back pain at any one time [146]. This has led to the over-prescription of opioid drugs to treat this condition in the US thus it is imperative that a better understanding of low back pain should be obtained in order to develop a more effective means of its treatment [147]. With ageing the IVD loses aggrecan and becomes more fibrous and dehydrated and more prone to defect development and subsequent degeneration [148]. This may make the degenerate IVD more vulnerable to the generation of piezoelectric effects that further contribute to pathological changes in the disc and age-associated pain generation. Studies need to be undertaken to assess this possibility. Electro-stimulation of the IVD has shown promise in the treatment of low back pain in degenerate symptomatic IVDs suggesting that disc cells are responsive to electro-stimulation.

Table III outlines proteoglycans which may be potentially influenced by electrostimulation. Each of the proteoglycans listed has specific functional properties of relevance to tissue repair and the functional properties of tissues. Much needs to be learnt of how each proteoglycan is affected by electrostimulation. Each proteoglycan will have to be focused on independently to determine how its structure and function is affected by electrostimulation. The GAG side chains of each proteoglycan are specific functional determinants of particular importance in

tissue function and development and the fine structure of the GAGs and their sulphation status will have to be carefully determined, the required methodology is available. While this represents a considerable body of work it will be insightful as to the functional properties of specific GAG structures in repair biology and tissue function.

7. Electro-stimulation and tissue repair

7.1 Nerves

Innervation plays significant roles in wound healing and modulates fibroblast and keratinocyte cellular activity. Sub-optimal levels of neuro-mediators are implicated in chronic wound development and excessive scar formation. Understanding the regulatory interactions between neuro-mediators, myofibroblasts and other skin cell types which facilitate normal re-innervation of the repairing wound site and how adequate levels of neuro-mediators is achieved during the healing process are critical if pathological healing or fibrosis/scarring is to be avoided. Myofibroblasts are key cells in normal repair of skin wounds [149]. Cardiomyocyte research shows that besides improving the conditioning of cardiomyocytes [150] electro-stimulation has the potential of specifically targeting mesenchymal stem cells *in vitro* to modify the differentiation of this cell type in a manner useful for the promotion of repair processes [109]. Electro-stimulation of resident stem cell populations in tissues represents an exciting new approach to repair biology[151] OBJ, it has even been suggested that electro-stimulation can drive a wave of regeneration into the infarcted myocardium[152] OBJ.

Nerves are a well-known electro-sensitive cell type with limited regenerative capacity. As already covered in this review a considerable number of studies have examined the impact of trauma on PNS/CNS nerve conductance and repair processes. Many novel bioscaffolds have been developed to stimulate neural re-growth *in vitro* with a view to developing protocols applicable to therapeutic application *in vivo* [153]. These bioscaffolds have electro-conductive properties conducive to use with electro-stimulatory methods.

7.2 Bone

Bone is a dynamic tissue and responsive to piezoelectric potentials generated within its microcrystalline calcified matrix. A number of composite bioscaffolds have been specifically developed for bone repair based on hydroxyapatite/chitosan [154]. Electro-conductive polymers have also been applied to bone repair strategies using pulsed electro-magnetic fields to stimulate bone cell proliferation and differentiation [155].

7.3 Diabetic ulcers and skin wounds

Diabetic ulcers and chronic skin wounds are painful conditions and can be difficult to treat clinically with poor reparative capability. Wireless micro-current stimulation has been employed for the treatment of diabetes-related wounds [156] with some success. Electrical stimulation has also been used to treat pressure ulcer healing in spinal cord patients [157], and combinations of ultrasound and electric current stimulation used to treat diabetic foot ulcers [158]. Neuromuscular electro-stimulation of lower limb wounds and electro-stimulation of chronic wounds have also been employed in an attempt to stimulate healing [159].

7.4 Neurological pain/scar revision of chronic percutaneous wounds

High voltage electro-stimulation has been shown to accelerate healing of skin graft-treated wounds [160]. Percutaneous direct current nerve stimulation has been evaluated as a potential means of alleviating neurological pain in soft tissue injuries [161]. Electro-stimulation has been used to assess if this treatment modality can improve healing by targeting resident skin stem cells to manipulate myofibroblasts and wound innervation to improve pain free healing of wounds [151].

7.5 Electro-stimulation for skin repair

The beneficial effects of electro-phototherapeutic procedures in skin wound repair has been reviewed [162] and beneficial aspects of electro-stimulatory procedures in skin wound repair subjected to a meta-analysis [163]. A bio-electric plaster has been developed for use in electro-stimulatory procedures to improve skin wound healing [164]. Electro-stimulation has been compared with hydrotherapy, ultrasound, negative pressure therapy and hyperbaric oxygen for skin wound repair [165].

