Unravelling the protective role of APRIL in the central nervous system

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Rôle neuroprotecteur de APRIL dans le système nerveux central

Unravelling the protective role of APRIL in the central nervous system

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This thesis manuscript will begin with an overview in English and a more detailed summary in French, which will be followed by four main chapters covering the introduction, the material and methods used, the results, and a discussion. Abstracts in French and English are presented on the last page of the document.

Summary

Chondroitin sulfate proteoglycans (CSPGs) are diverse, major constituents of the extracellular matrix (ECM) with key roles in the central nervous system (CNS) such as tissue structure, neural development, plasticity, and regeneration [1, 2]. They are especially studied for their role in inhibiting repair in the CNS [2–8]. Their upregulation following trauma in the CNS contributes to the formation of the glial scar, a cellular and biochemical barrier to regenerating neurons as well as myelinating cells [3, 4, 9, 10]. Additionally, CSPGs may play pathophysiological roles in modulating repair in neurodegenerative diseases [11]. For example, they have been shown to be upregulated in multiple sclerosis (MS) lesions [12–14].

The inhibitory activities of CSPGs are known to be mediated by their chondroitin sulfate (CS) chains [17–22]. The digestion of CS chains by chondroitinase ABC promotes neural regeneration and remyelination *in vitro*, as well as in the mouse spinal cord injury (SCI) model [4, 6, 10, 18, 23–26]. CS are sulfated glycosaminoglycans (GAGs) consisting of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine, and come in a variety of types depending on their sulfation pattern and epimerization [27]. Chondroitin sulfate A (CS-A) and B (CS-B, also known as dermatan sulfate) consist of mono-sulfated disaccharide units that differ by an epimerization, yielding either glucuronic acid or iduronic acid respectively. Chondroitin sulfate D (CS-D) and E (CS-E) on the other hand, are rich in disulfated disaccharide units, but differ from each other by the positions of their sulfations.

The inhibition mediated by CS GAGs depends on their ability to bind to a variety of receptors and ligands with roles in modulation of repair and regeneration. Among them, the best characterized are receptor-type tyrosine-protein phosphatase sigma (PTPRS), a member of the protein tyrosine phosphatase (PTP) family, Nogo receptor 1 (NgR1, also known as reticulon 4 receptor), a member of the Nogo receptor family, and semaphorin 3A (Sema3A), a member of the semaphorins family. CSPGs have been shown to mediate their inhibition of neural and myelin repair through PTPRS [8, 28–33] and NgR1 [34]. Other families of proteins with known affinity for sulfated proteoglycans and that regulate neural plasticity include the slits, and the ephrins [35–37]. Ephrin-B2 (EFNB2), a member of the ephrins subfamily of receptor protein-tyrosine kinases, has a role in the formation of the glial scar [38, 39], and Slit

guidance ligand 2 (Slit2, also known as slit homologue 2), a member of the Slit family has roles in axonal guidance and migration of oligodendrocyte precursors [37, 40, 41].

This study aims to show the therapeutic potential of blocking CS interactions with repair-inhibiting proteins of the CNS that mediate their activity. We show that differently sulfated CS GAGs have different binding preferences for various inhibitory proteins, eliciting the need for an agent capable of indiscriminate and stable binding to CS types. We have previously shown that a proliferation inducing ligand (APRIL, also known as TNFSF13) binds to CSPGs [42]. A recombinant, hexameric form of APRIL (APRIL-Fc) exhibits broad and strong affinities for CS GAGs. We show that APRIL-Fc is able to effectively block CS binding to repair-inhibiting partners and is able to neutralize the inhibitory effects of CS types on neurons in *in vitro* functional assays, as well as on oligodendrocyte progenitor cells in pilot experiments. In an *ex vivo* organotypic model, APRIL-Fc treatment boosted remyelination.

Key words: a proliferation inducing ligand (APRIL), chondroitin sulfate proteoglycan (CSPG), central nervous system (CNS), remyelination, spinal cord injury, multiple sclerosis

Résumé en français

Chapitre 1 : Introduction

Les chondroïtines sulfates protéoglycanes (CSPG)

Les chondroïtines sulfates protéoglycanes (CSPG) sont des composants majeurs de la matrice extracellulaire (MEC) et sont omniprésents dans le règne animal. Ils sont constitués d'un cœur protéique décoré par des chaînes glycosaminoglycanes chondroïtines sulfates (CS-GAG) [11, 43–45]. La MEC du système nerveux central (SNC) se distingue de celle d'autres types de tissus par des quantités plus élevées de GAGs, parmi lesquels les CS-GAGs sont les plus abondants [46–48]. Ces CS-GAGs ont des rôles variés comme par exemple dans le développement, la plasticité mais aussi la régénération. Ce dernier sera le sujet de cette étude [17, 49, 50].

La plupart des fonctions des CSPG sont médiées par leur CS-GAGs et ses motifs sulfatés ; ces derniers sont cruciaux pour la fixation de protéines partenaires [2, 17–22]. Les CS-GAGs sont composés de répétitions de disaccharides (N-acétylgalactosamine, GalNAc et acide glucuronique, GlcA). Ils forment différents types de CS-GAGs selon leur sulfation et épimérisation [27, 51, 52]. Les CS-A et CS-C sont mono-sulfatés à la position 4 ou 6 du GalNAc respectivement. Le CS-D est di-sulfaté aux positions 2 et 6 du GlcA et GalNAc respectivement, tandis que le CS-E est di-sulfaté aux positions 4 et 6 du GalNAc. Le CS-B, aussi appelé dermatane sulfate (DS), est une variante où le GlcA est épimérisé à un acide iduronique (IdoA) [27, 53–56]. Le CS-B est sulfaté a la position 4 du GalNAc comme CS-A, mais il peut aussi être sulfaté à la position 2 du IdoA [55].

Ces variations mènent à une diversité d'interactions avec des partenaires protéiques. Les chaînes de CS-GAGs peuvent alors avoir des préférences de liaison distinctes ou qui se chevauchent selon la présentation et la distribution des diffèrent CS-types, formant un 'code sulfaté' [20, 22, 37, 57]. En conséquence, il a été montré que les différent CS-GAGs n'ont pas les mêmes effets sur les cellules du SNC, étant soit inhibants, soit bénéfiques, ou même neutres. Cependant, différentes études se contredisent dans leur caractérisation de ces différences (voir tableau 3).

Les pathologies du système nerveux central : le rôle des CSPG

De nombreuses études ont démontré que les CSPG sont surexprimés dans les tissus nerveux endommagés ; c'est le cas pour des blessures traumatiques et neurodégénératives. Leur expression est considérée comme étant responsable de l'échec de la régénération. Nous allons considérer dans cette étude les exemples de la lésion de la moelle épinière et de la sclérose en plaques et discuter des rôles des CSPG dans ces pathologies.

Lésion de la moelle épinière : Les lésions de la moelle épinière sont majoritairement causées par le traumatisme physique, comme des chutes, accidents de véhicules, ou des blessures par balle [58]. Le tissu nerveux endommagé devient un site de transformation épigénétique et d'activité gliale, comprenant la mobilisation des astrocytes réactifs, microglies, et des cellules du système immune infiltrants. Ces effets contribuent à la formation d'un environnement inhibiteur, appelé « cicatrice gliale », qui constituera finalement une barrière à la régénération neuronale [59–61]. Parmi ces cellules, les astrocytes réactifs en particulier produisent des molécules inhibitrices comme les CSPG, des ephrines, et des semaphorines qui rendent l'environnement défavorable à la repousse des axones et à leur remyélinisation [2, 4, 7, 61–63].

Plusieurs études ont identifié les CSPG comme étant clés dans l'inhibition de la régénération suivant une blessure traumatique. Les neurones cultivés sur des substrats recouverts de CSPG présentent des défauts dans l'élongation axonale, des cônes de croissance dystrophiques, une croissance des neurites réduite, et une adhésion cellulaire perturbée [3, 15, 64–68]. Ces effets sont abolis par la dégradation des CS-GAGs par l'enzyme bactérienne, la chondroitinase ABC (ChABC). Les études *in vivo* ont aussi démontré l'efficacité de ce traitement pour augmenter la régénération dans des modèles des blessures traumatiques du SNC [18, 23, 69–71].

En plus de l'endommagement neuronal, les blessures traumatiques impliquent aussi la démyélinisation [72, 73], un trait dégénératif qu'on retrouve aussi dans la sclérose en plaques, le sujet du prochain paragraphe.

La sclérose en plaques : La sclérose en plaques est la maladie immunitaire la plus commune du SNC, et se caractérise par une attaque auto-immune sur les gaines de myéline qui entourent les neurones [74, 75], et en conséquence, par l'apparition des lésions ('sclerae') démyélinisées dans la matière blanche et grise du SNC [76–79]. La démyélinisation et l'inflammation du tissu nerveux conduisent finalement à une dégénération progressive des axones, ce qui se manifeste cliniquement par une variété de symptômes, comme la cécité, l'engourdissement, la perte de contrôle moteur et/ou des fonctions cognitives [74, 75, 80]. Typique des maladies neurodégénératives, la sclérose en plaques a une pathogenèse insaisissable, et n'a pas de remède.

Les CSPG sont surexprimés dans les lésions des patients, et s'associent avec l'astrocytose. C'est également le cas dans les moelles épinières des souris atteintes de l'encéphalomyélite auto-immune expérimental (EAE, modèle murin de la sclérose en plaques) [12, 14, 81]. Plusieurs études *in vitro* ont démontré que les CSPG inhibent l'adhésion et la croissance des cellules progénitrices d'oligodendrocytes (OPC), ainsi que leur différentiation, et leur capacité a myéliniser des axones [5, 24, 26, 82]. Comme dans le cas de l'inhibition des neurones, la dégradation des CS par ChABC abolit leurs effets néfastes.

Alors, comme pour les blessures traumatiques, les CSPG empêchent le recrutement des OPC aux tissus démyélinisés ainsi que leur différentiation, compromettant leur potentiel réparateur. Ces observations font des CSPG une cible intéressante dans la recherche de thérapies régénératives pour des pathologies impliquant la démyélinisation chronique, et jusque-là des études *in vivo* testant des inhibiteurs des CSPG dans les modèles de remyelination ont démontré une efficacité prometteuse (par exemple la xyloside [5], et la fluorosamine [82]).

Des récepteurs des CSPG connus

PTPRS : Le type récepteur tyrosine phosphatase protéine sigma (PTPRS) fait partie du sous-famille de type récepteur (RPTP) de la famille des phosphatases protéine-tyrosine (PTP) [83–86]. Cette sous-famille est importante dans la développement du SNC et des processus neuronaux variés [86–88]. Dans ce groupe se trouvent PTPRS et deux autres protéines,

leucocyte antigen-related (LAR), et RPTP-delta (PTPRD), qui forment la famille des LAR, et qui sont connues pour leurs interactions fonctionnelles avec les GAGs sulfates [89]. PTPRS est surexprimée dans des pathologies du SNC. Cette protéine s'accumule dans les axones dystrophiques qui ne peuvent pénétrer les lésions de la moelle épinière dans la souris [33], et elle est surexprimée par les OPC dans les modèles murins de la sclérose en plaques [8]. Le niveau d'expression de PTPRS est élevé dans les lésions de souris modèle EAE, ou lors d'un traitement lysolécithine (LPC) qui induit une démyélinisation focalisée. En interagissant avec les CSPG, qui sont eux aussi surexprimés dans ces modèles, il a été démontré que PTPRS joue un rôle dans l'inhibition de la réparation [31, 44, 89]. Plusieurs types des neurones exhibent une résistance à l'inhibition pas des CSPG *in vitro* s'ils n'ont pas une PTPRS fonctionnel [28, 65, 90–93]. PTPRS a aussi été impliquée dans la modulation des cellules déclinantes du SNC. Dans une étude, l'inhibition de PTPRS a inversé l'inhibition de croissance des OPC médiée par les CSPG *in vitro* [30]. Plusieurs études *in vivo* confirment cette observation, par exemple, les souris avec une PTPRS non fonctionnelle présentent une meilleure régénération après une lésion du nerf optique [94], ou de la moelle épinière [90, 92]. Les traitements bloquants les interactions entre PTPRS et les CSPG ont démontré un potentiel pour l'amélioration de la régénération neuronale, et aussi de la remyélinisation [30, 32, 33, 95].

NgR1 : La famille des récepteurs Nogo (NgRs) est constituée de trois membres, NgR1, NgR2, NgR3 [96, 97]. Ces protéines sont exprimées à la surface d'une variété de neurones [97–99], et interagissent avec des inhibiteurs associés à la myéline (MAIs), inhibant la régénération neuronale et la remyélinisation [100–104]. Une étude percutante par l'équipe de Dickendesher à l'université de Michigan a démontré que NgR1 et NgR3 sont aussi des récepteurs des CSPG [34]. Les neurones aux NgRs non fonctionnels ont montré une résistance à l'inhibition par les CSPG, et la double délétion de ces NgRs et de PTPRS a encore amélioré la régénération après une blessure par écrasement du nerf optique. Au sujet des maladies neurodégénératives, des études ont montré une surexpression des NgRs et de leurs partenaires dans les lésions des patients atteints de sclérose en plaques, et des souris modèles EAE [99, 101, 105–107]. Ciblant ces protéines et leur voies de signalisation dans l'EAE a promu la régénération axonale, et la remyélinisation [106, 108, 109], et les souris sans NgR1 fonctionnelle présentent un phénotype EAE atténué [106, 107].

Sema3A : Les semaphorines sont une grande famille de protéines sécrétées, transmembranaires, ou associées aux surfaces cellulaires, avec des rôles variés dans plusieurs tissus des invertébrés et des vertébrés [110, 111]. Semaphorin 3A (Sema3A) est un des membre avec des rôles clés dans le guide et la migration des neurones et OPC pendent le développement [112–115]. Il s'associe avec la matrice extracellulaire, en particulier avec les filets péri-neuronaux (PNNs) [116, 117]. Les CSPG sont des composants majeurs des PNNs, et ont été décrits comme les sites d'amarrage pour Sema3A [116, 118]. L'accumulation et la présentation de Sema3A sur les CS-GAGs peuvent médier les interactions avec des récepteurs comme neuropilin-1 (Nrp1) et plexin A1, exprimés par des neurones et des OPC. Cette coopération entre les CSPG et Sema3A augmente la répulsion des neurones et induit la dystrophie axonale *in vitro* [119, 120]. Plusieurs études dans les modèles de rat ont montré que Sema3A est surexprimée dans le SNC blessé [121–123]. L'accumulation de Sema3A dans les lésions corrèle avec l'incapacité des neurones à pénétrer ces zones, indicatif du rôle inhibiteur de Sema3A dans la régénération. Les semaphorines ont aussi été impliquées dans les maladies neurodégénératives [124]. La forte surexpression de Sema3A dans les lésions des patients atteints de sclérose en plaques [125, 126], et aussi des modèles rongeurs de démyélinisation [125, 127, 128], corrèle avec l'échec de remyélinisation. Validant ce lien, l'introduction de Sema3A dans les lésions démyélinisantes chez la souris [129] ou le rat [130] a eu comme effet d'inhiber le recrutement des OPC au site endommagé, et d'empêcher la remyélinisation.

Tout ceci montre que les CSPG travaillent main dans la main avec une variété de protéines de différentes familles avec des rôles inhibiteurs dans la régénération.

Des partenaires potentiels des CSPG

EFNB2 : Les ephrins (EFNs) sont des ligands transmembranaires qui exhibent une signalisation bidirectionnelle en interagissant en *trans* avec les récepteurs Eph. Dans le SNC les ephrines et leur récepteurs sont surexprimés sur les neurones, les astrocytes, et les OPC suite à une blessure [38, 39, 131–133]. Ces protéines sont surexprimées aussi dans et autour des lésions dans la sclérose en plaques [132, 134]. EFNB2, la protéine considérée dans l'étude actuelle, est impliquée dans l'inhibition de la réparation [39]. Cette protéine est exprimée par des astrocytes réactifs, et a un rôle clé dans la formation de la cicatrice gliale suite à la lésion

de la moelle épinière chez le rat [38]. Un récepteur de EFNB2 pertinent dans cette étude, EphA4, interagit avec les CSPG pour inhiber la croissance neuronale [135]. Il est possible que les CSPG puissent être impliqués dans la modulation d'activité des Ephs et leurs ligands. Dans cette étude nous allons creuser cette hypothèse en testant les interactions entre EFNB2 et différents types de CS. A notre connaissance, celle-ci sera la première investigation sur cette question.

Slit2 : Les slits sont une famille de protéines secrétées bien connues pour leurs rôles comme molécules-guides dans le développement du system nerveux. Ils sont exprimés par les cellules gliales ainsi que par les neurones, se lient aux récepteurs 'roundabout' (Robo) et influencent la croissance et la migration des neurones et des OPC [41, 136–138]. Les slits ont été étudiées pour leurs effets sur la plasticité neuronale [138, 139], et ont besoin d'interagir avec les protéoglycanes sulfatés comme corécepteurs pour effectuer leurs fonctions [36, 37, 140, 141]. C'est le cas pour Slit2, qui a besoin de syndecan-1 (un protéoglycane heparan sulfate (HSPG) qui contient aussi des motifs CS) pour ces effets inhibiteurs sur les neurones et OPC. Les protéines qui interagissent avec les HSPGs présentent souvent une affinité pour les CSPG (par exemple, la famille LAR des PTP, APRIL, et certaines semaphorines)., L'ajout de CS 'decoys' a aboli l'inhibition de la croissance axonale par Slit2, suggérant que les CSPG peuvent moduler les activités des slits, renforçant cette idée. Dans cette étude nous allons tester, pour la première fois à notre connaissance, des interactions entre Slit2 et différents types de CS.

APRIL: 'A proliferation inducing ligand'

'A proliferation inducing ligand' (APRIL, TNFS13), le treizième membre de la famille des facteurs de nécrose tumorale (TNSF), est une protéine sécrétée par les cellules myéloïdes, et a un rôle dans la différentiation et la survie des cellules productrices d'anticorps [142–144]. Deux récepteurs TNF ont été décrits pour APRIL : 'B-cell maturation antigen' (BCMA) et 'transmembrane activator, calcium modulator and cyclophilin ligand interactor' (TACI). APRIL a une affinité pour les glycosaminoglycanes (GAGs) sulfatés, ce qui est important pour sa fonction [145]. Par exemple, son interaction avec les HSPGs est critique pour la bonne costimulation des cellules B [142–144].

L'auto-immunité est le résultat d'une réponse immune adaptive vers un antigène de « soi », réponse souvent médiée par la production des auto-anticorps [146]. Ces anticorps

sont produits par des cellules B, et cibler cette lignée s'est révélé être une thérapie effective dans certain maladies, par exemple la sclérose en plaques (Ocrelizumab) [147–150].

L'atacicept, une forme de TACI soluble fusionnée à une immunoglobuline, a été conçue pour cibler les cellules B matures et les plasmocytes producteurs d'anticorps en agissant comme une barrière pour APRIL [151]. L'essai clinique en phase II désignée 'ATAMS' (atacicept in multiple sclerosis, IMP28063, ClinicalTrials.gov identifier: NCT00642902) a été mis en place pour évaluer les effets d'atacicept dans la sclérose en plaques [151, 152]. Dans une tournure inattendue, le traitement a abouti à une augmentation de l'inflammation et une exacerbation des symptômes, conduisant à l'arrêt de l'essai clinique.

Cette observation a indiqué un rôle neuroprotecteur pour APRIL dans le CNS. Pour tester cette possibilité, la surexpression et la délétion d'APRIL dans les souris EAE a été investiguée. Notre équipe a trouvé que l'absence d'APRIL aboutit à une exacerbation de la maladie, tandis qu'une surexpression a un effet protecteur [42]. Dans les biopsies des patients atteints de sclérose en plaques, APRIL s'accumule au niveau des lésions, et colocalise avec des CSPG, et en particulier le CS de type E. Nous pouvons constater que APRIL, en se liant au CSPG au niveau des lésions, peut avoir comme effet d'empêcher les CSPG d'interagir avec ces protéines partenaires inhibitrices.

Objective de l'Etude

L'étude actuelle a pour objectif de prendre avantage de l'affinité d'APRIL pour les CSPG. Un APRIL recombinant, fusionné à une immunoglobuline (APRIL-Fc) démontre une capacité à se fixer à différents types des CS. Nous allons évaluer le potentiel de APRIL-Fc comme inhibiteur des interactions entres les CSPG leurs partenaires protéiques connus (comme PTPRS et NgR1) ou potentiels (EFNB2, Slit2). De plus, nous allons tester APRIL-Fc comme traitement *in vitro* sur les neurones et les OPC inhibés par les CS. Enfin, nous allons utiliser APRIL-Fc dans un modèle *ex vivo* de démyélinisation, pour tester sa capacité à promouvoir la remyélinisation.

L'objectif de cette étude est de démontrer un potentiel thérapeutique pour APRIL-Fc dans le contexte de pathologies du SNC.

Chapitre 2 : Matériel et Méthodes

Ce chapitre décrira en détail les protocoles mis en place pour cette étude. Brièvement, on a produit une librairie ADNc dérivé de cerveaux de souris et de la lignée de glioblastome humain, U251. Les amorces conçues pour l'amplification des gènes candidats choisis pour l'étude (PTPRS, NgR1, EFNB2, Slit2) sont décrits dans tableau 5. Les amplicons ont été insérés par clonage moléculaire dans les vecteurs pCRIII contenant des séquences pour le fusionnement de tag-Fc. Ces plasmides d'expression ont été utilisés pour transfecter les cellules HEK293T pour la production des protéines recombinantes solubles, fusionnées à une immunoglobuline. Celles-ci ont été utilisées dans des tests ELISA pour des liaisons avec différents types de CS, et dans des tests de compétition impliquant APRIL-Fc.

Des tests *in vitro* fonctionnels ont requis l'extraction et culture des neurones et OPC des cortex de souris embryonnaires. Ces cellules ont été cultivées sur des lamelles recouvertes de CS, et traitées avec APRIL-Fc ou un contrôle. Les neurones ont été fixés 3 jours plus tard, tandis que les OPC ont été fixés 1 jour plus tard. Leur croissance a été mesurée et analysée sous imageJ.

Concernant les tests *ex vivo* utilisant les coupes de cervelets organotypiques des souris P10, les coupes de cervelets ont été soumises à un traitement LPC démyélinisant, puis cultivées en présence de APRIL-Fc ou un contrôle. Après fixation, l'imagerie confocale et l'analyse de la myélinisation ont été réalisées.

Chapitre 3 : Résultats

Nous avons cloné des versions solubles fusionnées à Fc de récepteurs connus pour leurs interactions avec les CSPG (PTPRS et NgR1), et aussi de protéines partenaires potentiels (EFNB2 et Slit2). Leur capacité à se lier aux différents types de CS (A, B, D et E) a été évaluée par ELISA. Ces protéines recombinantes ont toutes démontré une affinité pour des CS, en exhibant des préférences différentielles pour les différents types. Dans ces tests ELISA, APRIL-Fc a démontré une affinité pour tous les CS testés, et cette liaison a eu comme effet d'abolir efficacement la capacité des CS à fixer ces partenaires protéiques recombinants.

Pour voir si cette observation peut se traduire en un blocage fonctionnel d'activité inhibitrice des CS *in vitro*, nous avons cultivé des neurones dérivés des cortex embryonnaires

de souris sur les substrats recouverts de CS, et traités avec APRIL-Fc ou un contrôle. La croissance neuronale a été inhibée par les CS de types A, B et E, mais pas par le type D. Le traitement des CS par APRIL-Fc a efficacement neutralisé leurs effets inhibiteurs dans une manière dose-dépendante. Dans des expériences préliminaires, nous avons observé une inhibition de croissance des cellules progénitrices d'oligodendrocytes (OPC) dérivées des cortex de souris par les CS de types B, D et E. Ici aussi, APRIL-Fc démontre une capacité à abolir les effets des CS.

L'inhibition par les CSPG de la mobilisation des OPC et de leur maturation morphologique est un obstacle à la régénération de myéline dans le SNC. Les cultures organotypiques des coupes de cervelets démyélinisées par le traitement LPC est un modèle *ex vivo* de remyélinisation. Nous avons trouvé que l'expression de CSPG est augmentée dans la matière grise démyélinisée. Le traitement APRIL-Fc suivant la démyélinisation par LPC a eu comme effet d'accélérer la remyélinisation.

En conclusion, nos résultats démontrent l'efficacité d'APRIL-Fc comme un agent bloquant des CSPG, et un potentiel thérapeutique pour cette molécule dans les pathologies du SNC où la régénération est empêchée.

Chapitre 4 : Conclusions et discussion

En cohérence avec les études précédentes, nous montrons que les CS peuvent inhiber la croissance des neurones et les OPC *in vitro*. Dans le cas des neurones, l'étude actuelle est un complément à plusieurs autres qui ont testé les effets des différents types de CS (tableau 3). Nous démontrons les effets inhibiteurs des types A, B et E sur les neurones corticaux de souris embryonnaires, ainsi que l'inactivité du type D sur ces cellules. Il faut noter pour la variabilité dans la caractérisation des types de CS dans la littérature, qu'il y a des différences dans les procédures expérimentales utilisées. De nombreuses preuves suggèrent que les neurones des différents types ou espèces d'origines différentes peuvent répondre différemment à un CS. De plus, l'origine et la méthode de préparation du CS peuvent aussi influencer son activité [54].

Concernant les expériences préliminaires sur les OPC, notre étude est à notre connaissance la première à tester les différents types de CS sur la croissance de ces cellules.

Une observation intéressante est l'inhibition de la croissance des OPC dans la présence de CS-D, un type qui a régulièrement été rapporté comme neutre (comme dans notre étude) ou bénéfique pour les neurones (tableau 3). Inversement, les OPC n'ont pas été inhibés par le CS-A, une observation qui diffère de nos expériences avec les neurones. Parce qu'il a régulièrement été montré que les CSPG sont inhibiteurs à la fois de la régénération neuronale, et aussi de la mobilisation des cellules remyélinisâtes du SNC, nos observations éclairent les mécanismes à la base de la caractéristique à double tranchant des CSPG

Il est important de noter que les CS sont des molécules chargées négativement, et peuvent donc influencer l'adhésion cellulaire *in vitro*. Nous avons observé une perte d'attachement cellulaire et une augmentation de l'agrégation des neurones et des OPC quand ils sont cultivés sur des concentrations élevées de CS, en accord avec des études précédentes [82, 153]. Cependant, les effets inhibiteurs des CSPG ne peuvent pas être attribués uniquement à leurs charges anioniques. Il a été démontré que la neutralisation des charges anioniques, bien que bénéfique à l'adhésion cellulaire, n'inverse pas l'inhibition de croissance [82, 153].

L'objectif clé de cette étude a été d'évaluer APRIL-Fc comme agent bloquant des CSPG. Nous démontrons l'efficacité de cette molécule pour empêcher les effets néfastes de tous les types de CS testés de survenir sur les neurones et les OPC. Nous proposons un mécanisme moléculaire pour cette activité neutralisante. Par les expériences ELISA nous avons démontré que APRIL-Fc peut se lier aux différents types de CS, et peut interférer efficacement avec leur interaction avec des partenaires protéiques recombinants. Parmi ces protéines, nous avons découvert qu'un membre des ephrines, EFNB2, et un membre des slits, Slit2, ont la capacité de se lier à certains types de CS. Ceci peut être révélateur des interactions fonctionnelles entre ces protéines inhibitrices de régénération et les CSPG.

Dans un modèle *ex vivo* utilisant les cultures des coupes des cervelets organotypiques, nous voyons une augmentation d'expression des CSPG pendant 3 jours à la suite d'une démyélinisation par LPC. Nous rapportons que le traitement APRIL-Fc pendant cette période améliore la régénération de la myéline. La SNC a une capacité formidable de se remyéliniser suite a des blessures, et ceci se voit même dans la sclérose en plaques. Cette réparation intrinsèque se produit grâce à la mobilisation, au recrutement, et à la différentiation des OPC. Cependant, la remyélinisation finit par échouer dans les stades ultérieurs, non pour manque d'OPC disponible. Il semble que les OPC perdent leur potentiel régénérateur à cause des

altérations biochimiques dans l'environnement endommagé, particulièrement dans les lésions actives chroniques. Les preuves actuelles suggèrent que la surexpression des CSPG est principalement responsable, et cibler les CSPG dans des modèles de remyélinisation ont régulièrement démontré des effets bénéfiques. Puisque APRIL-Fc montre une capacité de bloquer des CSPG et leurs effets inhibiteurs *in vitro*, nous n'avons pas été surpris de trouver que le traitement des coupes de cervelets démyélinisés pendant une période de surexpression de CSPG ont exhibés une meilleure régénération. Le blocage des CS par APRIL-Fc peut prévenir leur interaction avec des protéines qui ont un rôle de médiation de leurs fonctions. Cela peut alors faciliter le recrutement des cellules réparatrices comme les OPC, et une remyélinisation sans entrave. L'étude de ces cellules et leur dynamique pendant le traitement présente une perspective intéressante.

