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**Modulation of Cell-Cell Interactions for Neural Tissue Engineering: Potential
Therapeutic Applications of Cell Adhesion Molecules in Nerve Regeneration**

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Abstract

Neural tissue engineering holds great promise in repairing damaged nerve tissues. However, despite the promising results in regenerating the injured nervous system, tissue engineering approaches are still insufficient to result in full functional recovery in severe nerve damages. Majority of these approaches only focus on growth factors and cell-extracellular matrix (ECM) interactions. As another important component in nerve tissues, the potential of modulating cell-cell interactions as a strategy to promote regeneration has been overlooked. Within the central nervous system, there are considerably more cell-cell communications as compared to cell-ECM interactions, since the ECM only contributes 10 % - 20 % of the total tissue volume. Therefore, modulating cell-cell interactions through cell adhesion molecules (CAMs) such as cadherins, neural cell adhesion molecules (NCAM) and L1, may be a potential alternative to improve nerve regeneration. This paper will begin by reviewing the CAMs that play important roles in neurogenic processes. Specifically, we focused on 3 areas, namely the roles of CAMs in neurite outgrowth and regeneration; remyelination; and neuronal differentiation. Following that, we will discuss existing tissue engineering approaches that utilize CAMs and biomaterials to control nerve regeneration. We will also suggest other potential methods that can deliver CAMs efficiently to injured nerve tissues. Overall, we propose that utilizing CAMs with biomaterials may be a promising therapeutic strategy for nerve regeneration.

Keywords: myelination, neuronal differentiation, neurons, oligodendrocytes, neural stem cells, scaffolds

1. Introduction

Neurodegenerative disorders and injuries to the nervous system can lead to permanent disabilities. In many cases, full recovery from serious injuries is difficult. The inhibitory environment that is caused by inflammation and glial scarring after injuries often hinders regeneration in the central nervous system (CNS). Comparatively, the peripheral nervous system (PNS) possesses better recovery capacity than the CNS. However, healing is still usually slow and sub-optimal in the presence of large lesions. Hence, although the outcomes of nerve injuries are devastating, current clinical treatments remain suboptimal [1].

In search for better therapeutic options, recent advances in regenerative medicine suggest that biomaterials and tissue engineering approaches offer hope to fully repair damages in the nervous system. In particular, tissue-engineering methods have been designed to deliver cells and signals, such as neurotrophic factors, for nerve regeneration [1-3]. In most of these approaches, the delivery of soluble neurotrophic factors and surface contact signals, in the form of extracellular matrix (ECM) proteins, have been the main foci. However, despite the promising results in regenerating the injured nervous system *in vivo* [4-7], these approaches were still insufficient to result in full functional recovery in severe nerve damages. As another important component in nerve tissues, the potential of modulating cell-cell interactions as a strategy to promote regeneration has been overlooked.

Indeed, engineering cell-cell interactions is less utilized even though there are considerably more cell-cell communications within the nervous system, especially within the CNS. In particular, the ECM only contributes 10 % - 20 % of the total tissue volume within the CNS [7, 8]. Furthermore, various functions such as neural development, synapse formation, neurite outgrowth and cell migration rely heavily on cell-cell interactions. Therefore, it is important to understand the role of cell-cell contacts in nerve regeneration. This information may serve as another potential avenue to enhance nerve regrowth.

Cell-cell interactions usually involve direct cell-cell contacts that are initiated and mediated by cell surface molecules or cell adhesion molecules (CAMs). CAMs are transmembrane proteins that are located on the cell surface. Although majority of the CAMs mediate homophilic interactions in which a CAM on the cell surface interacts with the same type of CAM on another cell surface, some CAMs are also capable of heterophilic binding with other CAMs, cell surface receptors, or ECM molecules [9]. This suggests that CAMs mediate other functions in addition to their cell adhesive capacity. Generally, the transmembrane CAMs link extracellular signals to intracellular response through their cytosolic domains. The signals are then transduced from the cytosolic domains to the connected intracellular signaling complexes and cytoskeleton, subsequently influencing downstream cellular responses. The importance of CAMs is especially emphasized in many genetic diseases in the nervous system [10, 11] where CAM deficiency leads to many neurological dysfunctions. For example, mutation of L1 cell adhesion molecule (L1CAM), a type of CAM found on neurons, leads to neurological diseases, such as X-linked hydrocephalus, MASA syndrome (MASA: mental retardation, aphasia, shuffling gait, and adducted thumbs; also known as CRASH syndrome or L1 syndrome), and spastic paraplegia type 1 [12]. Dysfunction in another type of CAM, close homolog of L1 (CHL1), is associated with intellectual disability and schizophrenia [13, 14]. On the other hand, the abnormal regulation of neural cell adhesion molecule (NCAM) is also suspected to be linked to neuropsychiatric diseases [15]. Therefore, CAMs are also expected to play crucial roles in neural regeneration [16, 17].