7.6 Electro-stimulation of bone marrow mesenchymal stem cells

Follistatin (FST) and the related proteins FSTL1 and FSTL3 modulate the activity of TGF-β family members by neutralizing activin which modulates cell proliferation, differentiation, immune responses, endocrine activities, wound repair, inflammation and fibrosis. Serum activin levels are elevated in cardiac heart failure patients and in cardiomyocytes following experimental myocardial infarction, implicating the activins in the etiopathogenesis of heart failure. FST is a key modulator of muscle development, differentiation and regeneration, and is thus implicated in the repair of mesodermal- and endodermal-tissues, through its ability to promote cell proliferation and limit fibrogenesis. Electro-stimulation stimulated FST production and was associated with cardiomyogenic differentiation of human mesenchymal stem cells. This differentiation system provides important insights into the molecular mechanisms that drive stem cell differentiation [109].

7.7 Brain: trans-cranial direct current stimulation for the treatment of alcohol craving

The findings of trans-cranial direct electro-stimulation in the treatment of alcohol dependence have been reviewed and subjected to a meta-analysis [166]. This study failed to find any positive findings of electro-stimulation procedures for the prevention of alcohol dependence.

7.8 Heart therapeutic electro-therapy

Cholinergic nerve stimulation holds significant potential as a bio-electronic therapeutic tool for heart disease, however the mechanisms of heart nerve directed regeneration remain unresolved. With a better understanding of the role of cholinergic innervation in the modulation of cardiomyocyte proliferation and inflammation during heart regeneration and adult cardiac tissue remodeling of infarcts may uncover an innovative bio-electronic-based therapeutic for the treatment of human heart disease and functional recovery of infarcted tissue regions following heart attack [167].

Myocardial infarction (MI) results in the death of cardiomyocytes triggering an immune response to clear cellular debris for the restoration of tissue integrity. In the adult heart, the immune system also results in scar formation in the infarcted zone, which repairs the damaged myocardium but compromises cardiac functional properties overall. In neonatal mice, heart regeneration and neo-angiogenesis following MI requires an influx of tissue macrophages into the damaged zone. Experimental macrophage depletion results in murine neonatal hearts being incapable of regenerating myocardial tissue but forming stabilising fibrotic scars instead [168]. This cardiac tissue had significantly reduced cardiac functional properties and this repair process did not elicit an angiogenic response essential for restoration of healthy myocardial tissue. Macrophages have a unique polarization phenotype secreting soluble factors that promote myocardial repair [168, 169]. An ambitious goal in modern cardiology is to develop a means of regenerating the injured myocardium following MI since the human myocardium has poor intrinsic regenerative capability [167, 169, 170]. Significantly, electro-stimulation studies in the repair of percutaneous skin wounds has demonstrated that the polarization status of macrophage populations is amenable to electro-stimulation and this stimulus also results in migration of macrophages into damaged tissue where they elicit a repair response [151, 160, 162]. Such electro-stimulatory procedures may also be suitable for the regeneration of MI tissue and the recovery of heart function following heart attack.

7.9 Electro-stimulation of the knee

Electro-stimulation of the knee following arthroscopic surgery results in reduced swelling in the knee joint region, improved range of motion, extensor and flexor strength, however the articular and meniscal fibrocartilages or knee ligaments were not specifically examined in this study [171]. A systematic review published in 2011 found little evidence of a significant effect for electro-stimulation vs sham or no intervention on pain in knee OA [172].

8. Electro-stimulation as a remedy for low back pain

Chronic pain is a major health problem and of major socio-economic impact. Low back pain of discal origin is considered the leading musculoskeletal condition affecting 80% of the \ global population some time in their lifetime. It is estimated that approximately 100 million US adults suffer from chronic pain, more than the number affected by heart disease, diabetes, and cancer combined. The economic cost of chronic pain in adults has been estimated at $560–630 billion annually. Chronic low back pain accounts for 22% of all cases of chronic pain and 35% of most persistent pain sites. The most common diffuse low back neuropathic pain is classified as nonspecific low back pain. Evidence suggests that electrical stimulation may modify both cause and perception of chronic pain. The development of a whole-body non-contact electro-magnetic sub-threshold stimulation device primarily affects peripheral nerves, spinal cord, muscles, joints, and bone with signal generated processed by the somatosensory cortex via many affected pathways, in line with the modern concept of central control of pain [173]. Targeted therapies have also been developed using electrical and magnetic neural stimulation for the treatment of chronic pain in spinal cord injury [174].

9. Future Research

9.1 Future developments in electro-stimulatory methodology

The ubiquitous distribution of PGs in the ECM, PCM and at the cell surface and their functional properties in tissues [12, 36, 175] and the link between GAGs/PGs/cells and electro-stimulation as a potential beneficial stimulus in tissue repair and regeneration is now well established. All cells are responsive to electro-stimulation and the GAG side chains of PGs should be considered electro-conductive conduits for electro-stimulation. Continued improvement in experimental electro-conductive bioscaffolds and electro-stimulatory devices and their application in regenerative or reparative strategies for neural tissues, diabetic ulcers, bone and chronic skin wounds and functional recovery of infarcted cardiac tissue following heart attack are expected and offer exciting possibilities and the probability of such methodologies becoming more widely available in the clinic.