Le mécanisme d'action d'APRIL-Fc le distingue des autres traitements ciblant les CSPG. Par exemple, la xyloside et la fluorosamine sont des inhibiteurs de la synthèse des CSPG, et la ChABC est une enzyme qui dégrade les CS. Des anticorps anti-CS-E ont démontré l'efficacité de bloquer les effets inhibiteurs d'un type de CS sur des neurones [65, 118], cependant, APRIL-Fc a le potentiel de bloquer plusieurs types de CS simultanément, et d'empêcher une plus grande variété d'interactions CS-protéines. Cela peut surmonter les problèmes de compensation et de redondance qui peuvent se produire en bloquant une seule voie de signalisation. L'équipe de Dickendesher a mis en évidence l' effet cumulatif de bloquer plusieurs partenaires des CSPG (NgR1 et PTPRS) [34].

Tout cela pris en compte, nous décrivons le potentiel d'un APRIL recombinant d'être un agent bloquant des CSPG, et proposons que cela présente une stratégie thérapeutique viable pour favoriser la régénération dans des pathologies du SNC.

Mots-clefs : a proliferation inducing ligand (APRIL), chondroïtines sulfates protéoglycanes (CSPG), system nerveuse central (SNC), remyélinisation, lésion de la moelle épinière, sclérose en plaques

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Chapter 1: Introduction

1 Chondroitin Sulfate Proteoglycans

Virtually all extracellular matrices of bilateral organisms, including multicellular animals, contain proteoglycans [154]. These are molecules comprised of a protein core covalently decorated with one or more glycosaminoglycan (GAG) chains that are often sulfated. Both the protein core and the composition of GAG chains attached to it come with some structural diversity, creating families of proteoglycans that can have differential distributions in tissues and organs, as well as varying functions [155].

GAG chains are constituted by repeating units of disaccharides and these can come in five types, hyaluronan (HA), heparan sulphate (HS) [156], chondroitin sulphate (CS)[45], dermatan sulphate (DS)[55], and keratin sulfate (KS) (figure 1) [157]. Of all the GAGs in the human body, HS are the most complex, diverse, and ubiquitous. CS-GAGs are the most abundant overall, and are especially important in the composition of the brain ECM [20]. DS are the predominant GAG in the skin, but are also found in various other tissues such as the brain and blood vessel walls [53]. KS are widespread in tissues such as the cornea, cartilage and brain, however compared to CS and HS, the functions of KS have so far not been as well characterized [158, 159]. Broadly speaking, a proteoglycan (PG) is classified as a HSPG, CSPG, DSPG, or KSPG depending on the type of GAG chains associated with it, though it is common to find PGs with more than one type of GAG. For example, the CSPG aggrecan contains KS, and the HSPG syndecan-1 can contain CS and DS (table 1) [154, 160].

In the current study we will focus on CSPGs and their roles in CNS pathologies.

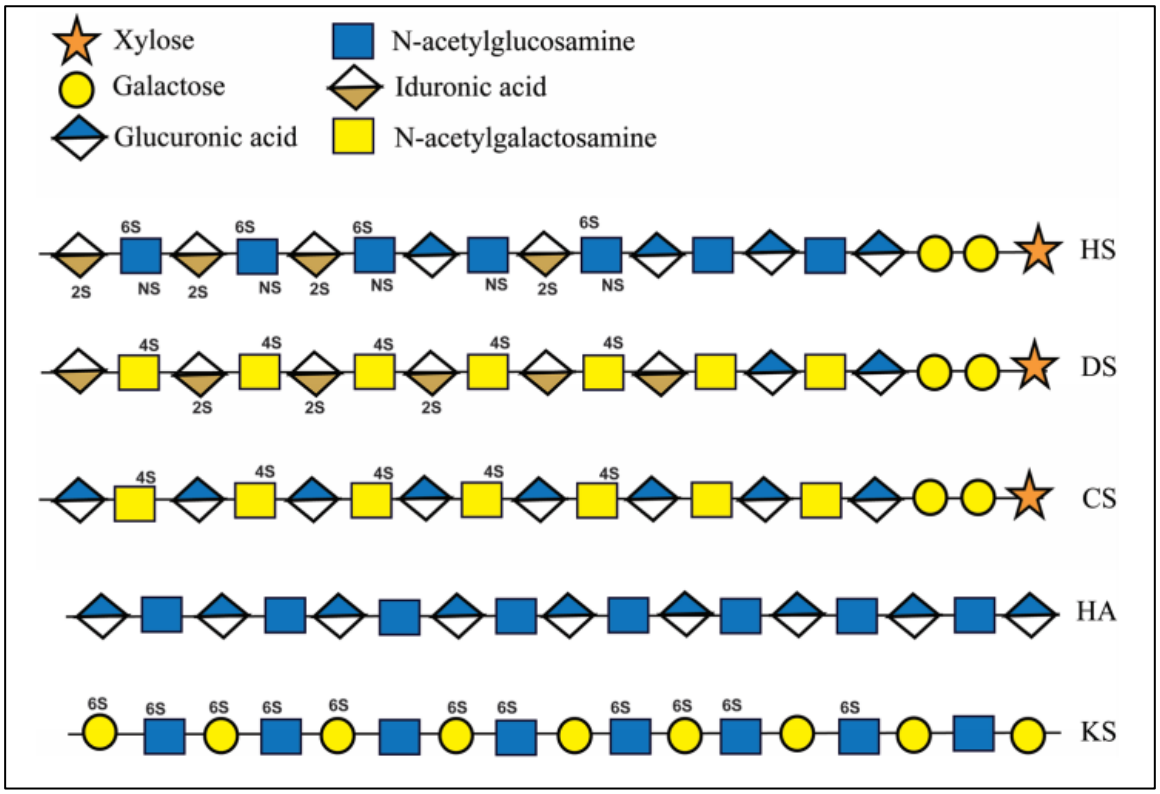


Figure 1: Composition of glycosaminoglycans.

Source: Couchman & Pataki, 2012 [154]. Heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS) share a common 'stub' consisting of a xylose unit, two galactose units, and a glucuronic acid. Their polymeric chains are constituted by different disaccharide units as shown, and are variably sulfated. The N-acetylglucosamine of HS can be sulfated at the 6 position (6S), but can also be deacetylated and N-sulfated (NS). Iduronic acid, which occurs in both DS and HS, can be sulfated at the 2 position. N-acetylgalactosamine residues are found in both DS and CS, and can be monosulphated or disulfated. Keratan sulfate (KS) is made of galactose and N-acetylglucosamine residues which can both be sulfated at the 6 position. Hyaluronan is comprised of repeating N-acetylglucosamine and glucuronic acid residues, but is not sulfated as its synthesis occurs at the cell surface.

Proteoglycan	Core Protein Size, kDa	Type of GAG Chains	Tissue Location
Glypicans			
Glypican 1	56	HS	GPI-anchored cell surface
Glypican 2	59	HS	GPI-anchored cell surface
Glypican 3	59	HS	GPI-anchored cell surface
Glypican 4	58	HS	GPI-anchored cell surface
Glypican 5	59	HS	GPI-anchored cell surface
Glypican 6	58	HS	GPI-anchored cell surface
Syndecans			
Syndecan-1	33	HS, CS/DS	Transmembrane, extracellular
Syndecan-2	23	HS	Transmembrane, extracellular
Syndecan-3	43	HS, CS/DS	Transmembrane, extracellular
Syndecan-4	22	HS	Transmembrane, extracellular
Lecticans			
Aggrecan	208–220	CS/KS	Extracellular
Versican (0/1/2/3 isoforms)	373/265/180/72	CS	Extracellular
Neurocan	145	CS	Extracellular
Brevican	96	CS	Extracellular
SLRPs			
Decorin	36	CS/DS	Extracellular
Biglycan	38	DS/CS	Extracellular
Fibromodulin	42	KS	Extracellular, intracellular
Lumican	38	KS	Extracellular
Keratocan	37	KS	Extracellular
Mimecan	25	KS	Extracellular
Others			
NG2/CSPG4	251	CS	Transmembrane
Neuropilin-1	130	HS/CS	Transmembrane
CD44 (19 isoforms)	37–81	CS/DS	Transmembrane, extracellular, intracellular

Table 1: Structural characteristics of human proteoglycans.

Adapted from: Couchman & Pataki, 2012 [154]. Proteoglycans come in a wide variety of sizes, types, and modifications. Glypicans are HSPGs anchored to cell surfaces by glycosylphosphatidylinositol (GPI) residues. Syndecans are transmembrane HSPGs that can also harbour CS and DS-GAGs in their polymeric chains. Lecticans represent the most important family of CSPGs of the central nervous system, and are usually associated with the extracellular matrix or cell surfaces. Decorin and biglycan are examples of DS-containing small leucine-rich repeat proteoglycans (SLRPs).

1.1 CSPGs and the ECM of the CNS

Chondroitin sulphate proteoglycans (CSPGs) are a major and ubiquitous component of the ECM in the animal kingdom, and are constituted by a core protein decorated by chains of CS-GAGs [11, 43–45]. The CSPGs of the CNS are majoritarily comprised of the lecticans family (aggrecan, brevican, neurocan, and versican) (figure 2), as well as neuron-glial antigen 2 (NG2), and phosphacan. Some of these, namely brevican, versican isoform V2, neurocan, and phosphacan are exclusively expressed in the nervous system [161–163].

Lecticans are secreted proteins with a shared affinity for hyaluronan, to which they bind and contribute to the formation of complex ECM structures called perineuronal nets (PNNs) (see figure 6) [164]. They differ from each other by their protein cores and number of attached GAG chains, which can be as few as one to as many as a hundred depending on lectican [2, 51, 165].

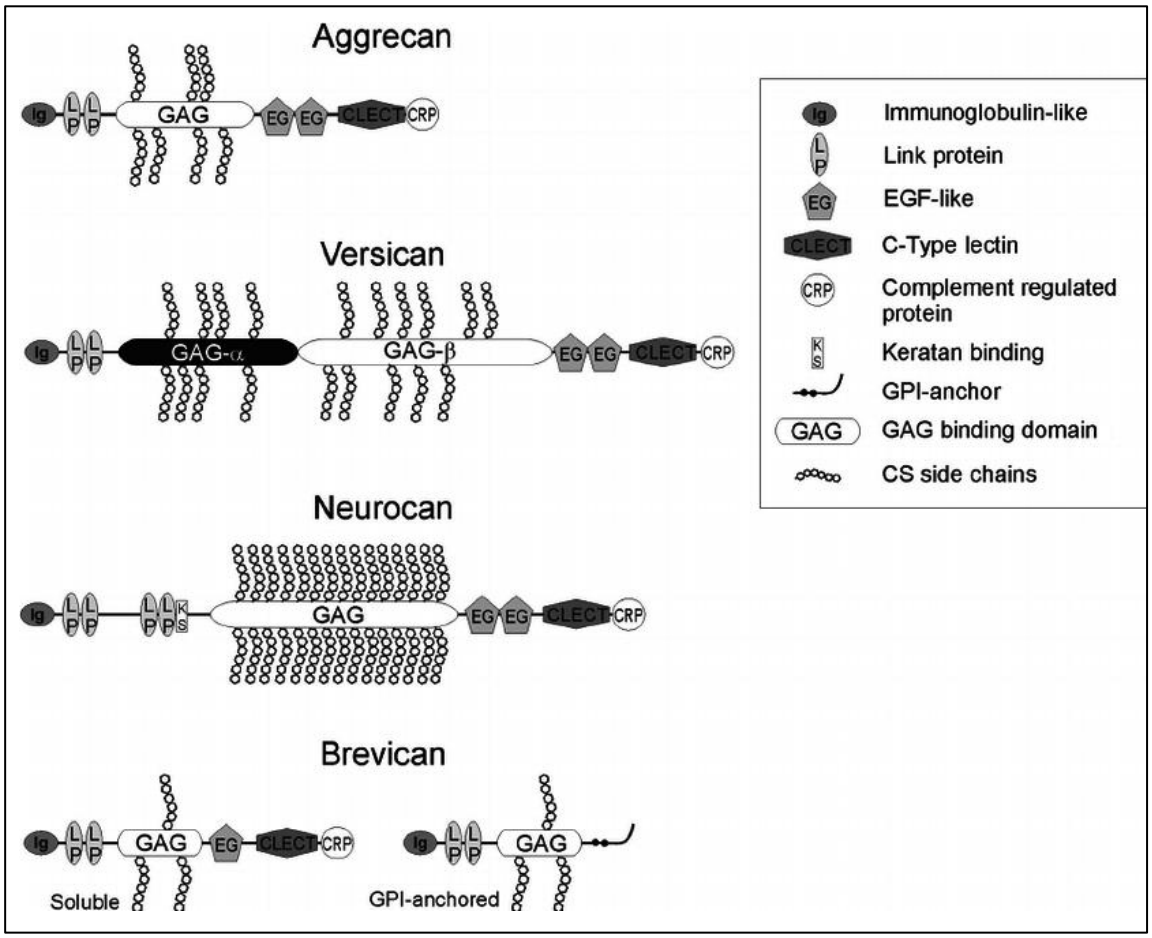


Figure 2: Schematic representation of the members of the lectican family.
Source: Grimpe & Silver, 2002 [166].

Other CSPGs can be membrane bound, such as NG2, which is a marker for neural progenitor cells, especially oligodendrocyte progenitor cells (OPCs) [166–168], and phosphacan, which is an extracellular variant of receptor-type tyrosine-protein phosphatase zeta, (PTPRζ/PTPRZ) [169].

Most cells of the human body bathe in complex information provided to them by proteoglycans like CSPGs written in a language consisting of a vast variety of proteins bound to their GAG chains. These include growth factors, cytokines, hormones, enzymes, repulsive and attractive guidance molecules, and viral proteins [20, 170]. The capture, sequestration, and presentation of bound factors serve as signals, modulating cell activities and processes. As such, CSPGs mediate a vast array of biological processes, such as cellular metabolism, cell migration, development, cancer progression, and the maintenance of the structural integrity of tissues to name but a few [2, 102].

The ECM of the CNS is distinct from that of other tissues, containing relatively high amounts of GAGs of which CS-GAG are the most abundant (table 2) [46–48]. These have various important roles such as in development and plasticity, as well as regeneration and repair which will be the focus of this study [17, 49, 50]. Most CSPG-mediated processes depend on the CS-GAGs and their sulphation motifs, which are crucial for binding to a wide variety of proteins [2, 17–22], though there have been reports of functions mediated by the protein cores of NG2 and versican V2 [67, 171], as well as neurocan and phosphacan [172].

Name	Core size[a]		GAGs		Cellular origin	CNS-specific
	Calculated[b]	SDS-PAGE[c]	Type	Number		
Aggrecan	244	370	CS	?	Neurons/astrocytes	No
Versican V0	371	≈550	CS	17–23	Neurons/astrocytes?	No
Versican V1	263	≈500	CS	12–15	Astrocytes?	No
Versican V2	180	400	CS	5–8	Oligodendroglial lineage	Yes
Neurocan	141	245	CS	3	Astrocytes/neurons	Yes[d]
Brevican	97	145	CS	0–5	Glial cells/neurons	Yes
Phosphacan (-KS)	172	400	CS/(KS)	3–4	Glial cells/neurons	Yes

[a] kDa
[b] Mature polypeptide
[c] Core glycoprotein after glycosaminoglycan removal
[d] Minor expression in peripheral nervous system

Table 2: CSPGs of the central nervous system.
Adapted from: Zimmermann & Dours-Zimmermann 2008 [163].

1.2 CS-GAGs: Biodiversity in structure and function

CS-GAGs are composed of repeating units of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) disaccharides, and come in a variety of types depending on their sulfation pattern and epimerization (figure 3) [27, 51, 52]. Various enzymes, such as glycosyltransferases and sulfotransferases, are responsible for the assembly and sulfation of these structures [173], and their genetic ablation can result in severe developmental defects and neurological disorders in both humans and mice [174–176]. There are five types of CS disaccharides in mammals. CS-A and CS-C are monosulphated at the 4 or 6 position of the GalNAc unit respectively. CS-D and CS-E are commonly referred to as the oversulfated CS-types; CS-D is disulphated at the 2 and 6 position of GlcA and GAlNAc, respectively, and CS-E is disulphated at the 4 and 6 position of GalNAc. CS-B, also known as dermatan sulphate (DS), is a variant where the GlcA is epimerized to iduronic acid (IdoA), creating a similarity to HS (differing from the latter by the lack of a glucosamine unit) [27, 53–56]. CS-B is sulphated at the 4 position of GalNAc like CS-A, but can also be sulphated at the 2 position of IdoA [55]. Different CS-types, including DS, often coexist in the same polymeric structures, for example in versican [27, 177].

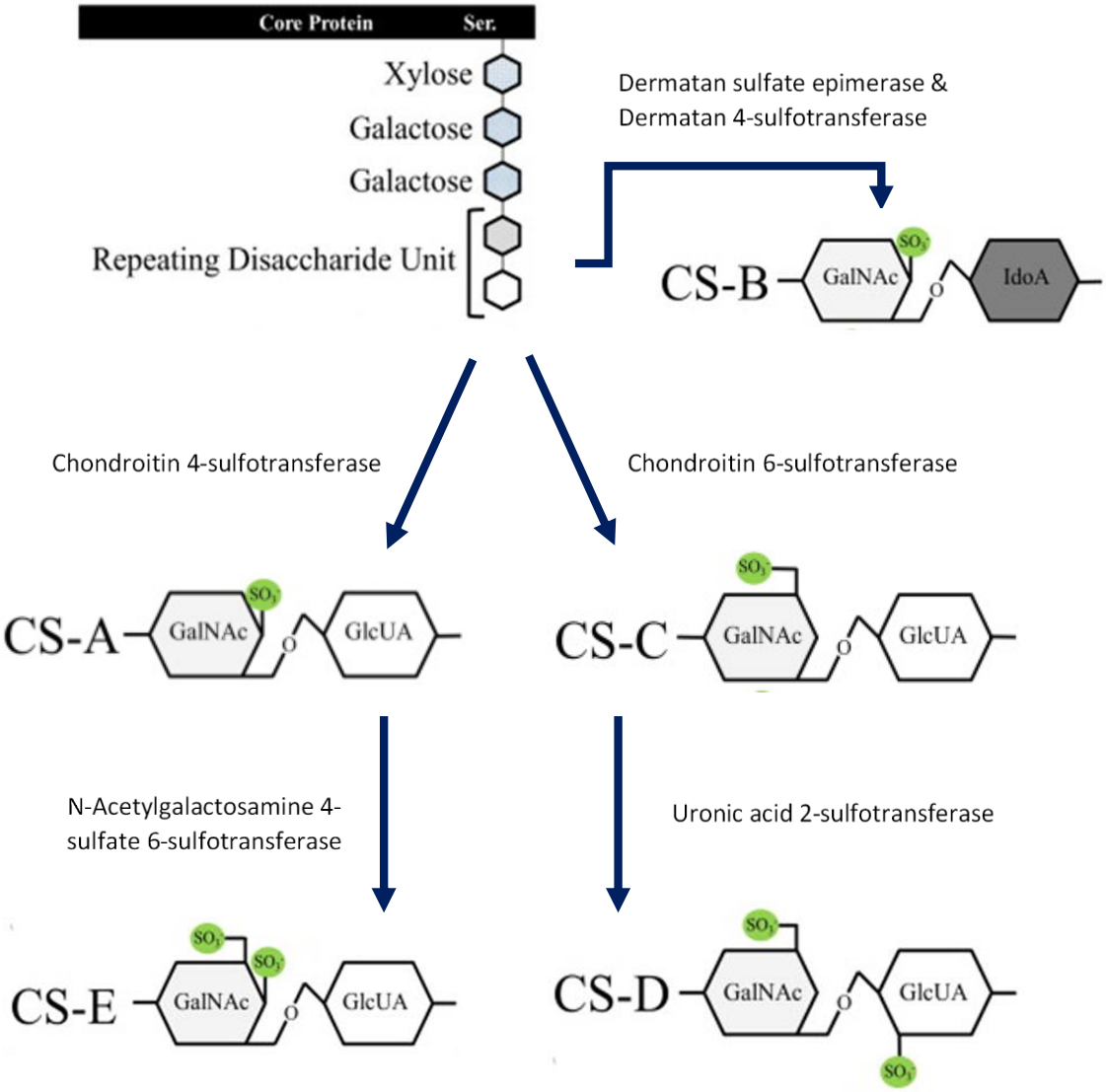


Figure 3: Chondroitin sulfate disaccharide types and their enzymes.
Adapted from Dyck & Karimi-Abdolrezaee, 2015 [102]. CS-A and CS-C are monosulphated by the indicated sulfotransferases at the 4 or 6 position of the GalNAc unit respectively. The additional sulfation of CS-A and CS-C gives rise to CS-E and CS-D respectively. CS-B, also known as dermatan sulphate (DS), is a variant where the GlcA is epimerized to IdoA; it is sulphated at the 4 position of GalNAc by dermatan 4-sulfotransferase.

These variances confer a diversity in interactions with protein partners. For example CS-E interacts with midkine (MK) and pleiotrophin (PTN) [178], while CS-B is a co-receptor for FGF-7 and hepatocyte growth factor (HGF) with roles in wound repair [53, 179]. Thus CS-GAG chains interact with various partners, and can exhibit different or overlapping binding preferences depending on the presentation and distribution of different CS-types, a 'sulfation code' [20, 22, 37, 57].

Likely in consequence of this, different CS-GAGs have been shown to have different effects on cells of the CNS, being either inhibitory, growth-promoting, or neutral in nature [2, 180]. However, studies have been conflicting in their conclusions when characterizing these differences (table 3). These contradictions may be explained by the differential effects of a CS-type depending on its source and preparation [54], as well as evidence that the effects of a CS-type may vary depending on the neuron subjected to it. For example, CS-E was reported to be beneficial, inhibitory, and neutral in rat hippocampal neurones, rat CGNs, and mouse CGNs, respectively (in separate studies) [64, 65, 181]. In addition, the effects of a CS can be modulated by the expression of certain receptors on the cell surface. As an example, J.M. Brown's team reported that CS-E-mediated inhibition of outgrowth is reduced in PTPRS-deficient neurons [182].

To add to the complexity granted by the variations in sulphation, the spatial and temporal expression of different CSPGs and their sulfation codes evolve during development, and in the context of injury or disease (table 4) [17, 52, 62, 161, 183–185].

Table 3: a summary of *in vitro* evidences for CS-type-specific effects on neurons.						
CS	**Reference**	**Source of CS**	**Assay type**	**Cells tested**	**Observations**	**Remarks**
CS-A	*V.P. Swarup 2013* [180]	Bovine trachea, Sigma	Neuron guidance on immobilised stripes of CS	Rat E18 hippocampal neurons	Neurons preferred to grow on CS-A over PLL only	CS were modified by thiolation
	J.M. Brown 2012 [65]	Whale cartilage, Seikagaku	Neurite outgrowth on CS-coated substrate	Chick E7 dorsal root ganglion cells (DRGs)	No effect	Concentrations up to 100 µg/ml used
			Growth cone collapse	Chick E7 DRGs	No effect	Concentrations up to 100 µg/ml used
			Axon crossing (spot assay)	Rat P5-9 cerebellar granule neurons (CGNs)	No effect	1 mg/ml of CS spotted
	L. Karumbaiah 2007 [186]	Bovine trachea, Sigma	Spot assay	Rat E18 cortical neurons	No effect on axonal crossing, fasciculation and cell attachment	CS-GAGs were biotinylated using biotin hydrazide concentrations of up to 2.8 mg/ml were used
	Wang 2008 [64]	Seikagaku	Spot assay	Mouse P5-8 CGNs	Repellent activity	Concentration used not cited
	A.M. Clement 1998 [187]	Bovine trachea, Sigma	Neurite outgrowth	Rat E18 hippocampal neurons	No effect	16 µg/ml of CS spotted
	A.M. Clement 1999 [181]	Not cited	Neurite outgrowth	Rat E18 hippocampal neurons	No effect	CS used at 5 µg/ml
	C. Ueoka 2000 [68]	Whale cartilage, and bovine tracheal cartilage, Seikagaku	Inhibition Assay of MK-mediated adhesion and outgrowth	Rat E17-18 cerebral cortical neurons	No effect	Concentrations up to 50 mg/ml used

CS-B (DS)	*V.P. Swarup 2013* [180]	Porcine intestinal mucosa, Sigma	Neuron guidance on immobilised stripes of CS	Rat E18 hippocampal neurons	Neurons preferred to grow on CS-B over PLL only	CS were modified by thiolation
	RJ Gilbert 2005 [68]	Sigma	Neurite extension in a 3D culture system with CS-GAGs immobilized on agarose hydrogels	Chick E9 DRG explants	Moderate but significant inhibitory effect	0.5 mg/ml of CS used
	Yu 2001 [66]	Sigma	Neurite Crossing of 3D agarose hydrogel	Chick E9 DRG explants	Significant inhibition	Dose dependent inhibition
	A.M. Clement 1998 [187]	Bovine mucosa, Sigma	Neurite outgrowth	Rat E18 hippocampal neurons	No effect	16 µg/ml of CS spotted
	C. Ueoka 2000 [188]	Porcine skin, Seikagaku	Inhibition Assay of MK-mediated adhesion and outgrowth	Rat E17-18 cerebral cortical neurons	No effect	Concentrations up to 50 mg/ml used
	M. Hikino 2003 [54]	Porcine skin, Seikagaku, Various DS preparations from lower marine organisms	Neurite outgrowth	Mouse E16 hippocampal neurons	Except for porcine skin CS-B, all other DS variants promoted neurite outgrowth	CS-B/DS from different organisms exhibited different effects on morphology 2 µg of CS coated
	F. Lafont 1992 [189]	Bovine mucosa, Sigma	Neurite outgrowth	Rat E14 mesencephalon neurons	Stimulated dendritic outgrowth	CS used at 10 µg/ml

CS-C	*V.P. Swarup 2013* [180]	Bovine, Pfaltz and Bauer	Neuron guidance on immobilised stripes of CS	Rat E18 hippocampal neurons	Repellent activity	CS were modified by thiolation
	J.M. Brown 2012 [65]	Shark cartilage, Seikagaku	Neurite outgrowth on CS-coated substrate	Chick E7 dorsal root ganglion cells (DRGs)	No effect	Concentrations up to 100 µg/ml used
			Growth cone collapse	Chick E7 DRGs	No effect	Concentrations up to 100 µg/ml used
			Axon crossing (spot assay)	Rat P5-9 cerebellar granule neurons (CGNs)	No effect	1 mg/ml of CS spotted
	Wang 2008 [64]	Seikagaku	Spot assay	Mouse P5-8 CGNs	No effect	Concentration used not cited
	F. Lafont 1992 [189]	Shark cartilage, Sigma	Neurite outgrowth	Rat E14 mesencephalon neurons	Enhanced neurite outgrowth	CS used at 10 µg/ml
	RJ Gilbert 2005 [68]	Seikagaku	Neurite extension in a 3D culture system with CS-GAGs immobilized on agarose hydrogels	Chick E9 DRG explants	No effect	0.5 mg/ml of CS used
	A.M. Clement 1998 [187]	Shark cartilage, Sigma	Neurite outgrowth	Rat E18 hippocampal neurons	No effect	16 µg/ml of CS spotted
	A.M. Clement 1999 [181]	Not cited	Neurite outgrowth	Rat E18 hippocampal neurons	No effect	CS used at 5 µg/ml
	C. Ueoka 2000 [188]	Shark cartilage, Seikagaku	Inhibition Assay of MK-mediated adhesion and outgrowth	Rat E17-18 cerebral cortical neurons	No effect	Concentrations up to 50 mg/ml used