Given the importance of CAMs, diverse CAMs have been identified to help in nerve regeneration and their underlying mechanisms were studied. However, the translational application of CAMs as therapeutics for neural regrowth remains underexplored. Hence, the aim of this review is to expand the potential application of CAMs to neural tissue regeneration. Strategies to restore the damaged nervous system generally involve inducing axonal regrowth, remyelination of the axons and differentiation of stem cells into the desired neural cell types. Therefore, we will review the CAMs that have been elucidated to play important roles in these neurogenic processes. Specifically, we have divided our

discussion into 3 categories, namely the roles of CAMS in neurite outgrowth and regeneration; myelination; and neuronal differentiation. Following that, we will discuss existing tissue engineering approaches that utilize these CAMs in modulating neural regeneration. We will also suggest other potential methods that can efficiently deliver CAMs to the injured nerve tissues. Overall, we propose that utilizing CAMs may be a promising therapeutic strategy for nerve regeneration.

2. Types of cell adhesion molecules in the nervous system

The CAMs in the nervous system that are involved in direct cell-cell interactions can be generally categorized into cadherins and immunoglobulin super family (IgSF). These CAMs that are found in the nervous system were reviewed in detail previously [9, 16, 18-20]. Table 1 summarizes the common CAMs that have been studied in the nervous system. Some of these CAMs have been isolated or modified into different forms that can be utilized for therapeutic applications.

Table 1: Major types of cell adhesion molecules in the nervous system

Subfamily	CAMs	Known cell-cell interaction	Soluble/Chimeric form tested for neural cells
Cadherins	Classical cadherins (NCAD, ECAD, RCAD etc.)	neuron-neuron [18], neuron-glia [18], glia-glia [21]	NCAD [22], NCAD90 [23], NCAD-Fc [24], ECAD [25], ECAD-Fc [26]
	Protocadherins	neuron-neuron [18], neuron-glia [27]	-
	Cadherin EGF LAG seven-pass G-type receptors (CELSR1, CELSR2, CELSR3)	neuron-neuron [28]	-
IgSFs	NCAM (NCAM1)	neuron-neuron [18], neuron-glia [29], glia-glia [29]	NCAM [30], NCAM-Fc [31]
	L1CAM (NgCAM/L1/NILE/8D9)	neuron-neuron [29], neuron-Schwann cell [32]	L1CAM [30], L1CAM-Fc [33]
	NrCAM	neuron-neuron [34], neuron-glia [35]	NrCAM-Fc [36]
	ALCAM (DM-GRASPP)	neuron-neuron [37], neuron-glia [38]	His-tagged ALCAM [39]
	Neurofascin (NF)	neuron-neuron [40], neuron-glia [41]	NF155-Fc [42], NF186-Fc [42]

Close homolog of L1 (CHL1, CALL)	neuron-neuron [40], neuron-glia [43]	CHL1 [44]
Contactin-1 (F3/ F11)	neuron-neuron [45], neuron-glia [41]	F3 [46]
Contactin-2 (Axonin-1 TAG-1, TAX1)	neuron-neuron [47], neuron-glia [48]	Axonin-1 [49], TAG-1 [50]
Nectins	neuron-neuron [18], neuron-glia [18]	Nectin-1 Ig3 [51]
Nectin-like molecules (Necls)	neuron-glia [18]	-

Among the CAMs, cadherins, NCAM and L1 are the most widely studied. The expression of cadherins is conserved in the nervous system, forming cell-cell adhesions through calcium dependent homophilic binding. On the other hand, both NCAM and L1 can interact in homophilic or heterophilic manner, depending on their function, by binding to other transmembrane molecules such as integrins. These CAMs play multiple roles in the nervous system and present interesting characteristics. For instance, Schwann cells (SCs) are prevented from migrating into the CNS by N-Cadherin (NCAD) mediated adhesion of SCs to astrocytes [21]. CAMs also play other important roles in the nervous system such as synapses formation, paranodal junctions formation, axon guidance and axon fasciculation [9, 52, 53].

Here, we will focus on the discussion of cadherins, NCAM and L1, which are the most well understood and commonly studied CAMs in nerve regeneration. Other CAMs that have been isolated and used as coatings or soluble factors for modulating neural cell adhesion will also be covered, where appropriate.

3. The roles of cell adhesion molecules in neurite outgrowth and regeneration

Among the studies on CAMs in the nervous system, the role of CAMs in axon regrowth is the most extensively studied as compared to myelination and stem cell neural differentiation. CAMs were

- 1 shown to induce nerve regrowth by different *in vitro* and *in vivo* models (Table 2). The mechanism,
- 2 potency and method of usage of these CAMs in supporting neurite growth will be discussed here.