9.2 How are tissue proteoglycans affected by electrostimulation

Table I outlines proteoglycans which may be potentially influenced by electrostimulation. Each of the proteoglycans listed has specific functional properties of relevance to tissue repair and the functional properties of tissues. Much needs to be learnt of how each proteoglycan is synthesized by cells subjected to electrostimulation. Each proteoglycan will have to be focused on independently to determine if its structure/function is affected by electrostimulation. Electrostimulation of cells may result in increased synthesis of proteoglycan species of modified structure. The GAG side chains of each proteoglycan are specific functional determinants of particular importance in tissue function and development and the fine structure of the GAGs and their sulphation status will have to be carefully evaluated, the required methodology is available and will be insightful as to the functional properties of specific GAG structures in repair biology and tissue function.

Table I

Proteoglycans that may be open to electrostimulation to promote repair of hard and soft connective tissues

Proteoglycan	GAG components	Functional properties

Aggrecan Versican	~100 CS, 20-30 KS chains 12-15 CS chains	HA/aggrecan/versican –link protein ternary complexes hydrate tissues and provide ECM stabilization , weight bearing and a hydrated matrix conducive to cell attachment and migration and tissue repair
Hyaluronan *	HA	Tissue hydration, regulation of cell signaling, cell proliferation/migration, modulation of inflammation
Decorin Biglycan Fibromodulin Lumican	1 CS/DS chain 2 CS/DS chains 3-4 KS chains 3-4 KS chains	Collagen fibrillogenesis, ECM organization, interaction with growth factors/cytokines and cellular receptor mediated regulation, focal adhesion, cellular migration in health and disease. Anti-angiogenesis, MMP-inhibition, chemokine matricryptic activity
Perlecan	3 HS chains in endothelial cells, hybrid CS/HS chains in chondrocyte, SMC and intervertebral disc cell KS in keratinocyte perlecan	HS-growth factor interactions, (FGF, VEGF, PDGF, pleiotrophin, midkine), promotion of angiogenesis/wound repair, matrix stabilization, disulphated CS modulates collagen fibrillogenesis
Syndecan family	1-2 CS or HS chains	Cytoskeletal linkage to ECM, tissue morphogenesis
Thrombomodulin	1 CS	Regulation of Protein C and Protein S which act as anti-coagulants inactivating Factors Va and VIIIa
CD44	CS	Widely distributed HA receptor, modulates cell adhesion
Serglycin in mast cells, cytotoxic T lymphocytes, basophils	CS, DS, HS, Heparin GAGs vary with cell type and tissue context	Storage and modulation of granule protease activity, regulation of TNFα in macrophages, release of immune and inflammatory mediators, histamine, prostaglandin, leukotrienes, regulation of immune response and focal inflammation
Bikunin	CS	Kunitz inhibitor domains regulate Ca2+ activated voltage gated ion channels. Stabilisation of HA by transfer of ITI HC chains which cross-link HA

Abbreviations : HA, hyaluronan; CS, chondroitin sulphate; KS, keratan sulphate; DS, dermatan sulphate; HS, heparan sulphate, FGF, fibroblast growth factor ; VEGF, vascular endothelial cell growth factor ; PDGF, platelet derived growth factor ; ECM, extracellular matrix; MMP, matrix metalloprotease; ITI, inter-α-trypsin inhibitor;HC, heavy chain. *While HA is a macromolecular GAG and not a proteoglycan by definition, it displays cell interactive properties that promote tissue repair.

10. Concluding remarks

Until recently, the therapeutic potential of electro-stimulation as a treatment modality in repair biology has been under-appreciated. So too have the diverse electro-regulatory roles of sulphated GAGs in connective tissue pathobiology. Of all classes of GAGs, KS -a previously rather neglected GAG- perhaps demonstrates this the best, having specific properties which convey regulatory properties not only in tissue development but also in many sensory processes. KS is the most sensitive proton detecting GAG and forms an electro-sensory gel which has important roles to play in electro-detection sensory processes in sharks, rays and

skate fish species. This is in keeping with the prominent occurrence of KS-PGs in the CNS/PNS in mammals and their emerging importance in neuro-regulatory and sensory processes in higher animals. The development of organic micro-electronics and electro-conductive biopolymers and their applications in repair biology are now offering great promise for the repair of tissues that formerly were considered difficult candidate tissues with low expectations of successful repair. This area in repair medicine has entered an exciting period with continuing improvement in the development of organic micro-electronics and application of electro-conductive polymers. Favourable outcomes in technically demanding and problematic tissues such as liver, neural/muscle interface and cartilage, previously considered to have meagre repair potential, now have reasonable expectations of functional recovery with the correct application of electro-stimulatory techniques. Much still needs to be learnt of how this methodology might be optimally applied to these tissues. However, the application of electro-conductive stimulation in an enhanced tissue environment provided by new generation electro-polymers offers considerable promise for functional tissue recovery in the near future.

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