CS-D	*V.P. Swarup 2013* [180]	Shark cartilage, Seikagaku	Neuron guidance on immobilised stripes of CS	Rat E18 hippocampal neurons	No effect	CS were modified by thiolation
	Wang 2008 [64]	Seikagaku	Axonal guidance assay	Mouse P5-8 CGNs	No effect	Result is only cited in discussion section
	A.M. Clement 1998 [187]	Shark cartilage, Seikagaku	Neurite outgrowth	Rat E18 hippocampal neurons	Modest (15 %) promotion of axonal elongation	16 µg/ml of CS spotted
	A.M. Clement 1999 [181]	Not cited	Neurite outgrowth	Rat E18 hippocampal neurons	Modest promotion of neurite length	CS used at 5 µg/ml
	Chikako Ueoka 2000 [188]	Shark cartilage, Seikagaku	Inhibition Assay of MK-mediated adhesion and outgrowth	Rat E17-18 cerebral cortical neurons	No effect	Concentrations up to 50 mg/ml used
	M. Hikino 2003 [54]	Shark cartilage, Seikagaku	Neurite outgrowth	Mouse E16 hippocampal neurons	Promotion of axonal elongation, no effect on number of neurites	2 µg of CS coated

CS-E	*V.P. Swarup 2013* [180]	Squid cartilage, Seikagaku	Neuron guidance on immobilised stripes of CS	Rat E18 hippocampal neurons	Neurons preferred to grow on CS-E over PLL only	CS were modified by thiolation
	J.M. Brown 2012 [65]	Squid cartilage, Seikagaku	Neurite outgrowth on CS-coated substrate	Chick E7 dorsal root ganglion cells (DRGs)	Inhibition of outgrowth	Concentrations less than 10 µg/ml were effective
			Growth cone collapse	Chick E7 DRGs	Increased growth cone collapse	Concentrations less than 10 µg/ml were effective
			Axon crossing (spot assay)	Rat P5-9 cerebellar granule neurons (CGNs)	Repellent activity	1 mg/ml of CS spotted
	L. Karumbaiah 2017 [186]	Squid cartilage, Seikagaku	Spot assay	Rat E18 cortical neurons	Inhibited attachment and exhibited repellent activity	CS-GAGs were biotinylated Concentrations of 0.7 mg/ml and above were effective
	Wang 2008 [64]	Seikagaku	Axonal guidance assay	Mouse P5-8 CGNs	No effect	Result is only cited in discussion section
	RJ Gilbert 2005 [68]	Seikagaku	Neurite extension in a 3D culture system	Chick E9 DRG explants	Potent inhibitory activity	0.5 mg/ml of CS used
	A.M. Clement 1999 [181]	Not cited	Neurite outgrowth	Rat E18 hippocampal neurons	Substantial promotion of neurite length	CS used at 5 µg/ml
	C. Ueoka 2000 [188]	Squid cartilage, Seikagaku	Inhibition Assay of MK-mediated adhesion and outgrowth	Rat E17-18 cerebral cortical neurons	Potent inhibitory activity	2.5 mg/ml of CS was effective
	M. Hikino 2003 [54]	Squid cartilage, Seikagaku	Neurite outgrowth	Mouse E16 hippocampal neurons	Promotion of axonal elongation, reduced number of neurites	2 µg of CS coated
	G. Dick 2013 [118]	PNN-GAGs extracted from adult rat brain	Neurite outgrowth assay using a CS-E blocking antibody	Adult rat DRG explants	Blocking CS-E improved neurite outgrowth	An indirect demonstration of inhibitory activity for CS-E

Table 4: A list of *in vivo* evidences for CSPG expression and sulfation in the injured CNS		
Study	**Injury type**	**Observations**
L.L. Jones 2003 [161]	Dorsal column spinal cord transection in the rat	Neurocan, brevican, and versican expression increased around the lesion site within days following injury, peaking at 2 weeks. Neurocan and versican upregulation persisted for 4 weeks, while brevican expression lasted for at least 2 months.
L.L. Jones 2002 [190]	Dorsal column spinal cord transection in the rat	NG2 was highly expressed by macrophages and oligodendrocyte progenitors within 24 hrs of injury, peaking at 1 week, and persisting for 8 weeks. Versican, neurocan and brevican were moderately upregulated after 7 days, while phosphacan was downregulated.
J.M. Levine, 1994 [191]	Puncture lesion of the adult rat cerebellum	Upregulation of NG2 expression adjacent to the lesion site that peaked at 7 days.
N. Harris, 2009 [62]	Cerebral contusion injury in the rat	Upregulation of neurocan and versican in the glial scar. High expression of neurocan, versican, aggrecan, and phosphacan in regions delimiting the contusion site 7 days after injury, mostly by astrocytes.
C.C. Stichel, 1995 [185]	Transection of post-commissural fornix of the adult rat	Rapid upregulation of decorin by astrocytes in a wide area around the lesion, followed by an accumulation of biglycan that was confined to the lesion site. Both proteoglycans persisted in the lesion site for 6 months after transection (decorin and biglycan are DS/CS containing small leucine-rich repeat proteoglycans, or SLRPs).
A. Buss, 2009 [63]	Biopsies of human patients with traumatic, complete SCI	In SCI, NG2 and phosphacan were found in the glial scar. Neurocan and versican were found only in the lesion epicentre.
Jae-Hyuk Yi, 2012 [184]	A controlled cortical impact injury over the left sensory motor cortex in mice, resulting in a traumatic brain injury (TBI)	Neurocan and NG2 expression was increased (and overlapped with an upregulation of 4-sulfated CS-GAGs) in a tight band surrounding the injury core within 7 days after injury, and this persisted for 4 weeks. Aggrecan and phosphacan expression was decreased in the regions surrounding the injury site. 2, 6-disulfated CS-GAGs (CS-D) were also reported to be upregulated in the injured cortex.
H. Wang, 2008 [64]	Dorsal hemisection of the mouse spinal cord	High astrocytic expression of 4-sulfated CS-GAGs delimiting the lesion within 24 hours following injury. Low levels of 6-sulfated CS-GAGs observed.
R.J Gilbert, 2005 [68]	Nitrocellulose filters implanted into adult rat cortices to stimulate glial scar formation	Chondroitin 4-sulfate (CS-A) was dominant in the uninjured brain, while chondroitin 6-sulfate (CS-C) and 4, 6-disulfate (CS-E) were overexpressed in the glial scar.
F. Properzi, 2005 [183]	Stab wound to adult rat cerebral cortex	CS-C synthesis and expression is increased around the injury site, but not other CS types. 6-sulphated CS-GAGs were highly expressed on the surface of OPCs.
H. Li, 2013 [18]	Transection of nigrostriatal pathway in the adult mice midbrain	DS and CS accumulated in the glial scar and the perilesional area of the injured brain.

1.3 Pathologies of the Central nervous system: the role of CSPGs

CNS pathologies can broadly be divided into traumatic, neurovascular, infectious and neurodegenerative. Traumatic injuries are majoritarily contusive in nature, but can also involve lacerations. Sharp physical forces that cause sudden compression, displacement, and shearing of nervous tissue will compromise neural networks and can cause a wide variety of debilitating symptoms depending on the extent and site of the injury [192]. Neurodegenerative disorders such as Alzheimer's disease (AD), multiple sclerosis (MS) and Parkinson's disease share many features, including an elusive cause for pathogenesis, the progressive degeneration of neurons, the lack of a cure, and a limited capacity for regeneration by the nervous tissue [193].

Innumerous studies have found that CSPGs are upregulated in and around damaged nervous tissues in both traumatic and neurodegenerative injuries, and they are believed to be at the heart of regenerative failure [17, 52, 62, 161, 183–185]. In the following section we will look at the examples of spinal cord injury and multiple sclerosis, and review the evidence for the role of CSPGs in these pathologies.

Spinal cord Injury

Spinal cord injuries (SCIs) are majoritarily caused by physical trauma, such as from falling, vehicle accidents, or gunshot wounds. The severity of the injury depends on location, and on whether it is classified as complete (causing a total loss of affected motor or sensorial functions) or incomplete injury (partial loss of function as not all nervous communication is severed by the traumatic event) [58].

The damaged neural tissue becomes the site of much epigenetic transformation and glial cell activity, including the mobilisation of reactive astrocytes, microglia, fibroblasts and infiltrating cells of the innate immune system which contribute to the formation of an inhibitory environment, a 'quarantine zone' delimited by cellular and chemical barriers, called the glial scar [59–61].

The latter is a protective response, isolating the damaged tissue and separating it from healthy tissue so as to prevent the spread of a potential microbial invasion and confine the immune response [194, 195]. Interfering with reactive astrocytes or their ablation disrupts scar formation, which resulted in an increased lesion size, dysregulation of local inflammatory immune responses, disordered repair of the blood brain barrier, increased neuronal loss, and worse clinical outcomes [194–197].

However, the glial scar ultimately becomes a barrier to neuronal regeneration. The upregulated production of a variety of inhibitory molecules like CSPGs, PTPRS, KSPGs, semaphorins, ephrins and myelin-associated inhibitors, especially by reactive astrocytes, create an environment unfavourable to regrowing axons and remyelinating cells [2, 4, 7, 61–63].

For this reason, such injuries are commonly associated with permanent debilitation, a grim perception brought about by the inability of CNS neurons to reextend across the damaged tissue and reform lost connections. This regenerative failure was first observed by Ramon y Cajal, who described severed axons apparently aborting repair at the glial scar, destined to degenerate [198].

Many more recent studies, however, have revealed that neurons of the CNS are intrinsically inclined to regenerate [61, 199, 200]. Their capacity to do so has been shown to depend on several factors, including the permissibility of the local environment, especially in and around the glial scar [7, 201, 202].

Many studies have identified CSPGs as key players in the inhibition of neuronal regeneration following injury. *In vivo* studies reporting that CSPGs are upregulated following injury [52, 64, 68, 161, 183, 184] give importance to *in vitro* findings demonstrating their inhibitory effects on cells of the CNS. Neurons cultured on CSPG-coated substrates exhibit many defects, including stunted axonal elongation, dystrophic growth cones, reduced neurite outgrowth, and disrupted adhesion [3, 15, 64–68]. Consistently, these effects have been shown to be abolished by the digestion of GAG chains with the bacterial enzyme chondroitinase ABC (ChABC). These observations have been translated into many *in vivo* studies demonstrating the effectiveness of ChABC treatment in traumatic CNS injuries of both the brain and spinal cord [18, 23, 69, 70]. For example, the digestion of CS-GAGs was shown

to improve functional recovery, including locomotion, following contusion [203], dorsal column crush [70], and hemisection SCI in rats [204, 205].

The main disadvantages of ChABC treatment are the invasiveness of applying the enzyme locally, the incomplete digestion of CSPGs, and the unwanted digestion of other proteoglycans that may be beneficial for healing. Nonetheless, the treatment demonstrates the potential of targeting CS-GAG chains for regenerative therapy.

In addition to neuronal damage, traumatic injuries also involve progressive demyelination [72, 73], a degenerative feature shared with MS, which will be the subject of the next section.

Multiple sclerosis

MS is the most common immune-mediated pathology of the central nervous system, driven by an autoimmune attack on the myelin sheaths that are essential for the normal saltatory conduction of action potentials along axons, and the latter's metabolic maintenance [74, 75]. The name comes from the multiple lesions ('sclerae' as in scars, also referred to as plaques) that appear in the white matter and grey matter of the spinal cord and brain [76–79].

The immune-mediated demyelination and inflammation of nervous tissue eventually lead to a progressive degeneration of axons, causing a wide variety of symptoms depending on the areas affected, such as blindness, numbness, loss of motor control and other cognitive functions [74, 75, 80]. Though the average life span is only reduced by 5-10 years [206, 207], the chronic disease is severely debilitating, and compromises the patient's independence and quality of life. Depression is also a sever secondary effect, and suicide rates are increased in MS patients [208]. The disease most commonly is diagnosed in young people between twenty to thirty years of age, and is two-to-three times more common in women than in men [207].

The majority of patients initially present a relapsing-remitting MS (RRMS), but up to half of these evolve into a progressive type if untreated (secondary progressive MS, SPMS) that is characterized by a continuous deterioration of symptoms and brain atrophy [209]. About 15% of patients exhibit a progressive type of disease at onset (primary progressive MS,

PPMS). A fourth type, progressive-relapsing MS (PRMS) is a rare form where patients have a progressive disease exhibiting relapses without remissions.

As is typical of neurodegenerative diseases, the mechanisms behind MS pathogenesis remain elusive, and the disease has yet no known cure. Current treatments are limited to being anti-inflammatory in nature, controlling the symptoms especially the frequency and severity of relapses, and thus slowing the progression of the disease [210], but these have had limited success in the case of progressive MS (though a recent breakthrough was made in the form of ocrelizumab, an antibody that selectively depletes CD20-expressing B-cells, and that was found to have some effectiveness in progressive MS [210, 211]).

Regeneration-promoting strategies have recently garnered much interest in light of evidence for extensive intrinsic remyelination in MS, which results from a mobilisation of resident NG2-positive oligodendrocyte precursor cells (OPCs), and their differentiation into myelinating oligodendrocytes (OLs) [210, 212, 213]. Such endogenous repair has been reported in both relapsing-remitting as well as progressive disease, and can be persistent in a long disease course [13, 212, 214–216]. Despite this, regeneration in the face of chronic CNS insult eventually loses its effectiveness [217], and it is this chronic demyelination and inflammation that leads to axonal degeneration, particularly in progressive MS, and lasting neurological disability in patients [218–220]. Reflective of this, OPCs can be found accumulating outside of early demyelinated lesions, but much fewer OPCs are found in chronic lesions, and are unable to differentiate [221], indicative of remyelination failure.

There is an anatomic variability to the extent of remyelination as well. When looking at lesions from the white and grey matter from the same patient, it was found that repair is favoured in the grey matter [222]. Tellingly, in a separate study myelinating oligodendrocytes were found to be more common in cortical lesions than in white matter lesions [13]. This correlated with fewer reactive astrocytes in grey matter lesions. In white matter lesions, reactive astrocytes were found to upregulate hyaluronan, and versican [13].

Other studies expand upon this observation. Hyaluronan [223], CSPGs including aggrecan, brevican, neurocan and versican, as well as DSPGs [12, 14, 81] have all been found to be upregulated in MS lesions, associated with astrocytosis, as well as in spinal cords of mice with experimental autoimmune encephalomyelitis (EAE, a mouse model for MS). In another

mouse model, CSPGs accumulated in lysolecithin-induced demyelinating lesions, and their clearance coincided with remyelination [5].

Many *in vitro* studies have demonstrated that CSPG-rich substrates inhibit OPC adhesion, process outgrowth, differentiation into OLs, and ability to myelinate cocultured neurons, and that enzymatic removal of the CS-GAG chains by ChABC treatment abolishes this activity [5, 24, 26, 82].

These findings shed light on the inhibitory roles of CSPGs in the eventual failure of regeneration in MS. Remyelination requires the mobilisation of OPCs to the damaged site, and their differentiation into OLs that extend and direct their processes to envelop 'unsheathed' axons. However, in response to the immune-mediated onslaught, reactive astrocytosis ensues and creates an inhibitory environment very much like the glial scar described previously.

As with SCI, the deposition of CSPGs is likely responsible for impeding the recruitment of OPCs to the demyelinated tissue and their subsequent differentiation, compromising their reparative potential. The exhaustion of local regenerative capacity and resultant chronic demyelination leads to axonal degeneration [219, 224]. The ability for neurons to reform the lost connections would likely also be inhibited by the persistence of the CSPG-rich environment, another common feature between MS and traumatic injuries.

This makes CSPGs an attractive target in regenerative therapy. Indeed, studies have demonstrated the potential of CSPG-targeting treatments *in vivo* to improve remyelination. The inhibition of CSPG synthesis in lysolecithin-demyelinated mice by xyloside [5] or fluorosamine [82] treatments both reduced CSPG burden in the lesioned spinal cord, and improved the recruitment of myelinating cells, and subsequent remyelination of lesions. Additionally, fluorosamine treatment in EAE mice resulted in a reduced upregulation of versican RNA, and a lower disease severity. The digestion of CS-GAG chains by ChABC also increased OPC migration and axonal myelination in a contusion SCI model [225], however given that ChABC requires a local application to be effective, it does not present a practical option for MS where lesions develop throughout the brain and spinal cord.

1.4 Known Partners of CSPGs

CSPGs are known to effectuate their inhibitory roles through the interaction with receptors expressed by cells of the CNS. The best characterized of these include members of the receptor-type protein tyrosine phosphatase (RPTP) family and Nogo receptor (NgR) family. In addition to these, CSPGs have also shown to work in tandem with secreted members of the semaphorin family. In the current study we focus on an example of each of these groups, namely receptor-type protein-tyrosine phosphatase sigma (PTPRS), Nogo receptor 1 (NgR1), and semaphorin 3a (Sema3A).

PTPRS

The enzyme called receptor-type protein-tyrosine phosphatase sigma, also known as protein-tyrosine phosphatase sigma (PTPRS, or PTPσ), is part of the receptor-type (RPTP) subfamily of the protein tyrosine phosphatase (PTP) family, which has over a hundred members with roles in a variety of cellular processes, including differentiation, division, oncogenesis, and neuronal plasticity (figure 4) [83–86].

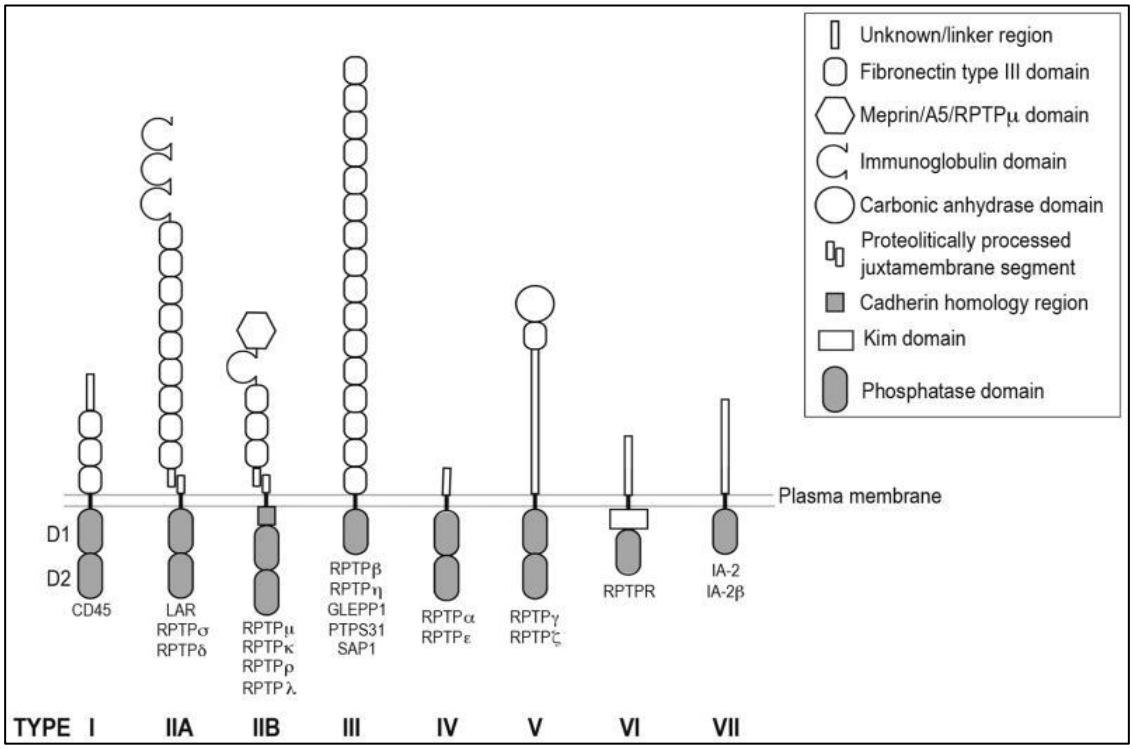


Figure 4: Structural schematisation and classification of human RPTP family members.
Source: Mohebiany et al. 2013, [227].

The RPTP subfamily is particularly important in the development of the CNS and various neuronal processes [86–88]. Within this group, PTPRS and two other members, leukocyte antigen-related (LAR, or PTPRF), and RPTP-delta (RPTPD), form the LAR family RPTPs, and have been shown to bind to sulfated GAGs with roles in neuronal guidance and extension [89]. These are transmembrane proteins consisting of two intracellular PTPase domains, and multiple extracellular Ig-like and fibronectin type III-like domains that can interact with the ECM [226].

PTPRS is expressed by neurons in the developing brain, and decreases gradually into adulthood until it is limited to areas of synaptic plasticity [86, 227, 228], but has also been found to be upregulated in CNS pathologies. It concentrates in dystrophic growth cones of axons reaching the outer regions of SCI lesions in mice [33], and was reported to be upregulated by OPCs in mouse models of MS [8]. PTPRS levels were found to be increased in the lesioned spinal cords of mice with EAE or LPC-induced focal demyelination, and correlated with the upregulation of CSPGs [8].

PTPRS was found to play an important role in repair in the CNS through the interaction of sulfated proteoglycans, but studies have shown that its function is dependent on the type of GAG that it interacts with [89]. HSPGs and CSPGs can both bind to PTPRS with similar affinities, and have been shown to share the same binding site on PTPRS's first Ig-like domain, resulting in competition between the two. Depending on whether PTPRS molecules are interacting with HSPGs or CSPGs, they will cluster or be spread out on cell surfaces respectively, owing to the different distributions of sulfated HS and CS motifs [89, 228–230]. This will likely explain the opposing effects of the two combinations. PTPRS binding to HSPGs has been shown to have an overall beneficial effect on axonal growth [89, 231], but this is reversed upon interacting with CSPGs [31, 44, 89].

Owing to the well-characterized role of CSPGs in the inhibition of repair and regeneration in pathologies of the CNS, their interactions with PTPRS have garnered much scrutiny [102]. CSPG-mediated inhibition of neurite outgrowth *in vitro* is reduced in several types of neurons deficient for PTPRS, including DRG neurons [65, 90], retinal ganglion cells [94], cerebellar granule neurons [28, 92], and cortical neurons [93]. PTPRS-deletion was found to specifically mitigate the effects of CSPGs, as inhibition of neuronal outgrowth by myelin-

associated glycoprotein (MAG, see section on NgR1 below) was not affected [90, 92]. The incomplete neutralisation of CSPG-inhibition in neurons lacking PTPRS suggests the involvement of additional CSPG-partners; this is expanded upon in the section on NgR1, below.

PTPRS' sister family member, LAR, has also been shown to similarly modulate neuronal growth by interacting with HSPGs and CSPGs [28, 89, 229, 232]. LAR family members were also found to be involved in the modulation of myelinating cells of the CNS. In one study, the inhibition of LAR and PTPRS was found to reverse the inhibition of survival, process outgrowth, maturation, and myelinating-ability of oligodendrocyte progenitor cells (OPCs) *in vitro* [30]. In a separate study, the CSPG-mediated inhibition of oligodendrocyte (OL) process outgrowth and myelination of neurons *in vitro* was reported to be abolished by the RNAi-mediated down-regulation of PTPRS, and in OLs lacking PTPRS [26]. These observations show that RPTP-CSPG interactions may be involved in impaired remyelination in the injured CNS, in addition to the inhibition of axonal repair.

Various *in vivo* studies have confirmed this, for example mice deficient in PTPRS have demonstrated enhanced regeneration following optic nerve lesion [94], and following SCI with axons entering into the CSPG-rich lesion penumbra [90, 92]. Deletion of LAR was also shown to improve axonal regrowth and recovery in mouse SCI model [233], and similar results were also reported in the peripheral nervous system; PTPRS-deficient mice showed improved sciatic nerve regeneration following injury [234], as well as better functional recovery following facial nerve crush injury [93].

Treatments targeting PTPRS and LAR translated these observations into promising results in mouse models. Silver's team developed two membrane-permeable blocking peptides against LAR and PTPRS called intracellular LAR peptide (ILP) and intracellular sigma peptide (ISP), respectively, that inhibited interactions with CSPGs [33]. Several subsequent studies found these molecules to be effective at reversing the effects of CSPGs in SCI models, regulating neuroinflammation, facilitating OPC mobilisation, differentiation and myelination, and promoting overall functional recovery [30, 32, 33, 95]. ISP and ILP treatments were found to be more effective when combined, highlighting that targeting multiple CSPG partners is more effective than blocking individual pathways.

In addition to SCI, ISP was also demonstrated to be effective in mouse models of MS [8]. ISP boosted remyelination in LPC-induced demyelination organotypic cerebellar cultures, and in EAE mice. ISP-treated EAE had reduced severity and progression, with smaller lesions and improved CSPG clearance observed.

NgR1

The family of Nogo receptors (NgRs), also known as reticulon receptors (RTNRs), is comprised of three members, NgR1, NgR2, and NgR3, which are 473, 420 and 445 amino acids long respectively [96, 97]. NgRs are majoritarily expressed by a variety of neurons of both the central and peripheral nervous systems [97–99]. They are composed of a signal sequence, eight leucine-rich-repeat domains that are flanked by LRR-capping domains, a C-terminal stalk region of about 100 amino acids in length, and a glycosylphosphatidylinositol (GPI) residue anchoring them to cell membranes [96].

NgRs act as receptors for certain myelin-associated inhibitors (MAIs), and can have overlapping and separate binding preferences. For example, NgR1 and NgR2 both bind to myelin-associated glycoprotein (MAG) [235, 236], while NgR1 selectively binds to Nogo66 (a functional domain of Nogo ligands that are expressed by oligodendrocytes and their myelin sheaths) [96, 98, 237]. Some neurons can co-express both the Nogo ligand and its receptor [99]. NgR3 is a relatively uncharacterized member of its family, exhibiting no binding to any of the aforementioned ligands. It may play the part of a co-receptor for NgR1 [34, 238].

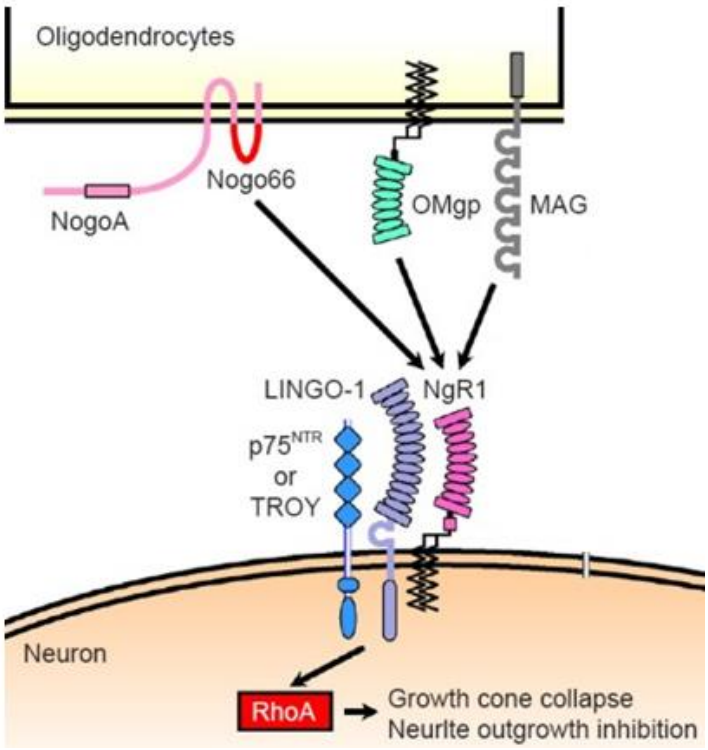


Figure 5: Schematic representation of molecular partners of NgR1.
Adapted from: Kurihara & Takei, 2015, [245]. Oligodendrocytes express MAIs such as NogoA and MAG. These induce growth cone collapse and inhibit outgrowth of neurons expressing NgR1 and its coreceptors.

Many *in vitro* and *in vivo* studies support NgR1's role in the inhibition of axonal regeneration and remyelination through its myelin-associated ligands [100–104]. The p75 or TROY coreceptors are necessary for this signalling pathway, along with the involvement of the adaptor protein LINGO1 (figure 5). Targeting this pathway has been shown to have beneficial effects, improving axonal regeneration *in vitro* and *in vivo* [34, 104, 239, 240].