Table 2: Overview of CAMs that were utilized to promote neurite growth

Type of CAM	Species / Cell type / Model	Format of CAM	Procedure	Concentration / Dosage	Outcome	Reference
NCAD	Chick retinal cells	Purified extracellular domain (NCAD90)	Adsorbed onto nitrocellulose coated culture dishes	0-10 µg/ml	- Dose-dependent - Number of adherent cells on NCAD90 > laminin	[23]
	Xenopus retinal ganglion cells	Purified extracellular domain (NCAD90)	Adsorbed onto polyornithine-coated glass coverslips	10 µg/ml	-Neurite length (>90 µm), -Effect comparable to laminin	[54]
	Chick retinal cells	NCAD-Fc	Affinity captured by IgG coated culture dishes	0-10 µg/ml	-Does dependent -Neurite length (49.9 µm) compared to laminin (41.5 µm)	[24]
	Rat embryonic cortical neuron	NCAD-Fc	- Chemically crosslinked - Affinity captured by IgG chemically linked to microporous OR microchannel alginate hydrogel	250 µg/ml	- Higher % of MAP2 ⁺ than GFAP ⁺ cells for biochemical conjugation compared to chemical conjugation - 2-fold higher MAP2 ⁺ / GFAP ⁺ ratio in microchannel than microporous scaffold	[55]
NCAM	Mouse cerebellar neurons	NCAM-Fc	Added to culture medium as soluble factor	0-10 µg/ml	- Dose-dependent - Maximal response at 5 µg/ml	[56]
L1	Mouse cerebellar neurons, Chick cerebellar neurons	Purified L1	Adsorbed onto nitrocellulose coated culture dish	0.1 mg/ml	-For chick neurons, higher % of neuron with neurites and longer neurite length on chick L1 (48%, 49 µm) and mouse L1 (49%, 41 µm) than laminin (6%, 37 µm) -For mouse neurons, % of neuron with neurites and neurite length were similar across coatings	[57]

Chick cerebellar neurons	L1-Fc	-Added as soluble factor -Affinity captured by IgG coated on PLL precoated culture slides	- 5 µg/ml (soluble) - 7, 35, 175 ng/ml (affinity capture)	- 2-fold increase in neurite length with soluble L1-Fc compared to control - 6-fold increase in neurite length with 175 ng/ml bound L1-Fc compared to 0 ng/ml and 7 ng/ml	[33]
Chick brain explant	Recombinant extracellular domain of L1	Adsorbed onto polyethylenimin coated coverslips	4-6 µg/ml	- >70% with neurites >100 µm, - Average neurite length 102.5 µm, shorter than soluble L1 and laminin (Substrate L1<soluble L1<laminin)	[58]
Rat spinal cord neurons, cerebellar neurons	L1-Fc	- Adsorbed onto PDL - Affinity captured by Protein A (PA) or PA/PDL coated polycarbonate	0-50 µg/ml	-Dose-dependent -2-fold increase in neurite length on L1/PA/PDL and L1/PA on film -2.5-fold increase in neurite length on L1/PA/PDL and L1/PA on microfibers	[59]
Rat hippocampal neurons, cerebellar granule neurons, DRG neurons	L1-Fc	- Adsorbed onto polystyrene - Covalently immobilized to pluronic-pyridyl-disulfide coating (PDS)	100 µg/ml	-Neurite length on L1-Fc > fibronectin and PDL	[60]
Rat cortical neurons	Purified L1	Covalently immobilized to GMBS treated-silicon wafer	100 µg/ml	-Neurite length on L1-PEG > laminin-PEG	[61]
-NG-108 cells - <i>in vivo</i> : rat (1 mm full transaction at optical nerve)	L1-Fc	-Coated onto nitrocellulose coated culture dish -Adsorbed onto chitosan coated PGA filaments	-10 nM (<i>in vitro</i>), -10 µM (<i>in vivo</i>)	- Longer neurite on L1-Fc (90.9 µm) than chitosan (47.5 µm) and BSA (22.3 µm) - 3.7-fold increase in number of cells with neurites on L1-Fc coated filaments compared to BSA	[62]

			- <i>In vivo</i> : Adsorbed onto chitosan coated nerve conduits		- <i>In vivo</i> : 2.4-fold increase in number of nerve fibers compared to without L1-Fc; promoted RGC nerve growth (2.5-fold)	
- Chick DRG neurons - <i>in vivo</i> : rat (spinal cord injury by contusion)	L1-Fc		- Coated onto PLL coated glass slide - <i>In vivo</i> : intrathecal injection with Alzet pump	- 4-200 µg/ml (<i>in vitro</i>) - 200 µg/ml, 0.5 µl/h for 2 weeks (<i>in vivo</i>)	- Dose-dependent (~6-fold increase in neurite length at 200 µg/ml compared to 4 µg/ml) - <i>In vivo</i> : 1.5-fold longer corticospinal axonal in L1 treated group than PBS control; better locomotor compared to PBS and IgG controls	[63]
Direct comparisons						
NCAD, ECAD	Chick embryonic retinal explant	NCAD-Fc, ECAD-Fc	Adsorbed onto nitrocellulose coated culture dish or PLL coated coverslips	0.25–0.50 µg (ECAD-Fc) 0.06–0.15 µg (NCAD-Fc)	-Neurites on ECAD was similar in length and density to that observed on NCAD, but different in growth cone morphology	[25]
NCAM, L1	Chick tectal neurons / mouse cerebellar granule cells	Purified NCAM, L1	Adsorbed onto nitrocellulose coated culture dish	- 0.11mg/mg (L1), - 0.14 mg/ml (NCAM)	-L1: Unfasciculated neurite outgrowth, longest neurites>200 µm in length on L1 (laminin <150 µm) after 18 h in culture. -Support attachment of neurons over astrocytes -NCAM: Weak attachment and not effective in producing neurites	[30]
NCAD, L1	Chick ciliary ganglion neurons	Purified NCAD, L1	Adsorbed onto nitrocellulose coated culture dish	0-50 µg/ml	-80% of CG neurons extended processes within 20 h, similar to laminin. -NCAD at 25 µg/ml (64% with neurites) was more effective than L1 at 100 µg/ml (35 % with neurites) -NCAD induced neurites formed faster, have more branches,	[22]

				shorter and more curved than laminin.	
Rat hippocampal neurons	Purified NCAD, L1	Adsorbed onto nitrocellulose-PLL coated culture dish	20 µg/ml (NCAD), 50 µg/ml (L1)	- >90% axons formed on NCAD after 24 h, compared to 70% on PLL. - Longer dendritic length (500 nm) than PLL (400 nm) and L1 (150 nm). - More dendritic branch points (3.5) compared to PLL (1.5) and L1 (1)	[64]
Chicken embryonic retinal ganglion cells	Purified NCAD, L1	Adsorbed on nitrocellulose coated culture dish	0.1 mg/ml (NCAD), 0.3 mg/ml (chick L1), 0.8 mg/ml (rat L1)	-Growth rate: laminin > NCAD > L1 > PLL -Defasciculated neurite growth on L1 and NCAD - No significant preference for substrate - No significant correlation with adhesion strength	[65]
Chick embryonic retinal explant	Purified NCAD, L1	Adsorbed onto PLL & nitrocellulose coated coverslips	100 µg/ml	-Weak preference for NCAD or L1 over laminin (100% neurons crossed from laminin to NCAD/L1, but only 75% from L1 and 60% from NCAD crossed to laminin).	[66]
Rat cerebral neurons	NCAD-Fc, L1-Fc	Affinity captured by IgG crosslinked to EDC/NHS functionalized PEDOT (conducting polymer)	10 µg/ml	-NCAD stimulated both axons and dendrites -L1 increased axons growth but inhibited dendrites -NCAD significantly increased all parameters from PLL in number of neurites, neurite length and number branches. -L1 doubled axonal length from PLL. -No further increase with dual	[67]

functionalization of NCAD+L1.						
Other CAMs						
ALCAM	Chick DRG neurons	His-tagged ALCAM	Immobilized by NTA-thiol coated gold nanodots on glass coverslips	1 µg/ml	- Neurite length and number of neurons with neurites were depending on density of CAMs on the surface	[39]
PTPµ	Chick embryonic retinal explant	Purified PTPµ	Adsorbed onto nitrocellulose coated culture dishes	2–4 µg/ml	- Faster growth rate on NCAD than on PTPµ. Lower density of neurite outgrowth on the PTPµ substrate in comparison with the NCAD substrate	[68]
NrCAM	Chick embryonic DRG neurons	NrCAM-Fc	Adsorbed onto polystyrene petri dishes	30 µg/ml	- Average neurite length: 145 µm for Nr-Fc, more potent than native Nr-CAM (84 µm), roughly similar to L1 (111 µm) - DRG and sympathetic ganglion cells extended long neurites on Nr-Fc, while dissociated tectal and forebrain cells had little neurite outgrowth on NrCAM.	[69]
Neurofascin	Mouse cerebellar neurons	NF155-Fc and NF186-Fc	Adsorbed onto petri dishes	30 µg/ml	- NF155 supported neurite growth - NF186 inhibited neurite growth	[42]
	Chick tectal neuron	NF22-Fc	Adsorbed onto petri dishes	100-250 µg/ml	- induced more neurite outgrowth than control and L1	[70]
CHL1	Rat embryonic hippocampal neurons	Soluble fractions of fragments from CHL1-transfected cells	Added to culture medium	40 µg /ml	- After 12 h, longest neurite length (35µm) increased by 30% -Total neurite length (60 µm) increased by 40% compared to L1 and control	[44]
Contactin-1 (F3)	Mouse DRG	Soluble F3	Added to culture medium	-	- After 24 h, average neurite length was almost 3-fold (944±245 µm) of the control with F3 (329 ± 100 µm)	[46]
Contactin-2 (TAG-1)	Rat DRG neurons,	Purified TAG-1	Adsorbed onto nitrocellulose	10-50 µg/ml	- 50 % of neurites with length over 170 µm (better than IgG,	[50]

	superior cervical ganglion sympathetic neurons		coated culture dish		not better than laminin)	
Contactin-2 (axonin-1)	Chicken embryo DRG	Purified axonin-1	Adsorbed onto nitrocellulose coated culture dish	80 µg/ml	-Longest neurite: $555 \pm 230 \mu\text{m}$ compared to $513 \pm 30 \mu\text{m}$ (L1) and $530 \pm 114 \mu\text{m}$ (Laminin) - Total neurite length: $2160 \pm 200 \mu\text{m}$ compared to $2040 \pm 140 \mu\text{m}$ (L1) and $1070 \pm 155 \mu\text{m}$ (Laminin)	[71]

3.1 Cadherins

3.1.1 Mechanism and potency

Cadherins play important roles in neurite outgrowth and nerve regeneration. This is particularly evident from the significant alteration in their gene and protein expressions after nerve injuries in the CNS [72, 73] and PNS [32, 74, 75]. Among the types of cadherins that are present in the nervous system, N-Cadherin (NCAD) is the best studied. Since NCAD binding requires calcium, NCAD-induced neurite outgrowth is also related to the presence of Ca^{2+} [19, 76]. The mechanism of NCAD-induced neurite outgrowth is mediated by catenins and p120, which are linked to the cytoskeleton [16, 77]. Besides that, NCAD also stimulates neurite outgrowth through fibroblast growth factor receptor (FGFR) activation and its downstream pathways [24].