However, an impactful study by Dickendesher's team at the University of Michigan found that NgR1 and NgR3 are receptors of CSPGs and mediate the latter's inhibitory activities [34]. GAG-dependent binding of soluble NgR1 and NgR3 was specific to injured optic nerve sections, coincidentally where CSPGs are known to be upregulated [34, 60]. Certain CS-GAGs (types B, D, and E) competed for NgR binding to neural tissue, and ChABC pre-treatment of the brain sections abolished NgR binding as well, further confirming that NgRs are interacting with CSPGs. Rat CGNs from mice deficient in both NgR1 and NgR3 exhibited resistance to CSPG-mediated inhibition, though this was not observed in CGNs from mice deficient in only either one of the receptors alone, indicating some functional redundancy between NgR1 and NgR3. The resistance of the double-mutant neurons to CSPGs was partial, and similar to that

of PTPRS-deficient neurons. In the same study, CSPG-mediated inhibition of CGNs is abolished when seeded in the presence of soluble NgR1-Fc or PTPRS-Fc, further demonstrating that both proteins are facilitating inhibition by CSPGs. This example presents an obstacle to targeting CSPGs, eliciting the need for a blocking agent capable of disrupting multiple CSPG-protein interactions. Underlining this, the study also reported that the combined loss of NgR1, NgR3, and PTPRS resulted in better axonal regeneration following retro–orbital optic nerve crush injury in mice, compared to the loss of any one receptor alone. Of curious note, the NgR-coreceptor p75 was not found to be necessary for CSPG inhibition, in contrast to the canonical pathways involving MAI ligands [34, 241].

Regarding traumatic CNS injury, a Nogo-A-blocking treatment and ChABC treatment were both found to have a similar effectiveness in improving axonal regeneration and recovery of locomotor functions in a rat SCI model [69]. Combining both treatments, however, produced even better results, highlighting the coexistence of a GAG-dependant and a GAG-independent regeneration-inhibiting pathway for NgRs. A separate study targeting NgR1 using lentiviral vector delivery of short hairpin RNA in a rat SCI model also yielded positive results, reporting improved neural regeneration, motor function recovery, remyelination, and lesion healing [242].

On the subject of neurodegenerative disease, studies have reported an upregulation of NgRs and their partners in MS lesions and in EAE mice, implicating NgR in the inhibition of repair [99, 101, 105–107]. Targeting NgR pathways has been shown to promote remyelination, axonal regeneration, and debris clearance in EAE [106, 108, 109], and mice deficient for NgR1 exhibited reduced disease severity and progression [106, 107].

Sema3A

Semaphorins are a large family of secreted, transmembrane or cell surface-associated proteins defined by their cysteine-rich semaphorin domains, and have a wide variety of roles in various tissues and stages of development of both vertebrates and invertebrates alike [110, 111]. Semaphorins also comprise the largest of the four general families of guidance molecules of the nervous system (the others being the ephrins (see section on EFNB2), the slits (see section on Slit2), and the netrins), with a variety of members exhibiting chemoattractant or chemorepellent activity, or even both [111, 114].

Semaphorin 3A (Sema3A) is a group 3 member with a key involvement in neuronal and OPC migration and guidance during development [112–115]. It is a secreted molecule that associates with the ECM, particularly with perineuronal nets (PNNs) [116, 117]. These organised, lattice-like complexes are found proximal to certain neuron types and closely associate with their synapses, with key roles in modulating development and restricting plasticity and repair (figure 6)[243, 244]. CSPGs of the lectican family are major components of PNNs, and have been found to be the scaffolds to which Sema3A docks to in the ECM [116, 118]. Kwok's team further characterized the involvement of the CS-E motif with the use of a CS-E blocking antibody that inhibited binding of a recombinant Sema3A to rat brain PNNs [118]. Furthermore, ChABC treatment abolishes Sema3A localisation to the PNNs, and enhances neuronal plasticity [116, 245].

Sema3A binding and accumulation on CS-GAGs could facilitate its signalling through its receptor components, neuropilin-1 (Nrp1) and plexin A1. Reinforcing this, a study showed that Sema3A binding to CS-GAGs enhances the repulsive effects of CSPGs on cortical interneurons expressing Nrp1 *in vitro* [120]. In an *ex vivo* experiment with mouse brain slices, neuron migration into the CS-Sema3A-rich striatal mantle zone is enhanced by ChABC or Nrp1-blocking treatments. Further, GAG-associated Sema3A is able to induce the clustering of neuronal Nrp1, and has an enhanced ability to induce axonal growth cone collapse [119]. Altogether these observations suggest that Sema3A and CS-GAGs work together to mediate the effects of PNNs.

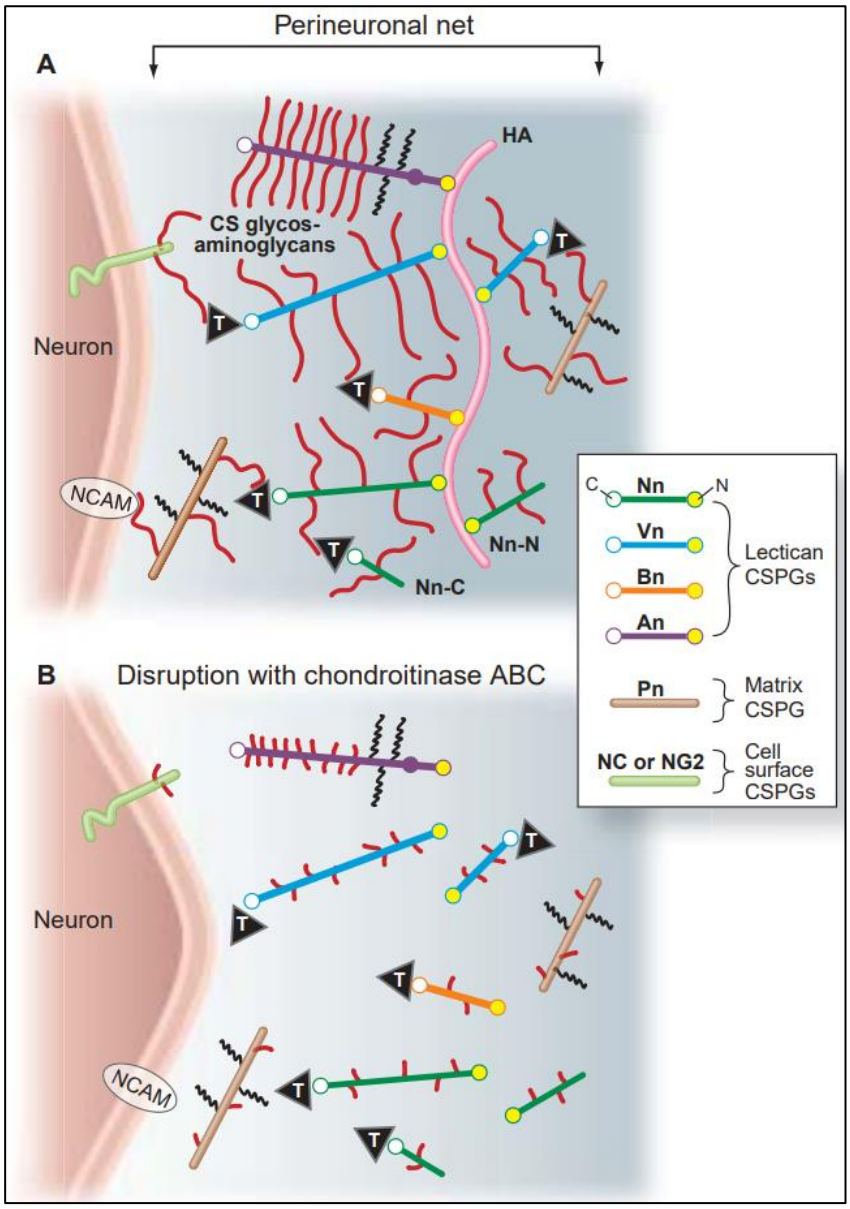


Figure 6: Schematic representation of the perineuronal net.

Source: Fox and Caterson, 2002, [249]. (A): Hyaluronan (HA, coloured pink) acts as a scaffold for the noncovalent attachment of members of the lectican family, aggrecan (An), brevican (Bn), neurocan (Nn), and versican (Vn). A matrix glycoprotein, tenascin, (T, black triangles) is able to interact with CS-GAG chains, as well as the C-terminal domains of some CSPGs, facilitating the formation of a large macromolecular lattice. Cell surface-associated CSPGs such as NG2 and phosphacan (Pn) are also represented. (B): The degradation of CS-GAGs by chondroitinase ABC treatment results in the disruption of the interactions that hold the structure together, facilitating neuronal extension and plasticity.

Though Sema3A has been extensively studied for its role in development, it has recently garnered much interest regarding its roles in the adult CNS, particularly in the context of injury [115]. Several studies in rat models of CNS injury have found that Sema3A is upregulated following injury in the CNS, after intramedullary axotomy of lumbar spinal cord motoneurons [122], transection of the thoracic dorsal columns [121], and complete transection or contusion lesions of the adult spinal cord [123]. Sema3A expression was accumulated at the lesion sites, and axons were unable to enter these regions, likely at least in part to their constitutive expression of Nrp1 and plexin A1 [121, 123]. These observations are reinforced by studies demonstrating the repellent activities of Sema3A on neurons *in vitro* and *in vivo* [246, 247]. A curious note is that injury to the peripheral nervous system (PNS) was reported to exhibit *down*-regulated Sema3A mRNA expression by neurons [248]. As this correlates with the well-known robust and spontaneous regenerative capacity of the PNS, this observation alludes to Sema3A's inhibitory role in CNS injuries that fail to repair.

In addition to these observations in traumatic injuries, semaphorins have also been implicated in neurodegenerative diseases [124], for example in Alzheimer's disease [249] and Parkinson's disease [250]. Adhering to the current study, Sema3A has been especially scrutinized for its role in impairing remyelination in MS. Several studies have reported the potent upregulation of Sema3A in demyelinated lesions in both MS patients (mostly in chronic active lesions) [125, 126] as well as rodent models of MS [125, 127, 128].

Sema3A expression in these lesions was closely associated with poor remyelination. Since OPCs express neuropilins and plexins [128], they are sensitive to semaphorin-guidance cues and this plays a role in development [112, 113]. However, in the adult CNS, this could be responsible for the impaired mobilisation of OPCs following injury.

Confirming the link, the addition of recombinant Sema3A to LPC-demyelinated lesions in mice [129], or ethidium bromide-demyelinated lesions in the rat [130] was found to inhibit OPC recruitment and remyelination. On the other hand, transgenic mice with reduced Sema3A expression [126] or truncated Sema3A-Nrp1 binding [128] saw improvement in these parameters.

Considering these evidences altogether, one can suggest that Sema3A and CSPGs work in tandem as a repulsive complex to create an environment inhibitory to neuronal regeneration and remyelination. In light of this, the effectiveness of ChABC treatment on

regeneration can also be owed to the release of Sema3A from their scaffolds in the ECM [119] in addition to disrupting PTPRS and NgR1 signalling.

It should be noted that Sema3A is far from being the only member of its family with potentially important roles in repair. Other semaphorins (Sema4D, Sema5A, and Sema7A) are also upregulated following CNS injury, and also modulate OPC recruitment or neuronal regrowth [123, 127, 251, 252]. Among them, Sema7A has been demonstrated to oppose the inhibitory effects of Sema3A, promoting regeneration in the CNS [125, 127, 128]. At least one other semaphorin apart from Sema3A exhibits CS-binding ability: Sema5A is a group 5 family member found to be both an attractive guidance cue, as well as a repulsive cue to axon elongation depending on whether it is interacting with HSPGs or CSPGs, respectively (echoing the similar phenomenon described for PTPRS) [253]. Similar to other CSPG-partner proteins described in this study, Sema5A has been shown to induce growth-cone collapse and inhibit neurite outgrowth [252].

Altogether these evidences show that CSPGs work hand in hand with a variety of proteins from different families, acting as a sort of bottleneck for many converging inhibitory pathways.

1.5 Potential Partners of CSPGs

CSPGs interact with a wide variety of proteins from different families, both membrane-bound and secreted, to effectuate their inhibitory functions. We chose to extend our study to include members of two other major families of neural guidance molecules: the transmembrane ephrins, and the secreted slits, to review and screen for their potential to partner with CSPGs to inhibit regeneration.

EFNB2

Ephrins (EFNs) are cell-bound, transmembrane ligands that exhibit bidirectional signalling by *trans* interactions with cell surface Eph receptors, the largest subfamily of receptor tyrosine kinases [131, 254]. The receptors and ligands are divided into two subfamilies, designated A and B; ephrin-As are anchored to cell membranes by a glycosylphosphatidylinositol (GPI) linkage, while the ephrin-Bs are transmembrane proteins with intracellular domains [255]. As a general rule, ephrins and Ephs interact with each other within their own subfamily, however there are notable exceptions, for example EphA4 which is well known to functionally interact with ephrin-B2 (EFNB2) (figure 7) [256, 257]. Naturally, such complexity gives rise to a variety of roles for this family in cell guidance, development, inflammation, cell adhesion, as well as angiogenesis and invasion in cancers [258, 259]. Early discoveries that members of the ephrin family were repulsive axon guidance signals motivated deeper investigations on the roles of these molecules in the inhibition of repair and regeneration [260]. In the CNS, ephrin ligands and receptors are upregulated on neurons, astrocytes, as well as OPCs following injury [38, 39, 131–133]. In addition, they have been shown to have an upregulated expression in and around active lesions in multiple sclerosis, influencing the immunopathogenesis of the disease [132, 134].

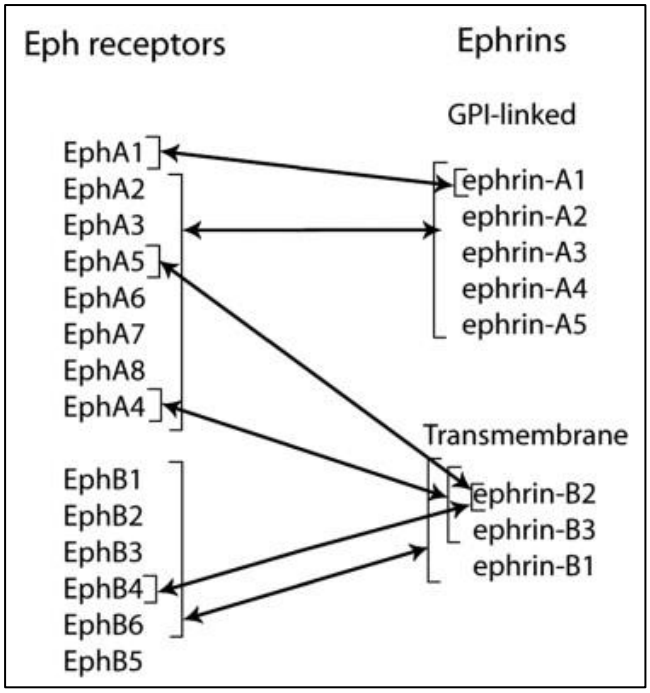


Figure 7: Eph receptor and ephrin ligand binding preferences.
Source: Coulthard et al. 2012, [262]. Note the promiscuity of class B ephrins, particularly ephrin-B2, in their interactions with EphA and EphB receptors.

Ephrin-B3 (EFNB3), a myelin based ligand known to have repellent activity in axonal pathfinding during development, was found to inhibit neurite outgrowth of cortical neurons *in vitro* [261]. It was later reported that EFNB3-defficient mice exhibited significantly improved axonal regeneration following optic nerve crush injury, highlighting a role in modulating repair for ephrins [262].

Several ephrins including EFNB3 and its close relative, EFNB2, were found to be upregulated on differentiating oligodendrocytes, and modulated their maturation and myelinating capacity *in vitro* [263]. Of note, their activity depended on the directionality of their signalling through various Eph receptors, adding a level of complexity to these pathways.

EFNB2, the chosen candidate of this family of proteins for this study, is also among the ephrins that are upregulated following CNS injury, and interacts with various Eph receptors, including EphA4 and EphB2, which have been implicated in the inhibition of repair processes [39]. EFNB2 expressed by reactive astrocytes was found to be a key player in the formation of the glial scar following spinal cord injury in rats. This was reinforced by the establishment of a conditional knockout mouse strain in which the EFNB2 gene was deleted in astrocytes, revealed that EFNB2 upregulation by reactive astrocytes is critical for the establishment of an inhibitory environment around CNS lesions [38]. Notably, it was suggested that the EFNB2 pathway works in tandem with CSPGs to accomplish this.

Of relevance to the current study, previous work reported that ephrin-A3 binds to heparin and heparan sulfate (HS), and that the latter was a modulating cofactor in the functional binding of ephrin-A3 to its receptors, EphA2 and A4 [264]. This was a significant find, as it raises the possibility that the complex and diverse ephrins-Ephs interactions could be moderated by sulfated proteoglycans in their environment. Advocating for this idea, another study reported that EFNB3 also binds to a sulfated proteoglycan on the surfaces of cells [35]. They set out to screen ephrins for binding to HS and heparin, and reported good affinity bindings for EFNB3, EFNA3, and a relatively lower affinity binding for EFNB2.

In a proteomic analysis screening for potential interactions with CS-A, some members of the ephrins and Ephs families were identified as binders, although it should be noted that the majority of proteins screened in this study were "*produced in baculovirus expression systems as N-terminal glutathione S-transferase fusion proteins and purified using glutathione S-Sepharose under nondenaturing conditions. These proteins, therefore, do not carry any of the posttranslational modifications, such as glycosylation side chains, that they may carry when synthesized in their normal human cell environment*" [265].

Of greater relevance, it was reported that EphA4, a receptor of EFNB2 and a well-established inhibitor of repair in the CNS, functionally binds to CSPGs and in particular CS-E to inhibit neurite outgrowth [135]. It was determined that CS-E binding induced the clustering of cell surface EphA4, which is necessary for the latter's phosphorylation. This suggests that CSPGs are involved in modulating the EphA4 binding to ligands like EFNB2. We aim to add to this theory by determining if the ligand EFNB2 interacts with CS-GAGs as well. In addition to the previously cited pathways, a putative blocking agent of CSPGs would theoretically interfere with the inhibitory functions of ephrins in the CNS. To the best of our knowledge, the current study is the first to investigate the potential interactions between a candidate ephrin, EFNB2, and a variety of CS-types.

Slit2

The Slits are a family of secreted proteins of about 200 kDa that are well known for their role as chemorepellent guidance molecules in the developing nervous system, but are also present in the adult. They are expressed by glial cells as well as neurons, and bind to the roundabout (Robo) receptors to influence guidance, branching, and migration of neurons and OPCs [41, 136–138].

Slit2 can undergo proteolytic cleavage *in vivo,* resulting in a N-terminal fragment (Slit2-N; ~140 kDa), and C-terminal fragment (Slit2-C; ~60 kDa)[40]. Full length Slit2 (Slit2-FL), as well as Slit2-N have been the subject of numerous studies describing their distinct and overlapping effects on neural plasticity [138, 139]. For example, both can repel olfactory bulb axons, however only Slit2-N can induce growth cone collapse. Additionally, while Slit2-N can promote the outgrowth of dorsal root ganglion neurons, Slit2-FL antagonizes this activity [40].

The role of the C-terminal fragment had long remained elusive, and was even speculated to have no function owing to the fact that it did not bind to Robo receptors [40]. A relevant bioactivity was only recently brought to light, when Slit2-C was found to functionally bind to PlexinA1, inducing growth cone collapse in commissural axons [266]**.**

The relevance of Slit2 to this study is highlighted by its well-characterized interactions with HSPGs. Indeed, HSPGs, in particular syndecan1, are necessary coreceptors for functional binding of Slit2-FL and Slit2-N to Robo receptors, and for their repellent activity [36, 37, 140, 141]. Slit2-C and full length Slit2, but not Slit2-N, have been shown to interact with glypican-1 *in vivo*, another major HSPG of nervous tissue [267, 268]. Slit2 and glypican-1 mRNA expression was reported to increase in reactive astrocytes delimiting lesions of the adult CNS in mice, leading to the speculation that, beyond its role in modulating plasticity, the protein may also play a part in the inhibition of repair.

As previously mentioned, molecules with functional binding to HSPGs are often also found to interact with CSPGs; to date, and to the best of our knowledge, only one study so far has described that this is the case for Slit2 as well [37]. Importantly, it was reported that decoy CS-polysaccharides abolished the Slit2-mediated repulsion of axons and migrating neurons, suggesting that CS-GAGs modulate Slit2 activity.

2 APRIL: A proliferation inducing ligand

2.1 Structure and physio-biology

A proliferation inducing ligand (APRIL, TNFSF13) is the thirteenth member of the tumor necrosis factor superfamily (TNSF) [269]. As is typical in this family, APRIL is produced as a homotrimer. It harbours a furin cleavage site in its extracellular domain proximal to the transmembrane domain, leading to its cleavage from the Golgi apparatus and secretion from the cell [270].

APRIL is produced by myeloid cells such as neutrophils and macrophages [143, 271, 272], and was first implicated in the proliferation of cancer cells [269]. APRIL's involvement in cancer was extended to colorectal cancers, multiple myelomas, and lymphomas [273–276].

APRIL plays a role in the B-cell lineage, primarily in the differentiation and survival of antibody-producing plasma cells [142–144]. Two TNF receptors have been characterized for APRIL: B-cell maturation antigen (BCMA) and transmembrane activator, calcium modulator and cyclophilin ligand interactor (TACI); these are shared with APRIL's close homologue, BAFF (B-cell activation factor, TNSF13b) [277, 278], though BAFF has a specific receptor, BAFF-R, as well [279].

APRIL, in contrast to BAFF, exhibits an affinity for sulfated GAGs that is rare in the TNFSF [145] (ectodysplasin A, EDA, is one other family member that interacts with GAGs [280]). It would turn out that APRIL binding to heparan sulfate proteoglycans (HSPGs) is important in its function. Not only is HSPG-binding critical for APRIL-mediated tumor-cell proliferation [281], it is also necessary for proper B-cell costimulation [145]. In the case of the latter, it would seem that HSPG mediates APRIL docking and oligomerization that is crucial for receptor-activation [145, 282]. This is also the case for certain other members of the TNFSF [283, 284], and is another example of the innumerous cellular functions that are mediated by sulfated proteoglycans and their repertoire of protein-interactions.

APRIL-binding to sulfated GAGs is mediated by several cationic sites, including a heparin-binding lysine-rich region near the furin-cleavage site at the NH_2-terminal domain of mature APRIL (figure 8)[145]. The deletion the aforementioned heparin-binding domain alone (APRIL H98) is sufficient to prevent heparin-dependent oligomerization, though not enough to abolish GAG binding altogether.

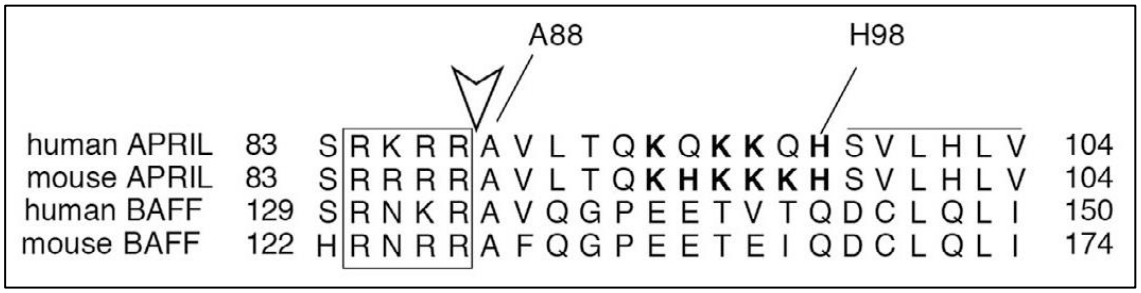


Figure 8: A comparison of an N-terminal region of mature APRIL and BAFF.
Source: Ingold et al. 2005, [145]. The furin recognition sequence is outlined by a box, and the arrow points to the cleavage site. APRIL A88 starts at the alanine outside this site. The bold letters highlight the basic amino acids involved in HSPG binding, which are absent in APRIL H98. This sequence is followed by the TNF homology domain, represented by a line on top of the alignment.

2.2 The ATAMS trial

Owing to its role in the maintenance of humoral immunity, it is not surprising that APRIL had come into the crosshairs of research towards autoimmune disease. Autoimmunity, the result of an adaptive immune response against a self-antigen, is commonly driven by the production of autoantibodies [146]. These are produced by B cells, which have been shown to have a variable importance in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and multiple sclerosis (MS)[285]. Therefore, a proven way to treat certain diseases is by targeting the B-cells; several such therapies are being considered, and some have had success as available treatments, for example in primary progressive MS (ocrelizumab) [147–150].

APRIL's contribution to autoimmune disorders is directly related to its role in the humoral response. It was reported that infiltration of APRIL-producing macrophages facilitate plasma-cell accumulation in tissue lesions in immunoglobulin G4-related disease [286].

Blocking APRIL delayed SLE in a mouse model, and APRIL-deficient mice exhibited lower disease severity, with lower autoantibody levels [287, 288]. To reinforce these observations, higher serum levels of APRIL levels were recorded in patients with SLE, and correlated with disease activity [289–293].

Finally, and most relevant to this study, APRIL and BAFF levels were reported to be upregulated in the cerebrospinal fluid of patients with MS and neuromyelitis optica (NMO) during relapse, correlating with disease severity [294]. Though previous research in MS had been dogmatically focused on T-cell activity, evidence has accumulated for the key pathological role of B-cells, generating several new avenues of research and potential treatments [150, 295, 296]. Combining these reports with the emerging success of B-cell targeted therapy [148, 149], it was logical that a strategy to target APRIL in the context of autoimmune disease should be considered.

Enter atacicept, an immunoglobulin-fused soluble form of TACI, designed to target mature B-cells and antibody-producing plasma cells by acting as a APRIL and BAFF blocking agent [151]. Atacicept demonstrated tolerability and efficacy in healthy volunteers and patients with SLE [297–299].

In light of these findings, the ATAMS phase II study (atacicept in multiple sclerosis, IMP28063, ClinicalTrials.gov identifier: NCT00642902) was set up to assess the molecule's effects in relapsing-remitting MS [151, 152]. In an unexpected twist, the treatment resulted in an increase in inflammatory activity and exacerbation of disease severity, leading to the halting of the trial. A similar scene played out with the ATON trial (atacicept in optical neuritis,, ClinicalTrials.gov Identifier: NCT00624468t.), where patients' disease evolved to clinical MS [300]. Importantly, in a separate phase II clinical trial (NCT 00882999) a BAFF antagonist had no effect on patients with multiple sclerosis, indicating that APRIL-blockade may have been specifically responsible for the failure of the ATAMS trial. Of note, these clinical trials clash with a preclinical study demonstrating the effectiveness of BAFF antagonism and atacicept in EAE mice [151], but echo a separate study which reported that atacicept increased inflammation and neuronal atrophy in rats with an EAE-variant that causes optic neuritis [301].

2.3 Previous work by the laboratory

The failure of the ATAMS trial reflects the difficulties of translating experimental observations into positive clinical outcome; it is far from the first bench-to-bedside disappointment. It did, however, point out a possible neuroprotective role for APRIL, which would be a novel, unexpected feature for the molecule. Indeed, when looking at the contrasting effectiveness of atacicept in autoimmune diseases of the central nervous system (MS/ON) versus the periphery (SLE), one can suggest an uncharacterized function for APRIL in the brain. This may explain why its blockade resulted in an exacerbation in MS.

To investigate this, the overexpression and deletion of APRIL was studied in mice with EAE. We showed APRIL deficiency results in a higher clinical score in EAE [42], echoing the observations in the ATAMS trial. Additionally, APRIL-transgenic mice overexpressing APRIL exhibited a reduced disease severity, even suggesting signs of prompter remission [302]. APRIL transcripts expression in the spinal cord temporarily increase with progression of EAE, and the origin of these transcripts was found to be infiltrating macrophages. Furthermore, treatment of EAE mice with recombinant APRIL lowered disease severity. Injected APRIL A88 accumulated in the spinal cord of EAE mice, but not healthy mice, likely due to the disruption of the blood brain barrier during the course of the disease. Importantly, APRIL A88 accumulated in the spinal cord, but APRIL H98 did not. The latter's compromised GAG-binding ability suggests that APRIL retention in the CNS might be mediated by GAGs.