By showing that neurite outgrowth is induced by NCAD present on the cell membrane, the foundation of utilizing NCAD as biomolecules to induce neurite outgrowth was laid. Functionally speaking, NCAD is essential in axonogenesis *in vivo* and mediates neurite outgrowth on astrocytes [54, 78]. When PC12 cells were cultured on 3T3 fibroblast cell line that was transfected with chick NCAD [79], NCAD expression induced higher percentage of PC12 cells with neurites, together with longer neurite outgrowth length after 48 h. Furthermore, NCAD induced longer neurite outgrowth than soluble nerve growth factor (NGF), and a combination of NCAD and NGF increased neurite length synergistically [79]. Since NGF is commonly used in neural tissue engineering to enhance neurite outgrowth [80], better outcomes could possibly be achieved by combining with NCAD. For example, NCAD may be introduced as a coating or encapsulated in the scaffold, together with NGF, for neural tissue engineering. Specific approaches will be further discussed in the subsequent sections of this review.

Similarly, other types of cadherins also play a role in neurite outgrowth. In particular, E-cadherin (ECAD) promotes neurite extensions from DRG neurons. When compared to NCAD, neurite extensions on ECAD coatings appeared similar in length and density. However, the morphology of growth cones

was different on ECAD, NCAD and laminin. Generally speaking, growth cones present on an ECAD substrate consist of large and broad lamellipodia with a few short filopodia, whereas NCAD had smaller lamellipodia with several short filopodial processes. In contrast, growth cones with small lamellipodia were observed on laminin [25]. These observations suggested that different signaling mechanisms may be involved but the implication of the observation remains unknown.

3.1.2 Method of Usage

Purified NCAD is commonly utilized as a coating to enhance neuron attachment and neurite extensions (Table 2). This shows that NCAD can be used as a biomolecule and opens the possibility of modifying biomaterials with NCAD. For example, NCAD may be applied to replace some existing coatings on biomaterials, such as poly-L-lysine (PLL), or NGF-incorporation that is commonly used for tissue engineering. As compared to PLL, NCAD is more permissive to neurite regeneration [64, 65]. However, relative to laminin, the extent of neurite outgrowth that are induced by cell-membrane derived NCAD appears similar, although NCAD appears to be more potent since lower dosages are often required to achieve similar extents of neurite outgrowth as laminin [22]. On the other hand, in terms of the rate of neurite regeneration, NCAD was less efficient than laminin [64, 65]. Notably, neurites that formed on NCAD typically had more branches that were shorter and more curved as compared to those found on laminin. Such morphological variations may be due to differences in adhesion strength and the mechanisms involved and between cell-cell and cell-matrix interactions on neurite outgrowth [22, 23]. As different mechanisms are involved, both NCAD and laminin may potentially stimulate neurite outgrowth through different pathways. Hence, an additive effect may be achieved by co-stimulating neurite outgrowth through both cell-cell and cell-matrix interactions. With the addition of growth factors such as NGF, combining these three factors may further stimulate neurite outgrowth when designing nerve regeneration strategies.

1 In addition to purified full length NCAD, the extracellular domain of NCAD alone is enough to
2 stimulate neuronal adhesion and neurite outgrowth. The smaller size of the extracellular NCAD makes it
3 beneficial for tissue engineering. For instance, the smaller size allows easier implementation without the
4 fear of reduction in bioactivity. In this regard, NCAD90, a natural proteolytic product of the parent
5 NCAD molecule, retained similar biological activity and resulted in similar neuron morphology as full
6 length NCAD [23]. When used as coatings, NCAD90 also supported retinal ganglion neuron adhesion
7 and neurite outgrowth in a dose-dependent manner [23, 54].

8
9 As a way to utilize and present NCAD in a more reproducible fashion, NCAD-Fc chimeric
10 protein was constructed from the extracellular domain of NCAD and the Fc region of human
11 immunoglobulin (IgG). Correspondingly, the Fc domain enables NCAD to be purified by protein A
12 sepharose with great yield [24, 81]. These Fc domains not only improve the solubility and stability of the
13 NCAD molecules [82], but also make it possible to immobilize NCAD onto culture dishes and
14 biomaterials [25, 26, 67, 83, 84]. As a result, neurite outgrowth from chicken retinal explant on NCAD-Fc
15 and ECAD-Fc-coated culture dishes appeared similar in length and density as compared to laminin [25].
16 Besides being introduced as a coating, the ability of soluble NCAD-Fc in stimulating neurite regeneration
17 from neurons suggests that soluble NCAD-Fc may be delivered into the pathological/lesion site to induce
18 neural regeneration *in vivo*. This possibility of utilizing NCAD-Fc in a soluble format further increases its
19 potential for clinical application. Specifically, when mouse cerebellar granule cells were cultured on 3T3
20 cells in the presence of soluble NCAD-Fc, neurite outgrowth was found to be dose-dependent on the
21 concentration of NCAD-Fc [24]. The soluble NCAD-Fc promoted neurite outgrowth even at a
22 concentration of as low as 5 nM as compared to neurons on 3T3 cells without NCAD-Fc, which did not
23 display any neurite regrowth [24].

24
25 NCAD-Fc chimeric proteins also allow the direct conjugation of these biomolecules onto other
26 surfaces and biomaterials for neuron adhesion and neurite outgrowth. Correspondingly, NCAD-Fc-

1 conjugated biomaterials can be an attractive platform for cell delivery and act as a bridge for neurite
2 growth across lesions. One such approach is to use a polyaspartamide protein linker to chemically modify
3 materials with Fc-antibodies so that NCAD-Fc may be biochemically immobilized. Correspondingly,
4 NCAD–tethered glass coverslips better supported the adherence of rat embryonic cortical neurons than
5 PLL-coated coverslips [85]. When this NCAD-Fc was conjugated within alginate hydrogel with micro-
6 channels, cortical neurons grew better and their growth direction could be controlled [55].

7
8 Despite the positive results of being utilized as surface coatings and soluble factors, the delivery
9 of cadherins *in vivo* has not been attempted. Instead, cadherins have been introduced *in vivo* in the form of
10 cadherin-overexpressing transfected cells. Indeed, the transplantation of neural stem cells (NSCs)
11 transfected with ECAD improved neurological functions of injured rats after spinal cord injury (SCI) [86].
12 At one week after transplantation, ECAD overexpression was found to significantly improve the Tavlov
13 scores of SCI rats as compared to non-transfected NSCs and NSCs transfected with empty plasmids. The
14 ECAD overexpression group also had more cells that expressed neurofilament (NF) [86]. These results
15 demonstrated the efficacy of ECAD in promoting regeneration after SCI. Hence, effective strategies to
16 deliver cadherins after nerve injuries can be promising regenerative methods.

18 **3.2 NCAM**

19 ***3.2.1 Mechanism and potency***

20 NCAM has three isoforms with an identical extracellular domain but different molecular weights,
21 namely NCAM-120, NCAM-140 and NCAM-180. NCAM-induced neurite outgrowth was suggested to
22 occur through homophilic binding, followed by the activation of lipid raft-associated kinases. These raft-
23 associated kinases then signal spectrin, which links the cell membrane to the cytoskeletal system [87, 88].
24 In addition to raft-associated kinases, NCAM, especially NCAM-180, also interacts with FGFR [87].
25 Both raft-associated kinases and FGFR then activate several downstream pathways such as the ERK-

1 MAP kinase pathway [77]. NCAM also potentially plays important roles in nerve regeneration as
2 demonstrated by their expression in nerve tissues in the CNS [89-91] and PNS [92].

3
4 When 3T3 fibroblasts were transfected with NCAM overexpression gene, PC12 cells seeded on
5 the transfected 3T3 fibroblasts had more neurite outgrowth than those that were seeded on 3T3 without
6 NCAM. NCAM also induced 2-fold and 2.5-fold increases in neurite lengths in normal medium and
7 medium with NGF respectively [79]. Using a similar approach, rat cerebellar neurons were seeded on 3T3
8 cells that were transfected with NCAM to determine the dose response of neurite outgrowth towards
9 NCAM. Interestingly, there appears to be a minimum threshold level of NCAM expressed on the 3T3
10 cells that is required for neurite outgrowth to occur. Once this minimum NCAM level is reached, neurite
11 regrowth increased exponentially with NCAM levels [93]. This indicates that for effective NCAM-
12 directed neurite outgrowth, high levels of NCAM may be needed. However, the exact effective threshold
13 level is dependent on cell type, substrate as well as post-translational modification of NCAM [93].

14
15 When compared to neurons on NCAD-transfected 3T3 cells, it appeared that the neurite
16 outgrowth response was linear with respect to NCAD levels, which is different from NCAM. Notably,
17 lower level of NCAM could stimulate neurite outgrowth when NCAD was co-expressed [94]. This
18 suggests that NCAD and NCAM may work synergistically to enhance neurite outgrowth.

19 20 **3.2.2 Methods of Usage**

21 Earlier studies have found that purified NCAM was ineffective in promoting neurite outgrowth
22 when these molecules were introduced as surface coatings for neuronal cell culture [30]. The authors
23 suggested that it may be difficult to purify NCAM in its active form or NCAM was unable to bind to
24 nitrocellulose-coated plates. However, when introduced as NCAM-Fc chimera in a soluble format,
25 effective promotion of neurite outgrowth was seen in a dose-dependent manner. This NCAM-Fc chimera
26 contained the extracellular portion of NCAM and enhanced neurite growth through the activation of

1 FGFR and GAP43 [31, 56]. Specifically, a low concentration of 1 $\mu\text{g/ml}$ of NCAM-Fc significantly
2 increased neurite length of mouse cerebellar neurons that were cultured on 3T3 cells and the maximal
3 response was observed at 5 $\mu\text{g/ml}$, where the neurite length was almost doubled as compared to cells that
4 were not treated with NCAM-Fc [56].