Immunohistochemistry of lesion biopsies from MS patients revealed that APRIL-producing macrophages surround chronic active lesions, and secreted APRIL is distributed throughout and just outside the lesions (for acute/chronic active plaques as well as slowly expanding lesions). Importantly, APRIL was found to colocalize with CSPGs expressed by astrocytes. These are closely related to the already well-established canonical co-receptor, HSPGs. APRIL binding to astrocytes was fully abolished by the preincubation of APRIL with heparin, which would confirm that binding is dependent on sulfated GAGs. This was further reinforced as APRIL-binding to astrocytes is inhibited by pre-treatment of cells with ChABC that digests CS-GAGs. Subsequent characterization of APRIL-GAG interactions revealed that APRIL specifically bound to CS-E, with an affinity similar to that of HS, in the nanomolar range.

3 Objective of study

The current project aims to take advantage of APRIL's ability to bind to CSPGs. A recombinant, crosslinked, hexameric APRIL (immunoglobulin-fused APRIL A88, hence forth APRIL-Fc) demonstrates indiscriminate and high affinity binding to various CS-types, in contrast to the physiologic trimer form.

In the current study, our main objective is to ascertain APRIL-Fc's potential as a CSPG-inhibiting molecule. APRIL-Fc's highly competitive binding to CSPGs has the potential to abrogate the latter's binding to a variety of protein partners that mediate its inhibitory roles in CNS pathologies, and we will assess this using recombinant forms of the well-characterized partners, PTPRS, NgR1,and Sema3A, as well as the potential partners Slit2 and EFNB2. Further, we will test APRIL-Fc in *in vitro* assays using neurons and oligodendrocyte progenitor cells in the context of inhibition by different CS-GAGs. Finally, we will test APRIL's ability to promote remyelination in an *ex vivo* organotypic model.

Chapter 2: Material and Methods

A majority of the protocols described in this chapter will also be published under the title 'Inhibition of chondroitin sulfate proteoglycans by APRIL', in the next edition of 'TNF Superfamily 2', from the lab protocol series, Methods in Molecular Biology, published by Springer Nature.

The first section of this chapter will detail the process of producing soluble, recombinant versions of the aforementioned proteins, with notes on troubleshooting when cloning particularly long DNA molecules (Slit2 is >5 kbp). We will also describe a straight-forward method of transient transfection and purification. In the second section we will detail our strategy for binding assays. We used ELISAs to screen for interactions between our recombinant proteins and CS-GAGs. Positive interactions were then challenged by the addition of APRIL-Fc to CS-GAGs in an 'inhibition assay', to evaluate the ability of APRIL-Fc to block CSPGs. The next section will detail our neuron and OPC outgrowth assays for the assessment of APRIL-Fc's ability to neutralize CSPG-mediated inhibition on neurons *in vitro*. Finally, the last section will describe *ex vivo* experiments involving organotypic cultures for assaying remyelination with APRIL-Fc treatment.

1 Production and purification of recombinant Fc-fused candidate proteins

Here we describe the methods for the construction of expression plasmids coding for soluble Fc-fused PTPRS, NgR1, EFNB2 and Slit2, and their subsequent use in protein production by transient transfection. An Fc-fused pCRIII plasmid was used as the vector.

1.1 Generation of mouse brain cDNA library

1. Establish primary mixed murine glial cultures from cortices of new-born mice.

   Following standard procedures, cortices must be carefully freed of meninges,

   chopped into small sections and dissociated by mild trypsinization and mechanical

   disruption before seeding.

2. Seed the cells onto poly-L-lysine (10 µg/ml, Sigma Aldrich) coated 25 cm2 flasks at the density of 5 x 10^5 cells/cm^2 in DMEM (Gibco) containing 10% FCS (PAA), 1% L-glutamine (Thermofisher) and 1% Penicillin/Streptomycin (Thermofisher). Change the medium every 3 days.
3. Extract total RNA using the RNAeasy mini kit (Qiagen) following the manufacturer's instructions. The extracted RNA should be treated with DNAse I (Thermofisher) to eliminate genomic DNA, then quantified and checked for quality by NanoDrop (Thermofisher) and agarose gel electrophoresis.
4. To convert the extracted RNA into cDNA, use the Moloney Murine Leukemia Virus (MMLV) SuperScript II Reverse Transcriptase kit (Invitrogen):
    a. Denature the RNA preparation by incubating for 5 minutes at 65°C (using a thermocycler is advisable).
    b. Put the solution on ice for 5 min, then incubate at 42°C for 50 min with the reverse transcriptase. Stop the reaction by incubating for 15 minutes at 70°C.

1.2 Polymerase chain reaction (PCR)

1. Primers should be designed with the appropriate restriction sites and a Kozak sequence for forward primers as illustrated in table 5. Generally, primers can be 20-30 nucleotides in length. Here, we designed primers for the extracellular domains of the membrane-bound proteins PTPRS, NgR1, and EFNB2, and for the whole coding sequence of Slit2, a secreted protein. It is important to omit the stop codon from the primer sequence as it is present at the end of the Fc-coding sequence in the plasmid, which will be downstream of the insertion site of a cloned sequence.

Table 5: Primer designs for amplification of sequences for soluble protein production.		
Molecule	**Accession Nº**	**Primer sequences**
Mouse PTPRS 1-855 aa (Gly)	Nucleotide: XM_006523881 Protein: XP_006523944	PF 5' GC GAATTC GCCACC ATGGCGCCCACCTGG AG 3' EcoR1 Kozak PR 5' CG GTCGAC GCCCTCCTCGCCGTCCAC 3' SalI
Mouse NgR1 1-446 aa (Gly)	Nucleotide: NM_022982 Protein: NP_075358	PF 5' GC GGATCC GCCACC ATGAAGAGGGCGTCCTCC 3' BamH1 Kozak PR 5' CG GTCGAC ACCCTCTGCGTCCCCTG 3' SalI
Human EFNB2 1-229 aa (Ala)	Nucleotide: NM_004093 Protein: NP_004084	PF 5' GC GGATCC GCCACC ATGGCTGTGAGAAGGGAC 3' BamH1 Kozak PR 5' CG GTCGAC TGCAAATAAGGCCACTTC 3' SalI
Mouse Slit2 1-1520 aa (Ser)	Nucleotide: AF144628 Protein: AAD44759	PF 5' GC GGATCC GCCACC ATGAGTGGCATTGGCTGG 3' BamH1 Kozak PR 5' CG GTCGAC GGAGGCACATCTCGCGC 3' SalI ^Stop codon omitted

2. For the PCR reaction follow the instructions of the polymerase manufacturer (Phusion high fidelity and TAQ platinum polymerase kits from Thermo-Fisher) and apply 35 cycles, however certain conditions will vary depending on the DNA sequence to be amplified, as illustrated in table 6. Indeed, finding the ideal conditions can involve a process of trial and error, and is especially difficult for very long sequences (see note 1).
3. Following a PCR reaction, 5 µl of the amplification product can be analysed by agarose gel electrophoresis against a DNA ladder.
4. Purify the rest of the amplification product using a PCR clean up Gel extraction kit from Macherey-Nagel.

Table 6: Specific PCR conditions and reagents for the amplification of candidate genes.				
Molecule	**PCR conditions**	**Template & primer**	**Polymerase**	**Additive**
Mouse PTPRS 1-855 aa (Gly)	Initial: 98ºC for 2 min Cyclic: 98ºC for 10 sec 65ºC for 30 sec 72ºC for 3 min	4 ng/µl of mouse brain cDNA 0.2 µM for each primer	Phusion High Fidelity	4 % DMSO
Mouse NgR1 1-446 aa (Gly)	Initial: 94ºC for 3 min Cyclic: 94ºC for 30 sec 60ºC for 30 sec 68ºC for 2 min	4 ng/µl of mouse brain cDNA 0.5 µM for each primer	Taq Platinum	-
Human EFNB2 1-229 aa (Ala)	Initial: 94ºC for 3 min Cyclic: 94ºC for 30 sec 55ºC for 30 sec 68ºC for 2 min	4 ng/µl of U251 cell line cDNA 0.5 µM for each primer	Taq Platinum	-
Mouse Slit2 1-1520 aa (Ser)	Initial: 98ºC for 40 sec Cyclic: 98ºC for 12 sec 65ºC for 30 sec 72ºC for 3 min	4 ng/µl of mouse brain cDNA 0.5 µM for each primer	Phusion High Fidelity	4 % DMSO

1.3 Molecular Cloning

Standard cloning procedures will apply for the insertion of the amplicon into an expression vector:

1. Purify the amplicon using a 'NucleoSpin Plasmid' purification kit (Macherey-Nagel). Note that with each purification step, a percentage of the product will be lost (around 20 %); it would therefore be important to have prepared an excess of the amplicon for the following steps.
2. Digest the purified amplicon with the appropriate restriction enzymes and purify the product by gel-extraction ('NucleoSpin Plasmid' purification kit). Proceed directly to ligation with the prepared vector (or else store the prepared insert at 4 ºC for use within one day or at -20ºC. Avoid freezing and thawing this preparation).
3. Digest the vector with the appropriate restriction enzymes and purify the product by gel-extraction. Dephosphorylate the product using Calf Intestinal Alkaline Phosphatase (CIAP)(Thermofisher) following the manufacturer's instructions. Purify the product.
4. Ligate using T4 DNA ligase enzyme (Promega) following the manufacturer's instructions (see note 2). The ligated product can be stored at -20ºC.
5. For transformation, use Max efficiency DH5α competent cells (Thermofisher) following the manufacturer's instructions (see note 3). Culture single bacterial colonies overnight in standard lysogeny broth (L.B.) medium containing the appropriate antibiotic for selection and perform a plasmid extraction using the 'NucleoSpin Plasmid' purification kit, following the protocol supplied.
6. Purified DNA can be quantified by Nano-drop and the insert can be verified by double-digestion with appropriate restriction enzymes followed by analysis on an agarose gel.

7. The nucleotide sequence of the cloned amplicon needs to be checked for errors introduced by the DNA polymerase. For this, Sanger sequencing services such as those of Eurofins Genomics are suitable.

1.4 pTOPO subcloning (TA cloning)

Following PCR-amplification, one may choose to insert the amplicon into a pTOPO plasmid by TA cloning (a TOPO® TA Cloning® Kit from Thermofisher is suitable for this procedure). This allows for an easier and more rapid insertion of an amplified product into a plasmid, without the use of restriction enzymes. The DNA can thus be sequenced more quickly, stored easily and for long durations, and is also immediately available for subsequent amplification by PCR or subcloning into expression vectors.

1. Purify the amplicon using a 'NucleoSpin Plasmid' purification kit. If a *Taq* polymerase was used for amplification, proceed to the ligation step (step 3).
2. If a proofreading DNA polymerase was used (as was the case for Slit2 and PTPRS), the amplicon will have blunt ends. As TA cloning relies on the hybridization of 3' adenine (A) overhangs of the insert with 5' thymine (T) overhangs of the pTOPO vector, we must add A-overhangs to the amplicon. For this, we will use a Taq DNA polymerase, which naturally add single adenine residues to the end of any template DNA they travel down. A purification step before the reaction is <u>critical</u> to remove all proofreading enzyme from the mix, or else the enzyme will reverse the overhang addition. Proceed to an A-overhang addition reaction:
    a. Prepare the following mix:

       14 µl of purified PCR product (a concentration of 50 ng/µl was sufficient, but the optimal amount to use may vary with the length of the amplicon)

       2.5 µl of Taq Platinum DNA polymerase buffer (Invitrogen)

       0.75 µl $MgCl_2$

0.5 µl dNTP (or else dATP)

0.5 µl of Taq Platinum enzyme (Invitrogen)

7 µl of DEPC-treated water

b. Incubate the reaction mix at 72ºC for 20 min using a heat block or thermocycler. Proceed directly to ligation step (A-overhangs gradually degrade during storage).

3. Ligate the prepared amplicon to a pTOPO vector using the protocol supplied with the TOPO® TA Cloning® Kit:

   a. Prepare the following reaction:

      4 µl of amplicon

      1 µl of salt solution

      1 µl of TOPO vector

   b. Incubate for 5-6 min at room temperature. Prepare a control where water is used instead of the amplicon to evaluate transformation efficiency later. The product is stable and can be stored at -20ºC for extended durations.

4. Proceed to bacterial transformation as described previously (section 1.3.5). 2 µl of the ligated DNA solution is sufficient for the transformation step. Subcloning efficiency DH5-alpha from Thermofisher are suitable for this cloning procedure.

1.5 Mutation or de-mutation by double PCR

If a single nucleotide was mutated during the cloning process, resulting in a code for a different amino acid, it is possible to perform a 'de-mutation' by double PCR to correct the error, rather than repeating the cloning from cDNA. The same principle can be used to introduce a mutation instead. The process relies on the fact that DNA polymerases use primers if about 15 nucleotides on the 3' end on the primer anneals perfectly to the template, but the 5' end can be altered to differ from the template (the same principle allows for the introduction of restriction sites to the flanks of an amplified sequence). Two pairs of primers can be designed to amplify two 'halves' of the sequence, where the mutation point is at the 3' end of one fragment, and at the 5' end of the other fragment (see figure 9). The mutation bearing extremities of both fragments must be identical to each other over about 15 nucleotides. After these fragments are created (using primers that corrected for the mutation), they can be mixed together and submitted through a few PCR cycles. The fragments will anneal by way of the overlapping region around the mutation site, and a full DNA sequence will be created by the action of the polymerase. This product can then be amplified using primers designed for its extremities.

1. Identify the cDNA sequence of interest and generate a sequence with the point mutation of interest using the genetic code.
2. Design the 3' sequence of the forward mutagenic oligonucleotide. Start at the mutation and include 15 nucleotides on the right (3'). If the sequence ends with A or T, and if next nucleotide is C or G, extend for one more nucleotide.
3. Use strand + of the mutated cDNA to design the 3' sequence of the reverse mutagenic oligonucleotide. Start at the mutation and include 15 nucleotides on the left (or 16 if this helps ending with a C or G).
4. Extend the forward mutagenic oligonucleotide on the left by 6 to 8 nucleotides and extend the reverse mutagenic oligonucleotide on the right by 6 to 8 nucleotides until the overlap between both oligos is exactly 15 nucleotides. If possible, have a C or G at the 5' ends.

5. Perform two separate PCRs to amplify the two fragments (called product A and B).

   Purify the amplicons by gel-extraction.

6. In PCR tubes, prepare mix 1:

   PCR product A, 5µl

   PCR product B, 5 µl

   5 x PCR buffer, 8 µl

   dNTP 2mM each, 5 µl

   H2O, 15.5 µl

   DNA polymerase, 0.5 µl (add last)

7. In a separate tube, prepare the reaction mix 2 (multiply volumes by the number of

   PCR tubes containing mix 1):

   Forward oligonucleotide at 10 µM, 5µl

   Reverse oligonucleotides at 10 µM, 5µl

   5 x PCR buffer, 2 µl

8. For the tubes containing mix 1, run the PCR program:

   Step 1: 3 min at 95 °C (initial denaturation).
   Step 2: 30 sec at 94 °C (denaturation).
   Step 3: 1 min at 52 °C (annealing).
   Step 4: 1 min at 72 °C (extension).
   Step 5: cycle to step 2, 2 times.
   Step 6: 5 min at 72 °C.
   Step 7: 5 min at 45°C (during this step, add reaction mix 2; 12 µl per PCR tube).
   Step 8: 30 sec at 98°C.
   Step 9: 30 sec at 55°C.
   Step 10: 1 min at 72°C.
   Step 11: cycle to step 8, 25 times.
   Step 12: 5 min at 72°C.
   Step 13: cool and at 10°C. Purify the product. It is ready for analysis and cloning.

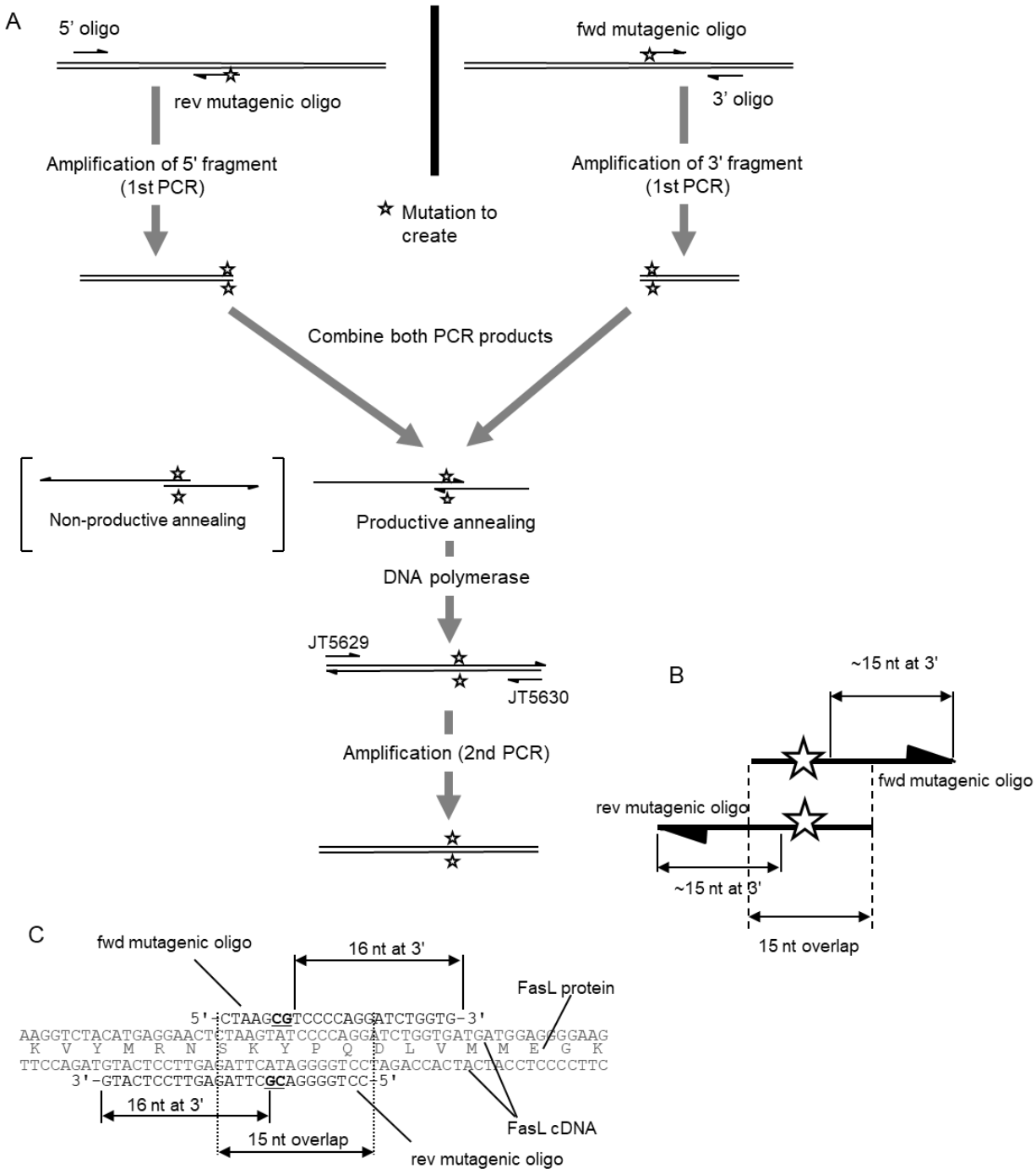


Figure 9: Principle of mutation/de-mutation by double PCR.

Adapted from the protocols of Pascal Schneider, University of Lausanne. A) Overview of the procedure. B) Specifications for the design of mutagenic oligonucleotides. C) Details of mutagenic oligonucleotide design with the example of FasL cDNA.

1.6 Transient Transfection of HEK293T cells

1. Culture HEK293T cells in 10cm dishes in D10 medium (composed of DMEM (Gibco), 10% FCS, 1% L-glutamine, 1% Penicillin/Streptomycin) at 37ºC using a humified incubator with 5% CO_2. Cultures should be at approximately 60-70% confluency on the day of transfection.
2. Gently wash the cells with PBS x1 once, and add 5 ml of a serum-free culture medium, Opti-MEM (Gibco). Return the dishes to the incubator while the transfection mix is prepared.
3. Prepare the transfection mix: for each 10cm culture dish, prepare a 2 ml Eppendorf tube with a 500 µl solution of Opti-MEM containing 15 µg of the expression plasmid, and 30 µg of polyethylenimine (PEI, Thermofisher)(1:2 ratio of DNA/PEI by mass). Mix gently by inversion, spin down, and incubate at room temperature for 15 minutes.
4. Gently, drop-by-drop, pipette the transfection mix over the HEK293T cells. Mix gently by swirling, and return the cells to the incubator for 6 hours.
5. After no longer than 6 hours (PEI is toxic) gently aspirate the medium, and wash the cells once with PBS. Add 10 ml of Opti-MEM and incubate for approximately 5 days (see note 4).
6. Collect the culture supernatant and discard the dishes. Centrifuge the supernatant at 1300 rpm for 4 minutes and discard the pellet (cells and debris). The supernatant can be stored at -20ºC.
7. Collected supernatants can be concentrated using Amicon® Ultra Centrifugal Filters (Merck Millipore) with appropriate cut-offs, by centrifuging at 4000 rpm for 5-10 minutes. The concentrated product is retained by the filter in the upper compartment, and its volume can be adjusted as desired by adding PBS.

1.7 Purification and validation of recombinant proteins

Purify the Fc-fused recombinant protein from the supernatant using a protein-G-sepharose loaded column (GE Healthcare) by affinity chromatography:

1. Wash the gel-loaded column with 3 volumes of elution buffer (Glycine-HCL pH2); do not let the gel remain in acidic pH longer than 20 minutes. Immediately equilibrate the column with Tris-buffered saline (TBS). Do not let the column dry at any time.
2. Allow the column to drain, then pass the supernatant through the column twice to maximise binding.
3. Pass 10 volumes of TBS to wash the column of non-specifically bound proteins. A vacuum pump at low power (40-60 mbar) can be used to speed up the washing step.
4. Meanwhile, prepare 9 glass tubes containing an appropriate amount of Tris pH 8 to neutralize 1 ml of elution buffer.
5. Elute the recombinant protein using 6 ml of elution buffer, collecting the flow through in fractions of 1 ml in the glass tubes. Do not leave the column with an acidic pH for longer than 20 minutes.
6. Immediately equilibrate the column with TBS.
7. Dialyze the purified product in PBS: this can be done by using the Amicon® Ultra Centrifugal Filters, washing the product with sequential 10 ml volumes of PBS (3 washes will suffice), and concentrating to a desired volume.
8. The product can be quickly quantified using a Pierce BCA Protein Assay Kit (Thermofisher) following the manufacturer's instructions.
9. Additionally, the quality of the preparation and the presence of the recombinant protein can be validated by standard SDS polyacrylamide gel (SDS-PAGE) and Western blot:

a. Denature samples in X1 reducing loading buffer solution by incubating at 95°C for 10 min, then place the samples in ice (3X reducing loading buffer solution: 187.5 mM Tris-HCl, 6% SDS, 0.03% Phenol Red, 10 % glycerol, 15 % β-mercaptoethanol, pH 6.8).
b. Migrate products and molecular weight ladders in reducing conditions on a standard SDS-PAGE.
c. Resolved proteins can be visualized with a standard, commercial silver staining (sensitivity of about 0.5 ng) or Coomassie blue staining (sensitivity of about 500 ng) kits.
d. For Western blot, transfer proteins to a PVDF membrane (Bio-Rad). Perform a blocking step with PBS, Tween 20 0.3%, BSA 5% at room temperature for 1hr. Incubate membrane at 4°C overnight in donkey α-human IgG-HRP (Jackson Immunology) diluted in blocking buffer 1.5 µg/ml. Wash 3 times with PBS Tween 20 0.1%. Perform revelation with the clarity western ECL substrate (Bio-Rad) and acquire the chemiluminescent signal with an imaging station, for example the ChemiDocTM (Bio-Rad).

2 Binding and Inhibition assays

The purpose of this section is to detail a method to screen for interactions between CS-GAGs and their potential protein partners, and the necessary protocol modifications to evaluate the inhibition of any positive interactions by a blocking agent.

2.1 Biotinylation of CS-GAGs

1. Dissolve 5 mg of solubilized CS-GAG (Sigma) 1 ml of 0.1 M MES, pH 5.5.
2. Mix with 25 µl of 50 mM biotin hydrazide (Pierce) in DMSO.
3. To this mixture, add 25 µl of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (to get 100 mg/ml in 0.1 M MES, pH 5.5), and incubate the solution overnight at room temperature with rotation.
4. Dialyze the solution against PBS, pH 7 (for example at 4°C for 36 hours, or similar). Products can be stored at -20°C. Cut-off value used: 5 kDa.

2.2 ELISA

1. Coat wells of a Maxisorb 96-well plates (Nunc) with 50 µl of purified Fc-fused recombinant protein at 10 µg/ml and leave overnight at 4ºC. Coat an appropriate irrelevant Fc-fused molecule prepared in a similar manner for a suitable non-CS-binding control (Thy1-Fc). Wash 3 times with PBS Tween 0.05%. Fc-fused PTPRS, NgR1, EFNB2, and Slit2 were prepared in our laboratory as previously described. Fc-fused semaphorin 3A was purchased from R&D Systems (catalogue # 1250-S3-025). APRIL A88-Fc was a gift from Pascal Schneider, University of Lausanne, Switzerland.
2. Add the blocking solution (PBS BSA 1%) and incubate for 1 hour at room temperature.
3. Addition of CS-GAGs for screening:
    a. Add 50 µl of biotinylated CS-GAGs at 1-50 µg/ml in PBS and incubate for 1 hour at room temperature (see note 5). Wash 3 times with PBS Tween 0.05%.

4. Add 50 µl of streptavidin-HRP (Thermofisher) at the dilution indicated on the bottle, incubate for 40 minutes at room temperature. Wash 5 times with PBS Tween 0.05%.
5. Add the tetramethylbenzidine substrate (TMB, BD Bioscience) and incubate until the solution turns blue (5-15 minutes). Do not wait for the negative control to turn blue before stopping the reaction.
6. Stop the reaction with 50 µl of sulfuric acid 2N. The plate is ready for spectrometric measurements at 480 nm using Victor3 1420 Multilabel Counter (PerkinElmer). Positive interactions are determined based on the strength of the absorbance signal, corrected using (subtracted by) the negative control.

Upon detection of positive interactions, this protocol can be modified into an 'inhibition assay' designed to evaluate a blocking agent. Replace step 3a with 3b-c:

3. Addition of CS-GAGs for blocking:
    b. During the blocking step, preincubate biotinylated CS-GAGs with APRIL-Fc for around 45 minutes at room temperature (various concentrations of the blocking agent should be tested). Preincubation with an equivalent molar concentration of Thy1-Fc can serve as a suitable negative control.
    c. Add 50 µl of 'blocked' CS-GAGs to coated wells and incubate for 1 hour at room temperature. Wash 3 times with PBS Tween 0.05%.

3 Neurite Outgrowth Assay

1. Prior to dissection, prepare culture plates as follows:
    a. Place sterilized 12 mm glass coverslips into a 24-well plate, and add 100 µg/ml poly-L-lysine (Sigma) so that it covers the coverslip. Incubate at room temperature overnight (under the hood with a dose of UV light is suitable), or for 1-2 hours at 37ºC. Wash the coverslips thoroughly with sterile water three times.
    b. Add a solution of CS-GAGs (1-10 µg/ml in PBS) to the coverslips, and incubate overnight at 4ºC. Wash once with PBS.
    c. Add the solution containing the blocking agent (APRIL-Fc or control), or PBS. Incubate for 1 hour at room temperature. Replace the solution with glial culture medium (DMEM, 10% horse serum) and proceed directly to cell seeding.
2. Using sterile dissection instruments, carefully dissect cortices of embryonic mice (E17) under a horizontal laminar flow hood with the help of a dissection microscope. Following typical dissection procedures, cortices must be carefully freed of meninges before being digested in 0.25% trypsin in Hanks' balanced salt solution (HBSS) at 37ºC for 15 minutes. Mix gently every 5 minutes.
3. Aspirate and wash 3 times with HBSS. Resuspend in 500 µl of glial culture medium (serum in this medium will promote cell attachment following seeding).
4. Mechanically dissociate the tissue by gentle up-and-down pipetting, first with a P1000 (about 10-15 times), then again with a P200 cone fitted onto the P1000 cone.
5. Seed the cells onto PLL-coated coverslips in a 24-well plate and incubate for 2 hours at 37ºC using a humified incubator with 5% CO_2.