5
6 Taken together, these studies show that NCAM is effective in stimulating neurite outgrowth when
7 expressed on cell surfaces at high levels. In comparison, soluble NCAM-Fc stimulated neurite outgrowth
8 even at low concentrations. This was not observed in cell membrane-expressed NCAM and coated
9 NCAM, suggesting that substrate-bound NCAM may not be as effective as soluble NCAM. Even though
10 NCAM may not be an effective coating to promote neurite outgrowth, soluble NCAM-Fc may be utilized
11 to enhance nerve regeneration.

13 **3.3 L1**

14 ***3.3.1 Mechanism and potency***

15 L1 within the cell membrane appears to have different binding sites for cell adhesion and neurite
16 outgrowth. Therefore, soluble L1 may stimulate neurite extensions through homophilic binding to cell
17 membrane L1 by interacting with sites that are specific to neurite growth [57]. Another suggested
18 mechanism is that soluble L1 may activate other cellular receptors such as FGFR [33, 58]. Since both
19 soluble and coated L1 can stimulate neurite outgrowth, both mechanisms may be involved. After L1 is
20 activated, the downstream signaling mechanisms then involve the Src and MAP kinase signaling
21 pathways, which in turn are associated with actin cytoskeleton through ankyrin-spectrin binding [40].

22
23 The high potency of L1 in stimulating neurite outgrowth makes it an attractive coating for axon
24 regeneration. In the earlier studies, L1 was purified from embryonic chick brain and coated onto
25 nitrocellulose-coated culture dishes. Following that, chicken tectal and mouse cerebellar neurons that
26 were grown on L1 extended unfasciculated neurites that were more than 200 μm in length after 18 hours.

1 The effects appeared better than laminin (<150μm) and NCAM (ineffective) [30, 57]. In addition, L1
2 supported neuron attachment over astrocyte attachment, which makes L1 an interesting candidate to both
3 promote neuronal adhesion while minimizing astrocyte binding. Similarly, for chick and mouse cerebellar
4 neurons, neurite length was also longer on L1-coatings than on same amount of laminin [57].

5
6 The potency of L1, in comparison to NCAD and laminin, may be cell-type dependent. As
7 compared to L1, NCAD was more effective in stimulating neurite extension from chick ciliary ganglion
8 neurons [22]. Additionally, chicken embryonic retinal ganglion cells also had slower neurite growth rate
9 on L1 than NCAD and laminin [65]. In contrast, for rat hippocampal neurons, the axons formed sooner on
10 L1 and NCAD than on PLL after 24 hours. The axons on L1 were also longer as compared to NCAD and
11 PLL [64]. This suggests that L1 is more effective than NCAD for some neurons in stimulating axon
12 growth and that both molecules should be compared for neural tissue engineering design of different
13 nerve tissues.

14 15 **3.3.2 Methods of Usage**

16 Similar to NCAD, the extracellular domain of L1 is enough to stimulate neurite outgrowth.
17 Correspondingly, the smaller size of the extracellular domain makes it easier to modify and apply L1 due
18 to its higher stability. In particular, recombinant extracellular domains of L1-coated onto
19 polyethylenimine coated coverslips supported neurite outgrowth from chicken brainstem explants.
20 However, the neurite length on recombinant L1 coatings was shorter than the same amount of laminin
21 coating and soluble L1 (5 μg/ml) [58]. This was suggested to be due to the L1 orientation that was
22 presented to the cells in another study that incorporated L1-Fc onto polymeric materials. In that study, as
23 compared to L1-Fc that was adsorbed on PDL, L1-Fc immobilized through affinity binding to protein A-
24 coated polycarbonate films and microfibers were able to stimulate longer neurites from rat spinal cord
25 neurons and cerebellar neurons [59].

1 In contrast, soluble L1-Fc appears to be more potent and its effects were shown in both cerebellar
2 and DRG neurons. Specifically, soluble L1-Fc enhanced neurite outgrowth from chicken cerebellar
3 neurons that were cultured on 3T3 fibroblast monolayers and on PLL/collagen or PLL/fibronectin-coated
4 substrates [33]. In particular, a two-fold increase in neurite length at 5 µg/ml of L1-Fc was seen as
5 compared to the control without soluble factors added. The effect of soluble L1-Fc on neurite extension
6 was dose-dependent, with a maximal response at 5 µg/ml. On the other hand, the effect of NCAD-Fc on
7 neurite outgrowth was not observed in the same study [33]. For chicken DRG neurons cultured on PLL,
8 neurite length also increased with L1-Fc concentration in a dose-dependent manner [63]. These results
9 demonstrate that soluble L1-Fc is a potential therapeutic agent that is more effective and stable than
10 purified L1. Therefore, incorporating soluble L1-Fc with biomaterials may be an attractive approach for
11 promoting neurite outgrowth *in vivo*.