6. Replace the medium with complete MACS culture medium (serum-free MACS Neuro Mediums supplied by Mylteniy Biotec, supplemented by GlutaMAX at 1/100 from Thermofisher, and B27 at 1/50 from Thermofisher). This medium lacks serum, preventing the proliferation of glial cells. Incubate at 37ºC for 3 days.
7. Aspirate the medium, wash once with warm PBS, and add PBS PFA 4 % sucrose 4% for fixation. Incubate at 37ºC for 30 minutes. Wash the coverslips with PBS 3 times.
8. Permeabilize the cells with PBS, Triton 0.2% for 5 min at RT. Wash twice.
9. Add the primary antibody (8 µg/ml β-tubulin III antibody clone Tuj1, R&D Systems; specific for neurons) diluted in PBS BSA 1% Triton 0.2% (blocking solution) and incubate at room temperature for 3 hours (see note 6). Wash twice.
10. Add the secondary antibody (8 µg/ml goat anti-mouse IgG2A-FITC, Thermofisher) in blocking solution and incubate in the dark at room temperature for 1 hour. Wash three times.
11. Mount the coverslips onto glass slides with Fluoroshield Mounting Medium with DAPI (Abcam, ab104139) and proceed to epifluorescence imaging.
12. Acquire photos of neurons from several representative fields at a magnification of x10 or x20 (Axio Imager M2 microscope (Carl Zeiss) with a plan-neofluar 10x/0.75 objective, or similar).
13. Proceed to analysis using the NeuronJ plugin in the ImageJ software to trace and measure neurite lengths of cells (see note 7). The averages for each measurement for a condition should be calculated using a minimum of 30 cells per experiment, and the percentage difference relative to the control (without CS-coat and with control treatment) can be calculated. Thy1-Fc was used as a non-CS binding irrelevant control, at a molar concentration equivalent to the highest concentration of APRIL-Fc used.

4 OPC Outgrowth Assay

To obtain OPC cultures, a primary culture of mix glial cells derived from mouse embryonic cortices must be prepared.

1. Using sterile dissection instruments, carefully dissect cortices of new-born mice under a horizontal laminar flow hood with the help of a dissection microscope. Following typical dissection procedures, cortices must be carefully freed of meninges before being digested in 0.25% trypsin in HBSS at 37ºC for 15 minutes. Mix gently every 5 minutes.
2. Aspirate and wash 3 times with HBSS. Resuspend in 500 µl of glial culture medium (this medium contains serum which will promote cell attachment following seeding).
3. Mechanically dissociate the tissue by gentle up-and-down pipetting first with a P1000 (about 10-15 times), then again with a P200 cone fitted onto the P1000 cone.
4. Seed the mixed glial cell suspension onto T25 flasks (about 2-3 cortices per flask), and incubated for 4-5 days in DMEM-10% horse serum.
5. By this point OPCs with distinct bipolar morphology can be seen growing on top of the astrocytic monolayer. Shake the flasks until OPCs are no longer associated with the monolayer, and centrifuge the supernatant briefly (1 minute at 500 rpm) to pellet the debris and any detached monolayer fragments. Resuspend the cells in serum-free complete MACS culture medium and proceed to seeding in 24-well plates prepared as described below.
6. Prior to shaking, prepare plates for secondary culture as follows:
    a. Place sterilized 12 mm glass coverslips into a 24-well plate, and add 10 µg/ml poly-L-lysine so that it covers the coverslip. Incubate at room temperature

overnight (under the hood is suitable), or for 1-2 hours at 37ºC. Wash the coverslips thoroughly with sterile water at least three times.

b. Add a solution of CS-GAGs (1-10 µg/ml in PBS) to the coverslips, and incubate overnight at 4ºC. Wash once with PBS.

c. Add the solution containing the blocking agent (APRIL-Fc or control) or PBS. Incubate for 1 hour at room temperature. Replace the solution with serum-free complete MACS culture medium and proceed directly to cell seeding.

7. Seed the cells onto PLL-coated coverslips in a 24-well plate and incubate for 24 hours at 37ºC using a humified incubator with 5% CO_2.
8. Aspirate the medium, wash once with warm PBS, and add PBS PFA 4 % sucrose 4% for fixation. Incubate at 37ºC for 30 minutes.
9. Wash the coverslips with PBS 3 times.
10. Add rabbit polyclonal anti-NG2 chondroitin sulfate proteoglycan antibody (Merck AB5320) at 1/250 (4 µg/ml) diluted in PBS BSA 1% and incubate at room temperature for 3 hours. Wash twice.
11. Add the secondary antibody (goat anti-rabbit IgG-PE, Thermofisher, 8 µg/ml) diluted in PBS BSA 1% and incubate in the dark at room temperature for 1 hour. Wash thrice.
12. Mount the coverslips onto glass slides with Fluoroshield Mounting Medium with DAPI (abcam, ab104139) histology mounting medium and proceed to epifluorescence imaging.
13. Acquire photos of OPCs from several representative fields at a magnification of x10 or x20 (Axio Imager M2 microscope (Carl Zeiss) with a plan-neofluar 10x/0.75 objective, or similar) and proceed to analysis using ImageJ software.
14. ImageJ can be used to determine OPC outgrowth by measuring cell perimeter, outgrowth surface area (total surface area minus the cell soma area), as well as using

the Scholl analysis feature to determine ramification indices. The averages for each measurement for a condition should be calculated using a minimum of 30 cells per experiment, and the percentage difference relative to the control (without CS-coat and with control treatment) can be calculated. Thy1-Fc was used as a non-CS binding irrelevant control, at a molar concentration equivalent to the highest concentration of APRIL-Fc used.

5 Ex vivo Organotypic Culture and Myelination Assay

5.1 Organotypic culture

P10 C57/Bl6 mouse cerebellar slices were prepared as already described by Hussain *et al.* [303] and Birgbauer *et al.* [304]. At least 3 animals and 9 cerebellar slices were used for each experimental condition.

1. Following decapitation, dissect brains and carefully remove meninges in cold Gey's balanced salt solution containing 5 mg/mL glucose (GBSS-Glc) in sterile conditions using a laminar hood and dissection microscope.
2. Extract cerebellums and cut them into 350 µm-thick parasagittal slices using a MacIlwain tissue chopper.
3. Lay out the slices onto membranes of 30 mm Millipore culture inserts with 0.4 µm sized pores (Millicell, Millipore).
4. Place inserts with slices in six-well plates containing 1 ml of medium composed of 50% basal medium with Earle's salts (Invitrogen), 2.5% Hanks' balanced salts solution (Life Technologies), 25% heat inactivated horse serum (Life Technologies), L-glutamine (1 mM) and 5 mg/mL glucose.

5. Culture the slices at 35°C in a humidified incubator with 5% CO_2. Refresh the medium every 2-3 days, and maintain the cultures for 7 days *in vitro*.
6. Demyelinate the slices by replacing medium with one containing 0.5 mg/ml lysolecithin for 16 h.
7. Wash twice with medium, and then culture the slices in the presence or absence of treatments (5 µg/ml APRIL-Fc or molar equivalent of Thy1-Fc).
8. Fix the slices after 3 days of treatment, or after 1, 2, 3, or 5 days for CSPG expression analysis in non-treated slices.
    a. Aspirate the medium and rinse briefly with PBS.
    b. Fix using 4ml PFA 4% for 40 minutes at RT
    c. Wash thrice for 10 minutes with PBS with shaking.
    d. Remove the slices from the Millicell inserts and store them at 4°C in PBS sodium azide 0.1% until use.

5.2 Immunofluorescence and analysis

1. Transfer the slices into 24-well plate wells (2-3 slices can share a well) with the help of a thin brush.
2. For blocking, incubated in PBS-GTA (composed of PBS Triton-X 0.25%, gelatin 0.2% (Sigma G9382), sodium azide 0.1%) and L-lysine monohydrochloride (0.1 M) (Sigma L5626) for 1 h at RT with shaking. Alternatively, PBS Triton-X 0.25%, 1% BSA can be used for blocking and diluting antibodies.
3. Replace the blocking solution with primary antibodies diluted in PBS-GTA:

    - Rabbit polyclonal anti-calbindin (CaBP) (Swant, CB38a) at 1/10,000 (0.1 µg/ml).

    - Mouse monoclonal IgG1 anti-MAG (Merck, MAB1567) at 1/1000 (1 µg/ml).

    - Mouse monoclonal IgM anti-CSPGs (CS-56) (Abcam, ab11570) at 1/200 (2.5 µg/ml).

    (see note 8**)**

4. Incubate with shaking overnight at 4°C.
5. Wash thrice with PBS and add appropriate secondary antibodies diluted in PBS-GTA:

   - Anti-mouse IgM conjugated to PE (SouthernBiotech) (2.5 µg/ml)

   - Goat anti-rabbit IgG conjugated to PE (Thermofisher) (5 µg/ml)

   - Goat anti-mouse IgG conjugated to FITC (Thermofisher) (10 µg/ml)
6. Incubate for 2 hours at RT in darkness with shaking.
7. Wash thrice. Carefully remove the slices from the 24-well plate with a thin brush and place them into a petri dish containing PBS. Carefully lay out the slices on glass slides with the help of the brush. Allow to dry for a few minutes, then mount using Fluoroshield Mounting Medium With DAPI.
8. Acquire images of cerebellar lobes (see figure 10-B) using a confocal microscope (LSM 510 META or similar) with 10x or 20x magnification.
9. Proceed to analysis using ImageJ software:
    a. For CSPG expression quantification, measure the mean fluorescence intensity of CS-56 staining in an area (defined by the lobe area).
    b. For quantification of myelination, choose a region of interest for each lobe and draw a virtual line transversally to the axonal tracts of Purkinje cells entering in the white matter trench. The percentage of myelinated axons can be determined as the ratio of MAG-positive fibres over CaBP-positive fibres crossing the transversal line (where 100% is set as the number of CaBP-positive fibres) (figure 10-A).

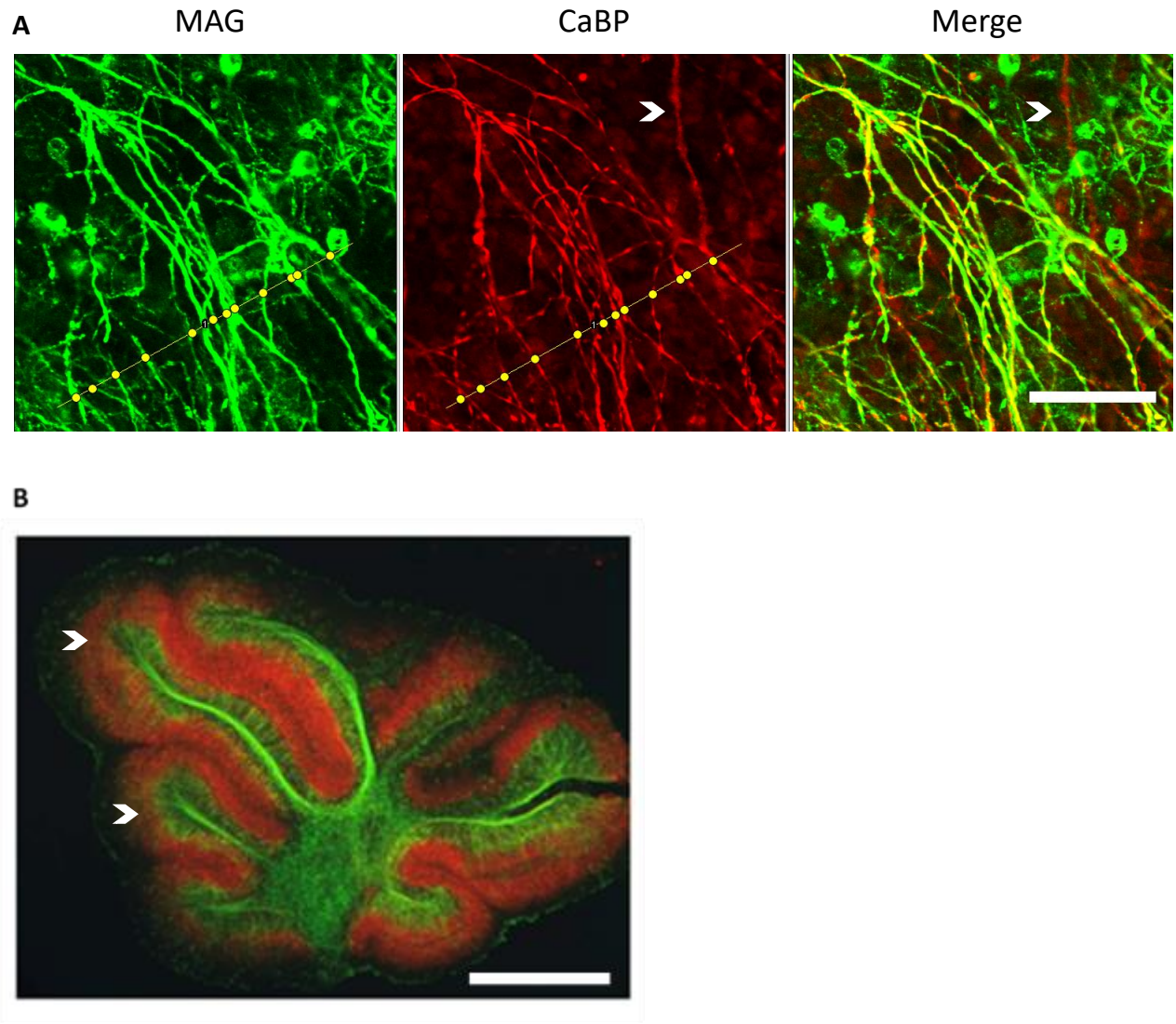


Figure 10: Quantification of myelination in organotypic cerebellar slices.
A) High magnification (200x) immunohistochemistry images of cerebellar fibres stained with MAG for myelin (green) and CaBP for axons (red). The quantification of myelination as a percentage of myelinated axons is defined by the number of MAG-positive fibres over the number of CaBP-positive fibres crossing a virtual line (yellow dots in left and middle panels). An unmyelinated axon is shown (white arrow). Scale bar = 50µm. B) Low magnification (40x) image of parasagittal cerebellar slice showing multiple lobes (white arrows). Scale bar = 0.5 mm.

6 Notes

1. The outcomes of PCRs are dependent on a multitude of variables. Further complicating the process is that the ideal set of variables will differ with the sequence to be amplified, owing for example to the length of the target sequence, or the richness of GC-nucleotides in it; these variables apply to the primers used as well. Some examples of important parameters are listed in table 6. For the purpose of this chapter, we list a few handy general rules for amplifying a complicated sequence from a cDNA library:
    a. The polymerase matters. We found that high fidelity polymerases such as Phusion high fidelity polymerase from Thermofisher are more reliable when amplifying long sequences of DNA (>3 kbp) and will make fewer errors; we found not more than 1 error for 5 kbp amplified. In our experience this polymerase performs better for very long sequences if DMSO is added and mixed into the reaction solution immediately before the reaction (optimal concentration may vary). Taq Platinum is reliable for lower length sequences to be amplified but is prone to introducing errors in the final product (1 error for about 1 kbp amplified). It is a suitable option for screening.
    b. Two of the most common problems with PCR is the production of non-specific products or getting no product at all. As a general rule, we found that adjusting the annealing temperature is often sufficient to fix both of these issues, which are often the result of falling either too short or too far off the optimal temperature for hybridization. Increase the annealing temperature to eliminate non-specific products or reduce it if there was no product (adjusting by increments of 5ºC is sufficient).

c. GC-nucleotide richness of the target or primers can also be obstacles to amplification. In the case of PTPRS which has a GC-rich DNA sequence, we obtained primer-dimers instead of the desired product. To overcome this, we increased the initial denaturation time, and adjusted primer-template concentration ratios to favour correct template-primer hybridization (see table 6). DMSO weakens hydrogen bonds between G or C base pairs to improve amplification of GC-rich sequences.

2. The ligation step with the T4 DNA ligase enzyme should be performed overnight in ice left to melt at room temperature (the gradually changing temperature guarantees an optimal reaction). The ideal molar concentrations of the insert and vector can differ depending on their relative lengths. Generally, a vector to insert molar ratio of 1:3 is suitable, though for long (>5 kbp) inserts a ratio of 1:1 can give better results.
3. Max efficiency DH5α competent cells were used for transformation, however we found that Subcloning Efficiency™ DH5α cells are also suitable. In either case, we recommend *not* using S.O.C. medium for the final steps; we found a significantly higher number of false positive bacterial colonies, and poor transformation efficiency using this medium compared to L.B. medium.
4. The 5-day incubation is a general rule. The viability of transiently transfected cells may deteriorate more rapidly due to factors such as confluency, or the toxicity of the produced molecule. In the case where cell viability has deteriorated to the point of detachment of the monolayer, it is better to collect the supernatant before the 5-day incubation is complete. Cell death is accompanied by the release of proteases that may degrade the desired product.
5. Polysaccharides like CS-GAGs have a poor affinity for plastic; it is for this reason we coat the candidate protein as bait and add the CS-GAG as prey for our ELISA protocols.

If it is desired that the CS-GAGs be coated instead, the plastic can be coated with streptavidin prior to the addition of biotinylated CS-GAGs. In this case, binding of Fc-fused proteins to the polysaccharide coat should be revealed with an anti-Fc antibody.

6. The neuron specific antibody presented in this protocol is *not* adequate for the analysis of specific neuron types, nor neurite types (for example axons only). TUJ1 (an anti-beta-tubulin III antibody) will stain *all* neurons, and *all* their neurites. Dissociated cortical tissue will contain several neuron types with various neurite morphologies; it is for this reason we measure total neurite length. If it is desired to measure, for example, axonal length specifically, we recommend either using specific antibodies (e.g. MAP2 for dendrites, or Tau for axons), or using hippocampal tissue, which is primarily comprised of pyramidal neurons with easily distinguishable axons.
7. The analysis of neurite lengths requires that neurons grow separate from each other to avoid overlapping processes. A few key steps are important to optimize the culture for this: Firstly, the number of cells seeded onto a well should be calibrated to have neurons that are not too close together nor too far apart (a seeding concentration that is too high will lead to overlapping neurites, and too low will hinder culture growth and viability). Test a range of seeding concentrations to find the optimal set up. It is equally important that the dissociation step before the seeding is performed correctly (too much mechanical dissociation can damage neurons, but too little will cause cellular clumps and poor spacing between cells). Check the dissociated cells under a microscope before seeding and continue to dissociate them if clumps are visible. Note that cell aggregation can also occur depending on the treatment of the coverslip prior to the seeding: CS-GAGs are negatively charged molecules, and higher concentrations will impede neuron attachment to the CS-coated substrate, as these cells prefer positively charged surfaces for attachment.

8. The CS-56 antibody recognizes chondroitin sugar moieties on proteoglycans of different molecular weights (its reactivity is abolished by chondroitinase ABC treatment of CSPGs, demonstrating its specificity for CS-GAGs) [305]. The reactivity of CS-56 is also inhibited by the addition of CS-GAGs of type A, C, and D, but not by types B or E [306], indicating a selectivity for specific types.

Chapter 3: Results

1 APRIL-Fc prevents binding of CS types to inhibitory receptors

CSPGs mediate their inhibitory effects by way of interaction between their CS-GAGs and several proteins of different families. In order to cast a wide net, in this study we opted to select known inhibitory proteins of the CNS from five different families with known affinities for sulfated proteoglycans. We selected the receptor-type tyrosine-protein phosphatase sigma (PTPRS), a member of the protein tyrosine phosphatase (PTP) family, Nogo receptor 1 (NgR1, also known as reticulon 4 receptor), a member of Nogo receptor family, semaphorin 3A (Sema3A), a member of the semaphorins family, Ephrin B2 (EFNB2), a member of the ephrins subfamily of receptor protein-tyrosine kinases, and Slit guidance ligand 2, (Slit2 also known as slit homologue 2), a member of the Slit family. The use of specifically designed primers (table 5) in PCR allowed the successful amplification of cDNA corresponding to the extracellular domains of the transmembrane proteins, PTPRS, NgR1, and EFNB2, as well as full-length Slit2, a secreted protein. Intricacies of the 'homemade' protocols used for this step are detailed in table 6, and in chapter 2. Amplicons were sequenced for the cloning of error-free sequences into Fc-encoding pCRIII vectors (kindly provided by Pascal Schneider). Vectors were used to transiently transfect HEK-293T cells. Supernatants were collected and purified on protein-G-sepharose loaded columns, and products were analysed by western blot. Bands corresponding to Ig-fused soluble proteins were observed at expected molecular weights (figure 11). Under reducing conditions, PTPRS-Fc, NgR1-Fc and EFNB2-Fc were detected at 130 kDa, 100 kDa and 60 kDa, respectively. For Slit2-Fc we observed a faint band at 240 kDa and a more intense band at 82 kDa, corresponding to full length Slit2, and the C-terminal cleavage product, Slit2-C, respectively. This mixture will collectively be referred to as Slit2-Fc for the purpose of this study.

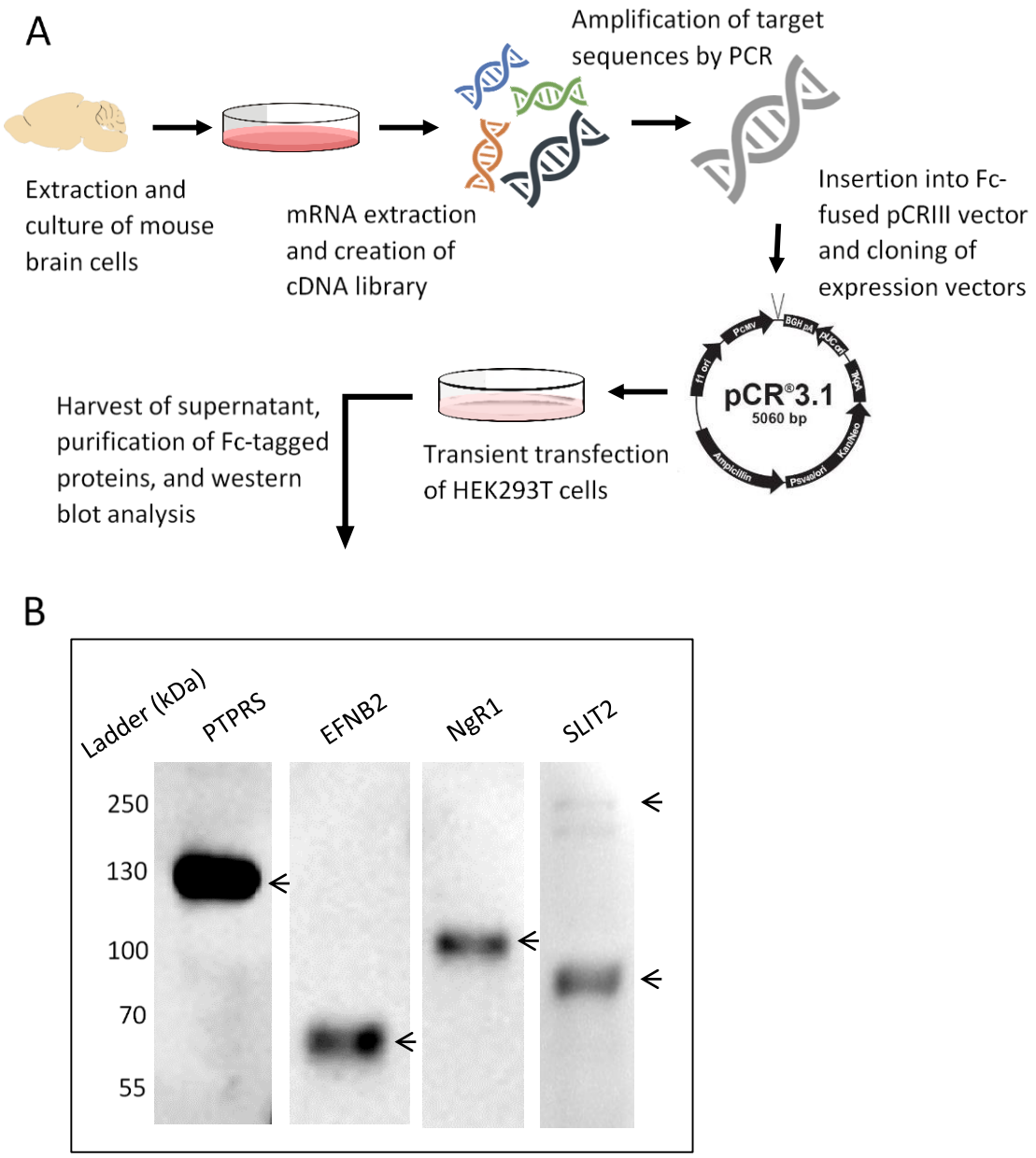


Figure 11: Production of recombinant proteins by HEK293T Cells.
A) Schematic overview of the process behind the production of soluble, Fc-fused PTPRS, NgR1, EFNB2 and Slit2. B) Western blot analysis of supernatants from transiently transfected HEK cells in denaturing conditions using goat anti-human Ig conjugated to HRP.

We first needed to confirm that our recombinant proteins could bind CS-GAGs. For this we performed ELISAs with Fc-fused APRIL, PTPRS, NgR1, Sema3A, EFNB2 and Slit2 testing for their binding capacities to different biotinylated CS-GAGs (figure 12-A). Thy1-Fc, a non-CS binding irrelevant protein, served as the negative control. CS-A exhibited significant binding to all tested proteins except Thy1, in order of strongest capacity to weakest being APRIL >PTPRS >NgR1 >Sema3A ≈EFNB2 >Slit2. CS-B bound to APRIL ≈PTPRS >NgR1 >Sema3A ≈Slit2. CS-D bound to APRIL >PTPRS. CS-E bound to APRIL >PTPRS >EFNB2 >Sema3A. This reflects the heterogeneous binding activities of different CS types. In order to determine if APRIL-Fc binding to CS-GAGs could compete with their binding to their partners, we preincubated CS-GAGs with APRIL-Fc or Thy1-Fc before performing ELISA (figure 12-B). Preincubation of CS-GAGs with APRIL-Fc significantly reduced the levels of binding to their binding partners by 80% in most cases, whereas preincubation with Thy1-Fc had no effect and was used to calculate percentage difference by inhibition. With the exception of the interaction of PTPRS to CS-E and CS-D, all other interactions were significantly blocked by a molar concentration of APRIL-Fc equivalent to 5x lower than that of the CS-GAG used. A greater molar ratio of APRIL-Fc to CS (10:1 for CS-E, and 1:2.5 for CS-D) was used for the inhibition of CS-E/CS-D—PTPRS interactions. To verify that interference by APRIL-Fc is not due to binding directly to recombinant proteins, we assessed their binding to APRIL-Fc directly (figure 13). BCMA is a known APRIL-receptor and was used as a positive APRIL-binding control. No binding between APRIL-Fc and our recombinant proteins was detected, arguing that APRIL-Fc inhibits interactions by direct binding to CS-GAGs. Overall these results demonstrate APRIL-Fc's broad and effective ability to block CS-GAG binding to several repair-inhibiting proteins of the CNS.

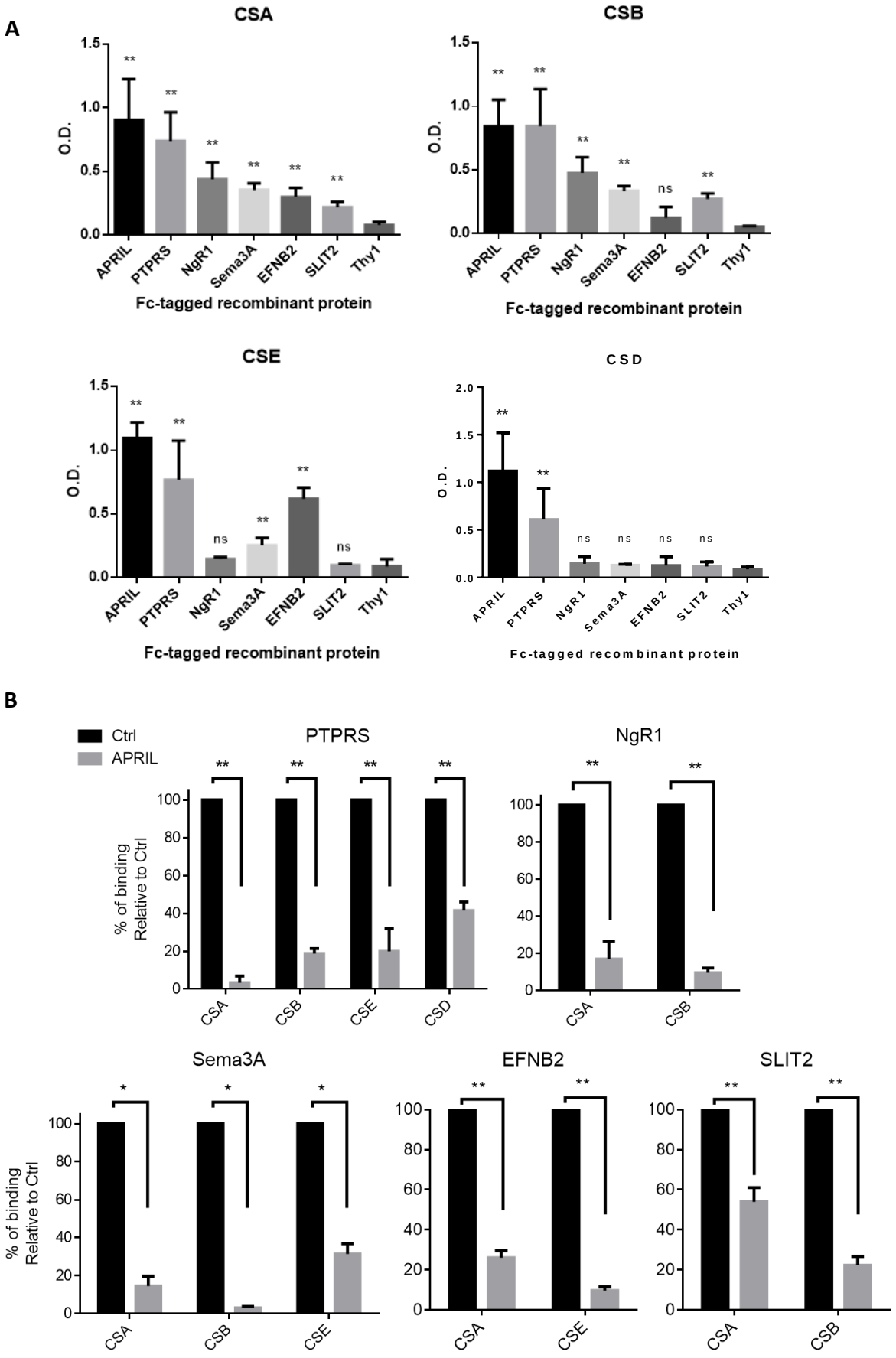

A
CSA
CSB
CSE
CSD
O.D.
Fc-tagged recombinant protein
APRIL
PTPRS
NgR1
Sema3A
EFNB2
SLIT2
Thy1
B
Ctrl
APRIL
PTPRS
NgR1
Sema3A
EFNB2
SLIT2
% of binding
Relative to Ctrl
CSA
CSB
CSE
CSD

Figure 12: APRIL-Fc prevents binding of CS types to inhibitory receptors

A) Binding of the indicated purified Fc-fused soluble molecules to different CS types was assessed by ELISA. B) Binding of inhibitory receptors to CS types that were preincubated with APRIL-Fc or Thy1-Fc control was assessed by ELISA. Inhibition is shown as a percentage of binding relative to control. Data are presented as mean +/- SD of at least 5 experiments. **p<0.01; ***p<0.001 versus Thy1-Fc (Mann Whitney Test); ns = non-significant. Thy1-Fc was used as a non-CS binding irrelevant protein. Inhibition is shown as a percentage of binding relative to that of Thy1-Fc preincubated CS-GAGs

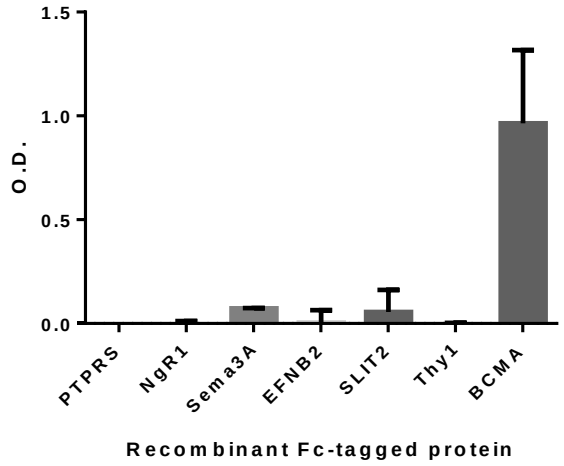


Figure 13: APRIL-mediated interference is not due to binding to recombinant proteins.

Binding of the indicated purified Fc-fused soluble molecules to APRIL-Fc was assessed by ELISA. BCMA was used as a APRIL-binding positive control. Data are presented as mean +/- SD of at least 3 experiments

2 APRIL-Fc prevents CS-mediated inhibition of neurite outgrowth

To answer the question if APRIL-Fc mediated inhibition of binding between CS-GAGs and their inhibitory receptors could translate to a loss of CS function, we investigated the effect of APRIL-Fc on the neurite outgrowth on CS-GAGs *in vitro*. We first evaluated the inhibitory activity of different CS-GAGs on neurite outgrowth (figure 15-A). Neurons grown on CS-A, CS-B, and CS-E had shorter neurites after 3 days, exhibiting a reduction of more than 50% compared to neurons grown on poly-L-lysine (PLL) alone. CS-D had no observable effect on neurite outgrowth. It is important to note that the inhibitory CS types exhibited differential effects on cell adhesion (figure 14). Due to a significant loss of cell adhesion and the formation of cell aggregations at higher concentrations of inhibitory CS-GAGs (>10 µg/ml for CS-A and CS-B, >1 µg/ml for CS-E), we used 10 µg/ml CS-A and CS-B, and 0.1 µg/ml CS-E for subsequent experiments to ensure adequate cell densities for measurements of neurite outgrowth. CS-E exhibited the strongest inhibitory activity on neuronal adhesion and outgrowth, resulting in the use of a lower concentration than other CS types for subsequent experiments. Of note, CS-D, did not have any significant effect on cell adhesion and aggregation at tested concentrations up to 10 µg/ml (figure 14). In order to assess if APRIL-Fc treatment could neutralize the inhibitory CS-GAGs, different concentrations of APRIL-Fc were added onto CS-coated coverslips before seeding of neurons. The application of APRIL-Fc resulted in a dose-dependent promotion of neurite outgrowth on CS-GAGs, up to levels of the control (figure 15-B). For CS-A, the lowest tested concentration of APRIL-Fc was sufficient to cause an observable recovery. At 15 µg/ml, APRIL-Fc abolished the effects of CS-A completely. CS-B mediated inhibition was only reversible by higher concentrations of APRIL-Fc (5-15 µg/ml). For CS-E, medium to high concentrations of APRIL-Fc (1.5-15 µg/ml) were sufficient to completely abolish inhibitory activity. As CS-E was coated at a 10x lower concentration than other CS types, this reflects ELISA experiments showing that a proportionally greater quantity of APRIL-Fc was necessary for the inhibition of CS-E interaction to PTPRS. Importantly, APRIL-Fc did not affect neurite lengths on PLL alone (figure 15-B). This would indicate that the pre-treatment of inhibitory CS-GAGs with APRIL-Fc resulted in a dose-dependent abolishment of their inhibitory activity, rather than APRIL-Fc having a direct effect on the neurons themselves. As a negative control, Thy1-Fc pre-treatment of coated CS-GAGs did not affect their activity.

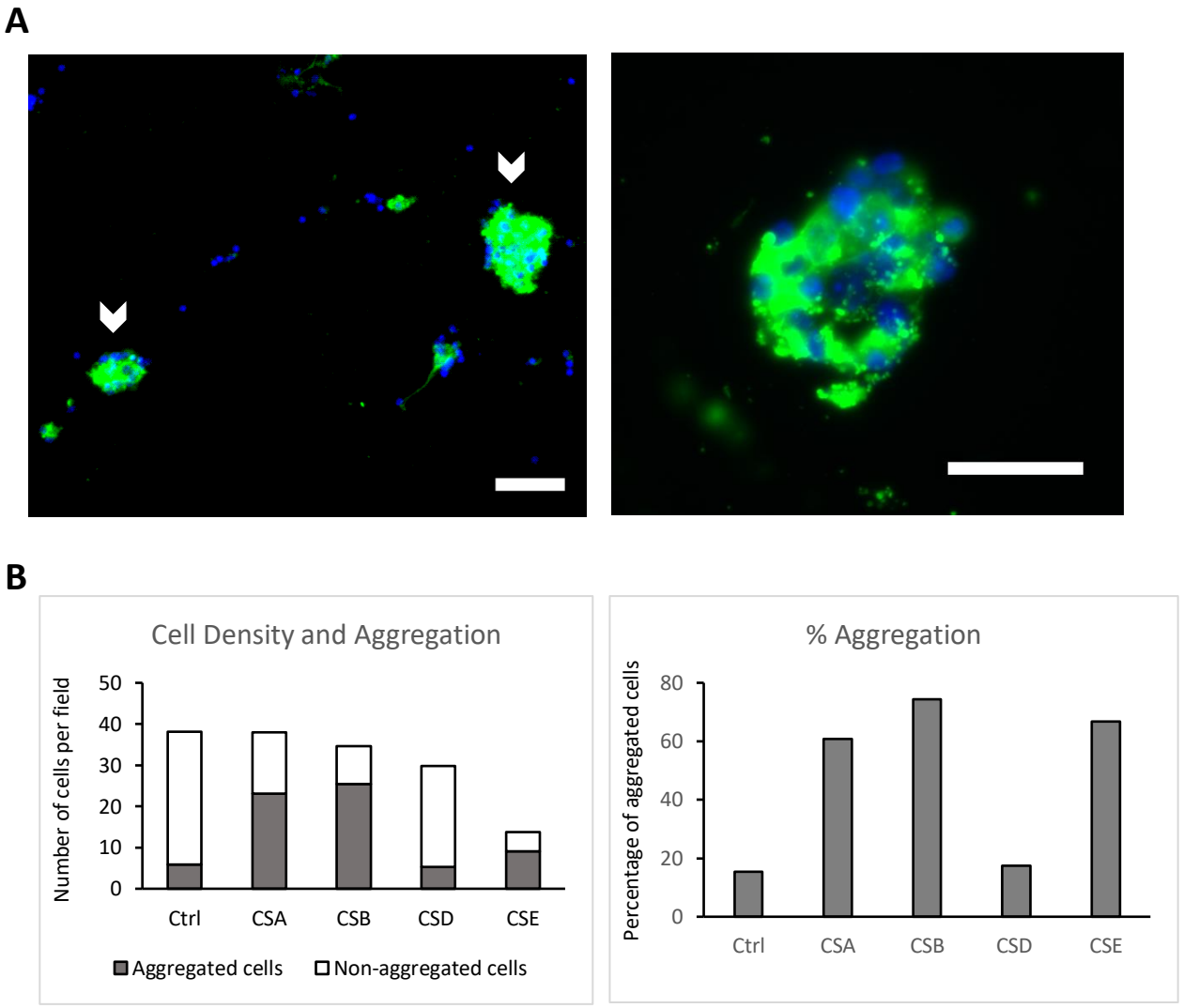
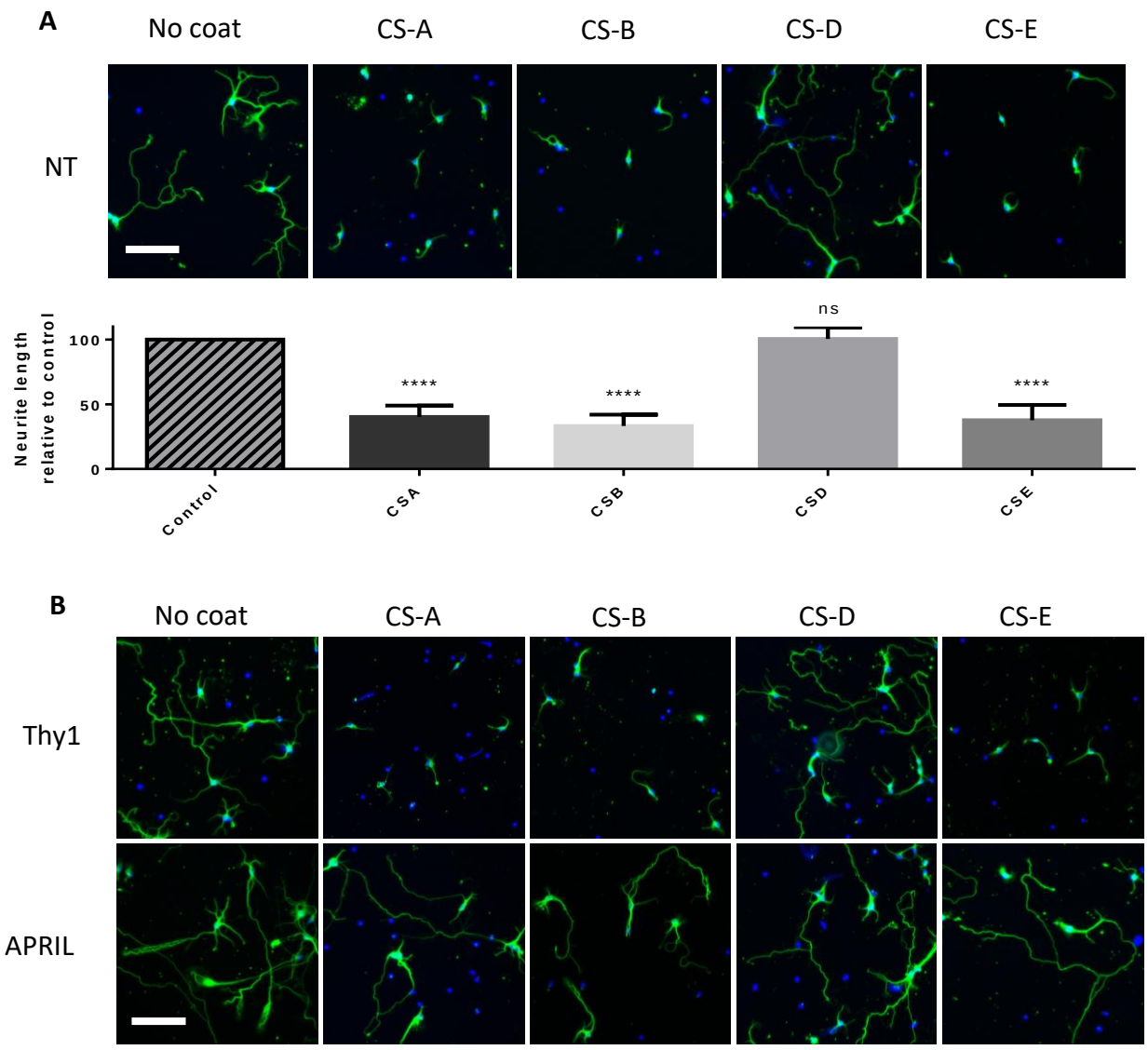


Figure 14: CS-GAGs affect cell adhesion and aggregation.
Embryonic mouse cortical neurons were cultured 3 days on either CS-coated or untreated substrate (control) and stained for β-tubulin III (green) and Hoechst 33342 (blue). A) Representative low magnification (100x) (left) and high magnification (200x) (right) images of neurons grown on higher concentrations of CS, resulting in the formation of aggregated cells (white arrows). Scale bar = 50µm. B) Representative quantification of cell density (left) and percentage of aggregated cells (right) on untreated (Ctrl) and CS-coated surfaces. n ≥ 2 experiments.


A
No coat
CS-A
CS-B
CS-D
CS-E
NT
Neurite length relative to control
100
50
0
****
****
ns
****
Control
CSA
CSB
CSD
CSE
B
No coat
CS-A
CS-B
CS-D
CS-E
Thy1
APRIL

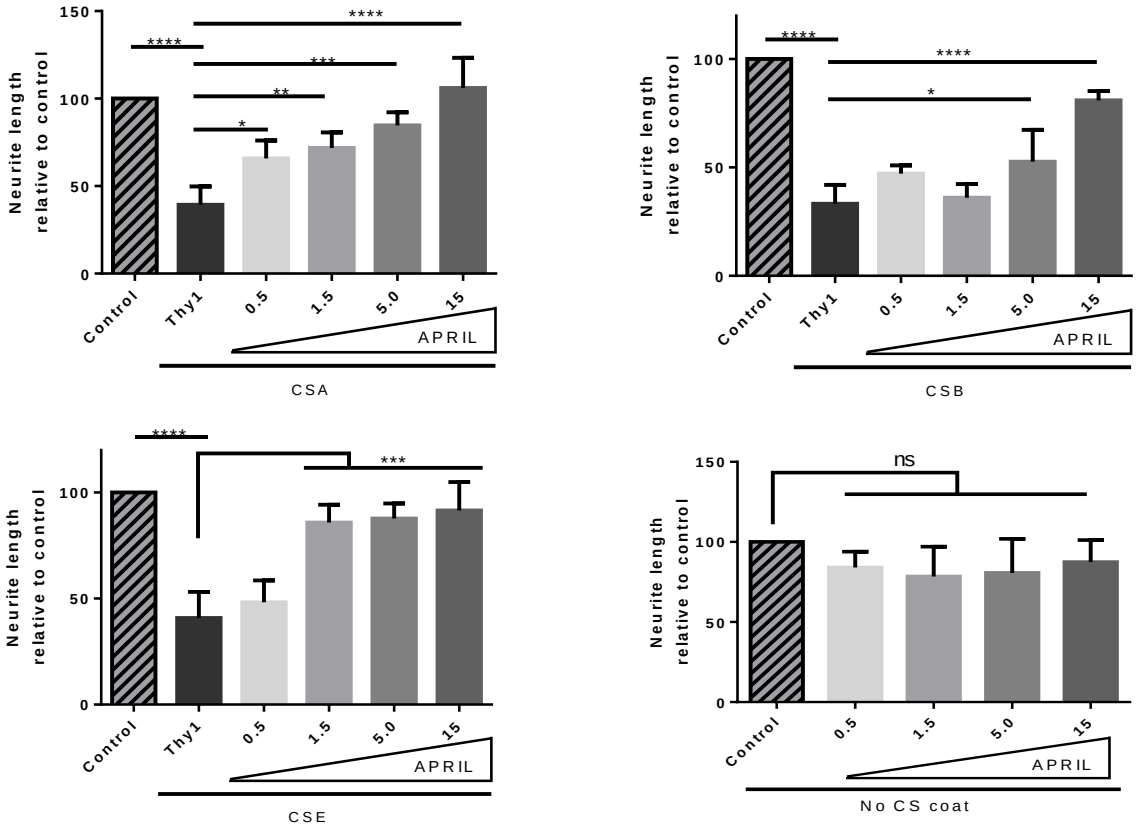


Figure 15: APRIL-Fc prevents CS-mediated inhibition of neurite outgrowth.

Embryonic mouse cortical neurons were cultured 3 days on either CS-coated or untreated substrate (control) and stained for β-tubulin III (green) and Hoechst 33342 (blue). A) Representative images of neurons grown in absence (left), or presence of CS-coat. Quantification of total neurite length per cell as a percentage of control-treated cells. Scale bar = 100µm. B) Representative images of neurons grown in absence (left), or presence of CS-coat with Thy1-Fc (negative control, top row) or APRIL-Fc (bottom row) treatment. Quantification of total neurite length per cell as a percentage of control-treated cells. Different concentrations of APRIL are shown in µg/ml. Data are presented as mean +/- SD of at least 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (One-way ANOVA). Data are presented as relative to average total neurite length without CS-coat and with control treatment. n > 30 cells measured per experiment. ns = non-significant. Thy1-Fc was used as a non-CS binding irrelevant control, at a molar concentration equivalent to the highest concentration of APRIL-Fc used. CS-A, CS-B, and CS-D were used at 10 µg/ml, while CS-E at 0.1 µg/ml.

3 Preliminary Results: OPC outgrowth assays

CSPGs have been demonstrated to inhibit OPC outgrowth *in vitro* in several studies [5, 24, 26, 82], however, to the best of our knowledge the actions of specific CS-GAG types have not been investigated. We evaluated the inhibitory effects of CS-GAG types on OPC outgrowth, as defined by their outgrowth surface area, cell perimeter, and process ramification on mouse embryonic cortex-derived OPCs. CS-A did not have any observable effects on OPC outgrowth or adhesion at concentrations tested (up to 20 µg/ml), while CS-E caused significant loss of cell adhesion at concentrations higher than 1 µg/ml (figure 16). OPCs cultured on CS-B and CS-D coated substrates exhibited stunted process outgrowth after 1 day, with a reduction of about 50% in their perimeter and outgrowth area, as well as a reduction in ramifications compared to cells grown on PLL alone (figure 17-A).

We set out to determine if APRIL-Fc treatment could abolish CSPG-mediated inhibition of OPC outgrowth. As with our experiments with neurons, CS-coated coverslips were treated with APRIL-Fc before seeding of cells. APRIL-Fc abolished outgrowth inhibition by CS-B and CS-D (figure 17-B). Of note, a much lower concentration of APRIL-Fc was necessary for this effect compared to that used to alleviate inhibition of neuronal outgrowth (1.5 µg/ml versus 15 µg/ml of APRIL-Fc necessary to abolish CS-B inhibition of OPC and neuronal outgrowth, respectively). APRIL-Fc treatment alone did not affect OPC outgrowth. Here we show for the first time that, as is the case with neurons, CS-GAG types exhibit differential inhibitory activities on OPC outgrowth, and that these are blocked by APRIL-Fc.

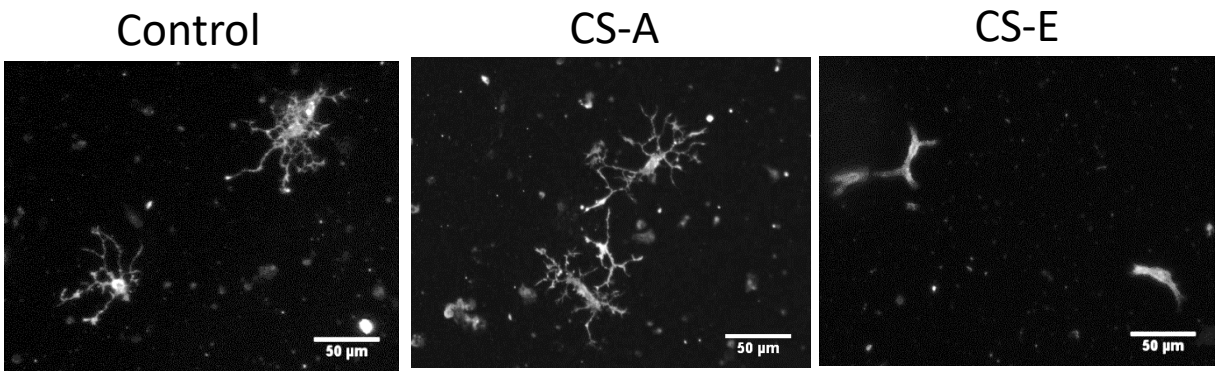
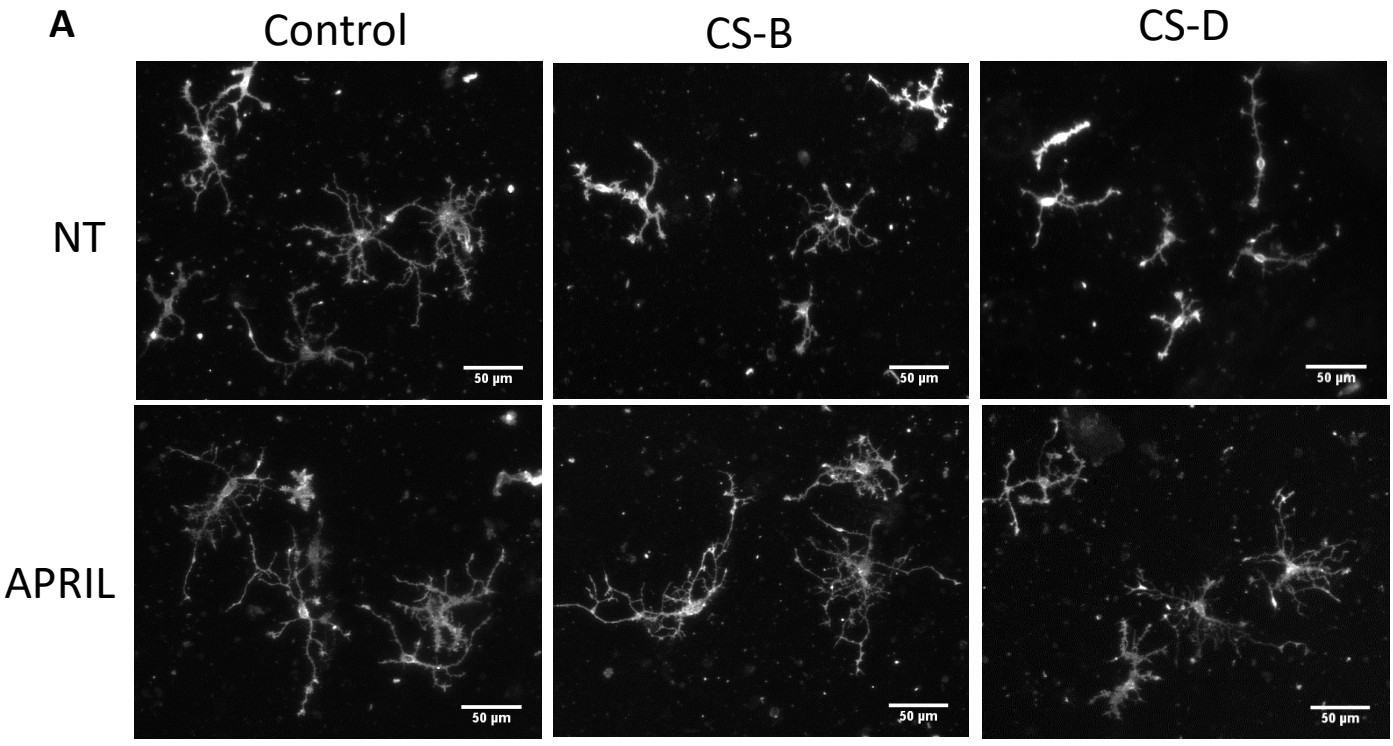


Figure 16: The differential effects of CS-A and CS-E on OPCs.
Representative images of embryonic mouse cortical OPCs cultured for 24 hours on either CS-coated (either 20 µg/ml of CS-A, middle, or 1 µg/ml of CS-E, right) or untreated (control) substrates, and stained for NG2. A severe loss of cell attachment was observed on CS-E-coated surfaces, while no observable effect was seen on CS-A-coated surfaces. Representative of n ≥ 2 experiments. Scale bar = 50 µm.



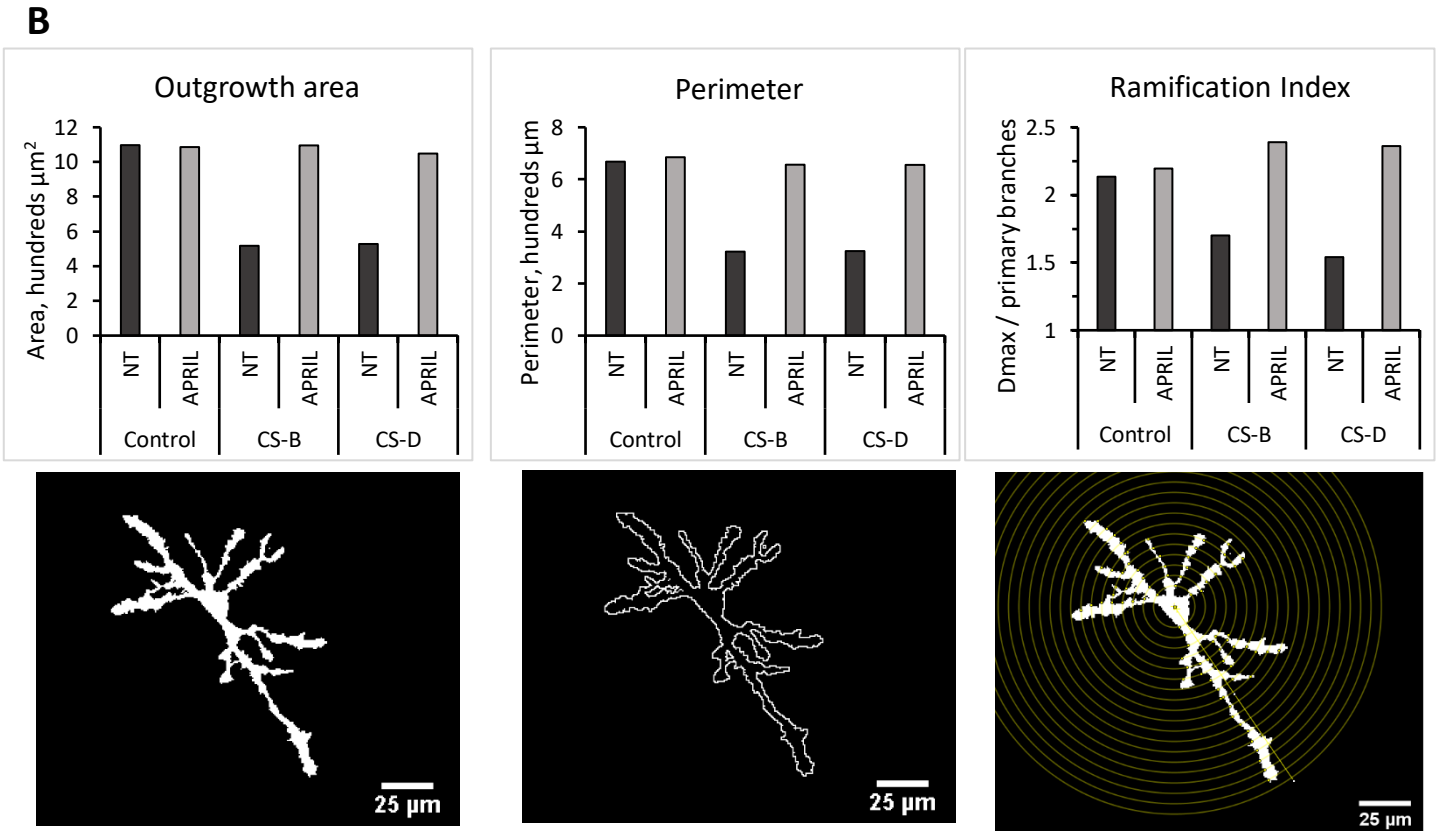


Figure 17: APRIL-Fc prevents CS-mediated inhibition of OPC outgrowth.
A) Representative images of embryonic mouse cortical OPCs cultured for 24 hours on either CS-coated (either 2 µg/ml of CS-B, middle, or 10 µg/ml of CS-D, right) or untreated (control) substrates, and stained for NG2. Substrates were pre-treated with APRIL-Fc (1.5 µg/ml, bottom row) or with PBS alone ('NT', top row). Scale bar = 50 µm. B) Quantifications and representative images of OPC outgrowth in terms of outgrowth area (left), perimeter (middle), and ramification index (right). The outgrowth area is calculated as the surface area of the cell minus the surface area of the cell soma. The perimeter is calculated as the area of the cell outline. The ramification index was acquired by Sholl analysis and is equal to the ratio of the dendrite maximum (Dmax) to the number of primary branches of the cell. Data represent a single experiment and is presented as a mean of n ≥ 30 cells measured per condition.

4 APRIL-Fc promotes myelination in an *ex vivo* organotypic model

A well-established *ex vivo* model of remyelination utilizes P10 mouse pup-derived cerebellar slice cultures demyelinated by lysolecithin (LPC) [303, 304]. An overnight (16-18 h) LPC treatment causes progressive widespread disruption of myelin sheaths in the cerebellar white matter (figure 18-A), peaking at day 3. These demyelinated axons of Purkinje cells of the cerebellar lobes spontaneously remyelinate in the days following the LPC treatment, with significant restoration of myelinated axons observed by day 5.

In a pilot experiment, we assessed the spatial-temporal expression of CSPGs following LPC treatment in cerebellar lobes using the mouse monoclonal anti–pan-CSPG antibody, CS-56 (red staining). As previously reported in similar models [5, 8], CSPG expression increased in response to LPC treatment (figure 18). This upregulation is closely associated with the extent of demyelination, peaking at day 3 and disappearing in remyelinated tissue by day 5. In a same slice, lobes more severely demyelinated by LPC were more intensely stained with CS-56, while lobes that escaped demyelination were relatively clear of CS-56 staining, underscoring that CSPG upregulation is a response to local damage, and not a tissue-wide response to LPC treatment.

To assess whether APRIL-Fc-mediated release of OPC inhibition by CSPGs *in vitro* could translate to an accelerated remyelination, cerebellar slices were treated with LPC and then cultured in medium alone (non-treated, NT), in the presence of 5 µg/ml of APRIL-Fc, or an equivalent molar concentration of an irrelevant control (Thy1-Fc) for 3 days, then stained for myelin (MAG, green) and axons (CaBP, red). Following LPC-demyelination, APRIL-Fc treated slices exhibited a greater percentage of myelinated axons (~70% myelination versus ~45% seen in Thy1-treated or non-treated slices) (figure 19). The irrelevant treatment did not significantly differ from the non-treated control.

In light of preliminary *in vitro* data, one can suggest APRIL-Fc promotes the mobilisation of local myelinating cells and their reparative potential by binding to CS-GAGs and blocking their interactions with inhibitory protein partners. Altogether, these observations show a double-edged regeneration/remyelination-promoting role for APRIL-Fc.

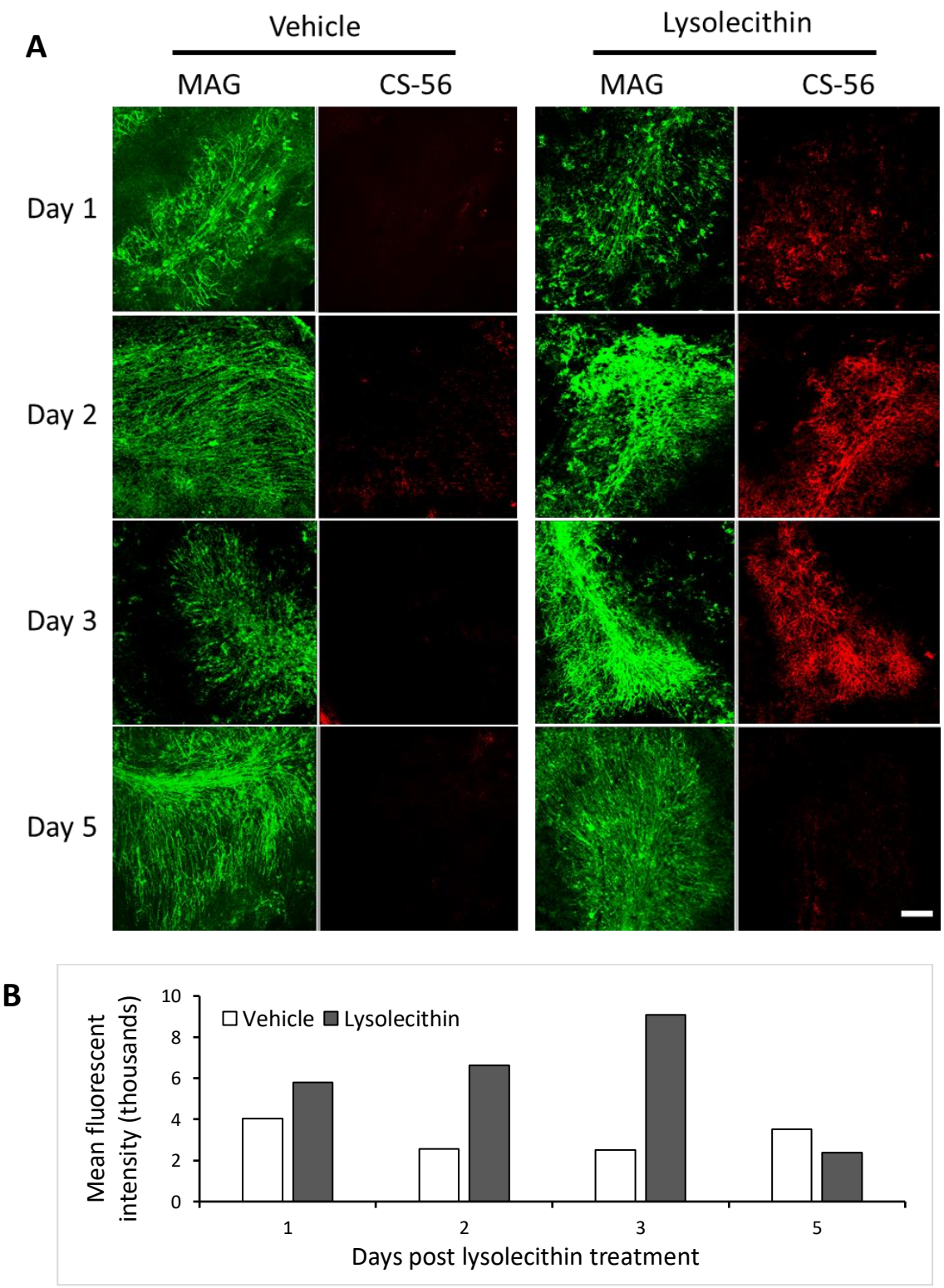
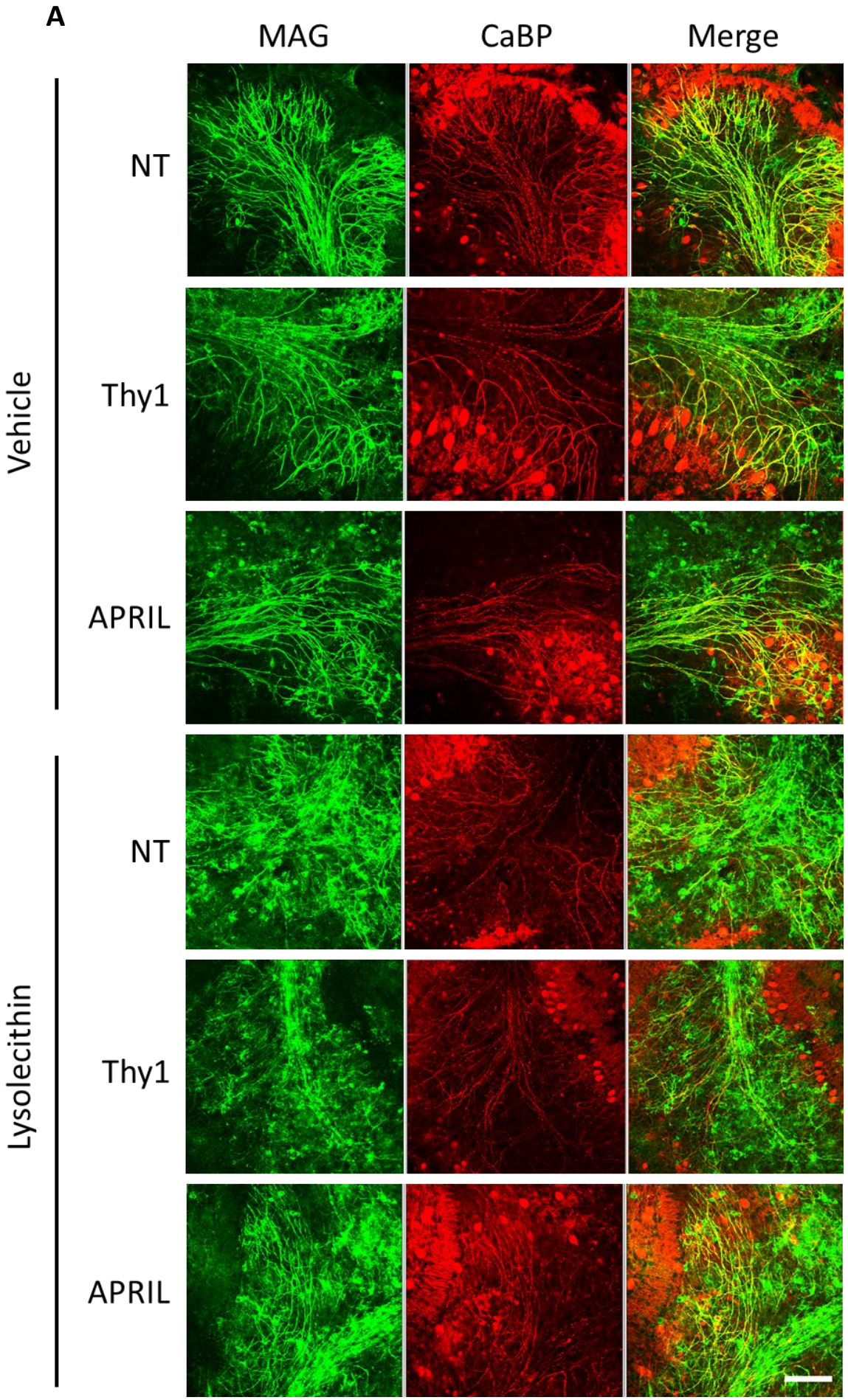


Figure 18: CSPG expression is upregulated following cerebellar demyelination.
Parasagittal slices of postnatal day 10 (P10) mouse cerebellum were treated with lysolecithin or methyl chloride (MeCl) (vehicle) and then cultured for different durations for a pilot experiment. A) Representative immunohistochemistry images of cerebellar lobes stained with MAG (green) for myelin and CS-56 (red) for CSPGs. Scale bar = 100µm. B) Quantification of CSPG expression by mean fluorescence intensity of CS-56 staining cerebellar lobe white matter. Data are presented as mean of 6-9 lobes per condition from a single experiment.


A
MAG
CaBP
Merge
Vehicle
NT
Thy1
APRIL
Lysolecithin
NT
Thy1
APRIL

B

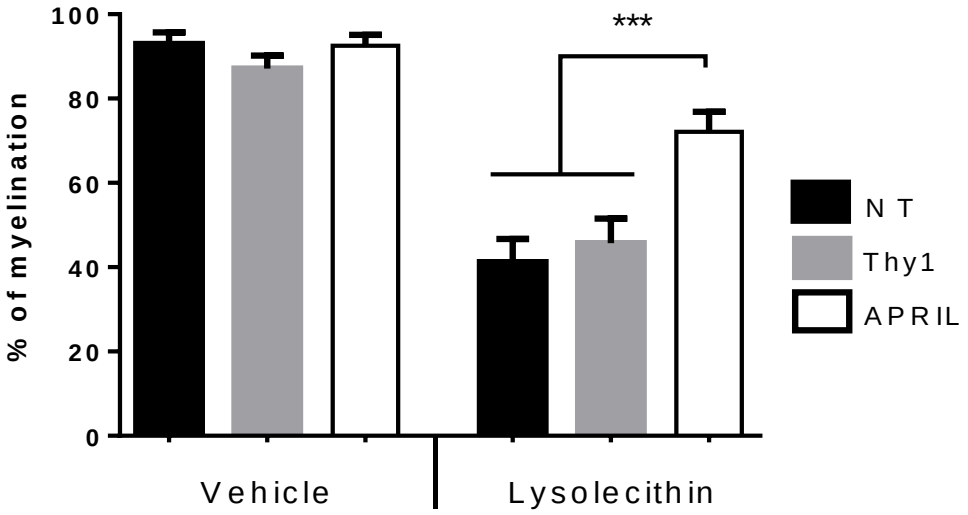


Figure 19: APRIL improves myelination in LPC-treated organotypic slice cultures.
Parasagittal slices of postnatal day 10 (P10) mouse cerebellum were treated with lysolecithin or MeCl (vehicle) and subsequently cultured with medium alone (non-treated, NT), or medium containing control treatment (Thy1), or 5 µg/ml APRIL-Fc for 3 days. A) Representative immunohistochemistry images of cerebellar lobes stained with MAG for myelin (green) and CaBP for axons (red). Scale bar = 100 µm. B) Quantification of myelination as a percentage of myelinated axons, defined by the number of MAG-positive fibres over the number of CaBP-positive fibres. Data are presented as mean +/- SEM of n >18 lobes per condition from 3 independent experiments. ***p<0.001 (Mann Whitney test). 2-3 lobes were measured per cerebellar slice, and 2-3 cerebellar slices measured per experiment. Thy1-Fc was used as an irrelevant control, at a molar concentration equivalent to that of APRIL used.

Chapter 4: Conclusions, discussion and perspectives

In line with previous work, we show that CS-GAGs are able to inhibit the growth of neurons and myelinating cells *in vitro*. In the case of neuronal outgrowth assays, the current study is an addition to several others who have tested the effects of specific CS types (table 3) We demonstrate the inhibitory effects of types A, B and E on embryonic mouse cortical neurons, as well as the inactivity of type D on these cells. To remark on the variability in the characterization of CS types in literature, we must take note of the variability in the experimental procedures used. Ample evidence suggests that neurons of different types or species of origin respond differently to a specific inhibitory cue, and this could be explained by the variability in expression of receptors like PTPRS [182]. In addition, it has been shown that the source of the CS-GAG used can alter its activity, as well as the manner in which it is prepared as a reagent. To provide an example for the latter, we found that a biotinylated preparation of CS-E had the opposite effect on OPCs compared to the non-biotinylated form, promoting process outgrowth instead of inhibiting it (data not shown).

Regarding our pilot *in vitro* functional experiments on myelinating cells, our study is, to the best of our knowledge, the first to test the effects of several CS types on the process outgrowth of NG2+ OPC cells. A significant observation lies in the differences between cortical OPCs and neurons in their response to exposure to specific CS types. For example, CS-D, a type that has consistently been found to be neutral or else beneficial to neuronal growth, exhibited an inhibitory activity towards OPCs. Conversely, OPCs grew well on CS-A at concentrations as high as 20 µg/ml, but neuronal outgrowth was strongly inhibited. This would suggest that the presentation of multiple sulfation patterns by CSPGs allow them to influence different cell types simultaneously. To vulgarize the idea, one can imagine CSPGs as a signal source 'broadcasting on different frequencies', providing instructions to cells in the vicinity that would behave differently depending on which frequency they are tuned to (defined by the expression of different protein receptors). As CSPGs have consistently been found to inhibit both neuronal regeneration as well as the mobilisation of remyelinating cells, our observations shed light on the mechanisms behind this double-edged feature of CSPGs.

It is important to note that CS-GAGs are negatively charged molecules, and thus influence cell adhesion *in vitro*. We observed a loss of cell attachment and an increase in aggregation at higher concentration of CS, in line with what was previously observed in both neurons and OPCs [82, 153]. However, the inhibitory effects of CSPGs cannot be attributed to their charges alone. It has demonstrated that neutralizing the anionic charge, though beneficial to cell adhesion, does not lift the inhibition of process outgrowth [82, 153].

The key objective of this study was to evaluate APRIL-Fc as a CSPG-blocking agent. We demonstrate the effectiveness of this molecule in relieving both neurons as well as OPCs from CS-mediated inhibition, and further we show that this applies for any and all CS types tested.

We offer a molecular mechanism for this neutralizing activity, demonstrating by ELISA experiments that APRIL-Fc binds strongly with all tested CS-GAGs and subsequently disrupts their ability to interact with recombinant protein partners. CSPGs are able to interact with a wide variety of proteins from different families to effectuate their inhibitory actions on repair in the CNS. In addition to the well-characterized CSPG receptors, we show that CS-GAGs are able to interact with members of the ephrin and slit families as well. Recombinant Slit2-Fc interacted with CS-A and CS-B, while EFNB2 interacted with CS-A and CS-E. Of note, CS-E was previously found to be important in the activity of EphA4, a receptor of EFNB2. This presents the possibility that, as in the case for Sema3A and its receptors, CS-E may be playing the role of a coreceptor for the interaction of an ephrin receptor and ligand. It should be stated that the commercially available recombinant Sema3A that was used in this study is known to have a reduced ability to bind to CS-E, due to mutations in its sequence.

A curious remark is that CS-D, a type that showed limited diversity in protein binding in our experiments, was also the type found to be neutral in neuronal outgrowth assays. Its inhibitory activity on OPC outgrowth may likely depend on other interactions, and PTPRS is indeed a prime candidate to be among them. Furthermore, the fact that CS-D and CS-E are both disulphated CS types but have differing protein partners and effects underscores the importance of the distribution of sulphate motifs, rather than the disaccharide's overall anionic charge.

In an *ex vivo* demyelinating model using organotypic cerebellar slice cultures, we show that APRIL-Fc treatment during the stages of CSPG-upregulation boosts the regeneration of myelin in 3 days following LPC treatment. The CNS has a remarkable ability to remyelinate

following injury, and this can be seen in multiple sclerosis (MS) [210, 212, 213]. It is generally accepted that this intrinsic repair is owed to a mobilisation and recruitment of OPCs, and their subsequent differentiation into myelinating oligodendrocytes. However, remyelination eventually fails in later phases of MS, though not for a lack of OPC numbers available around the damaged region, but rather due to their failure to migrate into the lesions and differentiate [126]. It would appear that OPCs lose their regenerative potential upon certain biochemical alterations that occur in chronically active lesions**,** and there is ample evidence to suggest that the upregulation of CSPGs in particular is to blame [8, 225]. Targeting CSPGs in demyelinating models has consistently shown to improve repair.

In a pilot experiment, we showed that CSPGs are upregulated following LPC-demyelination in the mouse cerebellum, and are cleared with remyelination. This closely mirrors what was previously observed in the demyelinated mouse spinal cord [5]. As APRIL-Fc treatment exhibited effective CS-blocking activity in pilot *in vitro* assays, it was promising to find that the treatment promoted myelination in demyelinated cerebellar slices during a period of CSPG-upregulation. Blockage of CS-GAGs by APRIL-Fc may prevent their interactions with partners present on local myelinating cells, facilitating the latter's reparative functions. The characterization of these cells and their behaviour in the context of APRIL-Fc treatment presents an interesting perspective for further study, and may further elucidate the mechanisms behind the modulation of remyelination by CS-GAGs and APRIL-Fc. Alternatively, or perhaps as a result of accelerated repair, CSPGs may also be cleared from the environment at an expedited rate. At this stage it is difficult to say whether APRIL-Fc treatment could be directly responsible for a downregulation of CSPGs, or whether this is a natural consequence of facilitated regeneration.

APRIL-Fc's action as a blocking agent allows it to join the ranks of other prospective CSPG-targeting treatments. Previous studies have sought to create CSPG-targeting agents, with various degrees of success. In one paper, a small HS-binding compound, surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide), was also found to have an affinity for CS [307]. When tested in mouse models of MS, surfen treatment was found to reduce inflammation and clinical score in EAE mice [308], reflecting the possible role of APRIL in MS that was disrupted by atacicept. However, the same study found that surfen treatment had a negative effect on remyelination in the LPC model.

In another study, heparin-binding growth-associated molecule (HB-GAM) was found to bind to CSPGs, and reverse inhibition of neurite outgrowth by CSPGs *in vitro* [25, 309]. In addition, HB-GAM promoted neurite regeneration in the injured mouse cerebral cortex and spinal cord. This candidate abrogated binding of CSPGs to one of its receptors, PTPRS, and was demonstrated to promote neurite growth by interacting with cell-surface glypican-2 while docked on CS.

The inhibition of CSPG synthesis in lysolecithin-demyelinated mice by xyloside [5] or fluorosamine [82] treatments both reduced CSPG burden in the lesioned spinal cord, and improved the recruitment of myelinating cells, and subsequent remyelination of lesions. Additionally, fluorosamine treatment in EAE mice resulted in a reduced upregulation of versican RNA, and a lower disease severity. Though these molecules act as inhibitors of CSPG synthesis rather than as blocking agents, it nonetheless demonstrates the potential in targeting CSPGs.

Anti-CS-E antibodies have demonstrated the effectiveness of stereometric blockage in relieving the inhibition of neuronal outgrowth by CS [65, 118]. This in particular reinforces the appeal of APRIL-Fc, which lies in its indiscrimination, i.e. its pan-CS affinity and competitive inhibition of a great variety of CS-protein interactions tested.

As CSPGs act as a convergent point for many repair-inhibiting pathways, APRIL-Fc's potential to block several of these simultaneously offers to overcome the problem of compensation and redundancy that may occur from blocking one pathway alone. Evidence for the effectiveness of targeting multiple CSPG-partners can be found in Dickendesher's impactful study on NgRs and PTPRS [34]. Perhaps this would explain the particularly high sensitivity of CS-A-mediated inhibition of neurons to APRIL-Fc treatment, as CS-A was observed to interact with the widest range of protein partners tested.

There are a limited number of studies on APRIL's role in the CNS. Davies' team of Cardiff University reported that APRIL could modestly enhance mouse dopaminergic and hippocampal axon growth in a BCMA-dependent manner during a window of embryonic development [310, 311]. In another study, APRIL-deletion in mice appeared to reduce scar-formation following contusive SCI, though this was attributed to a reduced immune response at the injury site.

Though the current study focuses on a recombinant, crosslinked (hexameric) form of APRIL, it stems from the adverse effects of APRIL blockade in the ATAMS clinical trial [152], and the subsequent discovery of an accumulation of physiological APRIL in MS lesions with a putative protective role [42]. These observations highlighted a novel role for this TNSF member in neurodegenerative disease, but also paved the way for therapeutic potential. In the form used for this study, APRIL-Fc retains the ability to interact with its receptors, BCMA and TACI. A single substitution mutation (R231A) is sufficient to disrupt receptor binding without affecting HS-GAG binding [282], creating a variant that could theoretically circumvent potential immune-related side effects.

Taken altogether, we demonstrate the effectiveness of a recombinant APRIL molecule as a CSPG-blocking agent, and propose that it presents a viable therapeutic strategy to promote regeneration in the CNS.

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Résumé / Abstract

Abstract in English

Chondroitin sulfate proteoglycans (CSPG) are major constituents of the extracellular matrix, and are well established as obstacles to neural regeneration and remyelination in the central nervous system. As such, they are promising targets for therapy in neurological pathologies where repair is impaired, such as spinal cord injuries, and multiple sclerosis. Since CSPG mediate their inhibitory functions by interacting with signalling protein partners through their variably-sulfated chondroitin sulfate (CS) chains, blocking these epitopes presents a path to promoting repair. The aim of this study is to evaluate a proliferation inducing ligand (APRIL) as an agent to block the inhibitory effects of CSPG. We show that different CS types interact with a wide variety of inhibitory proteins, and that these interactions are blocked by APRIL. APRIL-Fc is able to neutralize the inhibitory effects of CS types on cortical neurons in *in vitro* functional assays, as well as on oligodendrocyte progenitor cells in pilot experiments. APRIL also promoted remyelination in an *ex vivo* organotypic model of demyelination, where CSPG are upregulated. Altogether, we demonstrate the potential of a recombinant APRIL for releasing the brakes on repair in the CNS.

Key words: a proliferation inducing ligand (APRIL), chondroitin sulfate proteoglycan (CSPG), central nervous system (CNS), remyelination, spinal cord injury, multiple sclerosis

Abstract en français

Les chondroïtines sulfates protéoglycanes (CSPG) sont des composants majeurs de la matrice extracellulaire, et sont bien connus pour leur rôle en tant qu'inhibiteurs de la régénération neurale et de la remyélinisation dans le système nerveux central. A ce titre, ils représentent une cible thérapeutique prometteuse dans les pathologies neurologiques où la régénération est entravée, comme dans des lésions de la moelle épinière ou la sclérose en plaques. Puisque les CSPG fonctionnent en interagissant avec des partenaires protéiques à travers leur chaines chondroïtine-sulfates (CS), cibler ces derniers en bloquant leurs épitopes peut ouvrir une voie pour promouvoir la régénération. L'objectif de cette étude est d'évaluer une 'a prolifération inducing ligand' (APRIL) recombinante comme agent bloquant des effets inhibiteurs des CSPG. Nous montrons que les différents types de CS testés interagissent avec une variété de protéines inhibant la régénération, et que ces interactions sont bloquées par APRIL-Fc. Dans des cultures des cellules corticales de souris, APRIL-Fc a aboli les effets inhibiteurs des CS sur la croissance des neurones, et des effets similaires ont été observés pour des cellules progénitrices d'oligodendrocytes dans des expériences préliminaires. Finalement, APRIL a boosté la remyélinisation dans un modèle *ex vivo* organotypique, où les CSPG sont surexprimés à la suite d'une démyélinisation. En conclusion, ces données démontrent le potentiel d'une APRIL recombinante de relâcher les freins sur la régénération dans le SNC.

Mots-clefs : a proliferation inducing ligand (APRIL), chondroïtines sulfates protéoglycanes (CSPG), system nerveuse central (SNC), remyélinisation, lésion de la moelle épinière, sclérose en plaques