12
13 In addition, the effect of L1 on neural cells is also selective, which can be exploited to support
14 neurite outgrowth while reducing the growth of other cell types. Specifically, L1-Fc coated on
15 polystyrene plates or immobilized on pluronic-pyridyl disulfide selectively promoted the adhesion and
16 neurite outgrowth of neurons in the presence of glial cells [60]. L1-Fc also supported neurite outgrowth
17 from hippocampal and cerebellar neurons but prevented the adhesion of astrocytes, fibroblasts and
18 meningeal cells. Therefore, L1 was used to coat neural electrodes to reduce microglial coverage [95] and
19 promote neurite outgrowth [61, 96]. Similarly, electrically conducting polymer coated with NCAD and
20 L1 also synergistically promoted neurite outgrowth [67]. In neural tissue engineering, this unique feature
21 of L1 may be utilized for better nerve regeneration. For example, by preventing the adhesion of astrocytes,
22 fibroblasts and meningeal cells, L1 may be able to reduce glial scar formation while promoting neurite
23 outgrowth at the same time.

24
25 The promising *in vitro* results has led to trials in utilizing soluble L1-Fc to regenerate the injured
26 spinal cord *in vivo*. Using a rat contusion SCI model, L1-Fc solution was infused intrathecally for 2 weeks

after injury. Thereafter, at twelve weeks after injury, L1-Fc treated rats showed longer corticospinal tract growth at the injury site and better locomotor function recovery in terms of BBB scores [63]. In addition to L1-Fc, gene delivery by adeno-associated virus to induce L1 expression has also led to better recovery after SCI, with enhanced regeneration of 5-HT axons and corticospinal tract axons at five weeks after compression injury [97]. Besides SCI, L1-Fc coated nerve conduits also promoted axonal regeneration and remyelination in a transected optic nerve model [62]. Altogether, these promising *in vivo* results suggest that the combined delivery of L1-Fc with tissue engineering scaffolds may be clinically viable treatment methods for damaged nerve tissues.

3.4 Summary

Since both CAMs and ECMs could stimulate neurite extension, there could be synergism on neurite outgrowth by different CAMs and ECMs. Correspondingly, NCAD, integrin $\beta 1$, and L1 have been suggested to work synergistically [98], and L1 could be modulated in the presence of NCAM [99]. NCAM and NCAD were also able to work synergistically on neurite outgrowth [94]. Furthermore NCAD, NCAM, and L1 induce neurite outgrowth by activating different pathways (NCAD: β -catenin [77], NCAM: FAK [16, 77], L1: Src [16]) which then converged through the FGFR [100] and MAPK/ERK signaling pathways [16, 100]. These common pathways suggest that these CAMs may interact with each other. Hence, these results suggest that the CAMs may have synergistic effects in promoting neurite outgrowth but more investigations are needed. While CAMs may work together, comparisons between CAMs to determine which is superior remain inconclusive. When neurons were presented with two different substrates, observations on the growth cones illustrated a weak preference for NCAD or L1 over laminin but no difference was observed between the two CAMs [66]. This suggests that neurons may prefer to migrate on CAM-coated surfaces as compared to ECM-coated substrates.

CAMs in substrate-bound form, soluble form and transfected cellular format were able to promote neurite outgrowth. NCAD and L1 could stimulate neurite growth in both soluble and substrate-

bound formats, with slightly different neurite morphologies. For NCAM, soluble NCAM is effective in stimulating neurite outgrowth but there is a lack of evidence to show its effectiveness as surface immobilized coatings. In addition, the response of neurons towards CAMs is dose-dependent with interesting dose response that depends on the types of CAMs and how the CAMs are presented. This information should be considered for practical applications to increase the chances of success in neural tissue engineering approaches (Figure 1).

Figure 1. Existing use of CAMs in promoting neurite outgrowth. Some CAMs are used as soluble factors while others are substrate-bound to modulate cell adhesion onto the CAMs. While some of the CAMs were only studied with a single delivery method, we believe that the CAMs, in general, can be delivered by scaffold in soluble and substrate-bound forms.

1 The studies on CAMs have demonstrated their effectiveness in promoting neurite outgrowth.
2 Some of these studies have made comparisons with commonly used coatings, such as laminin and PLL,
3 and showed that CAMs may be superior in promoting neurite outgrowth from certain neurons. Some of
4 the studies have also moved on to investigate the effectiveness of CAMs when delivered into an animal
5 model with SCI and showed that CAMs can promote functional recovery even in an inhibitory
6 environment. Nevertheless, it remains a question whether CAMs can accelerate axonal regeneration over
7 neurotrophic factors or promote nerve regeneration synergistically with neurotrophic factors.

9 **4. The roles of cell adhesion molecules in remyelination.**

10 Demyelination is often seen after traumatic nerve injuries and demyelinating diseases, such as
11 multiple sclerosis, optic neuritis, and neuromyelitis optica. Demyelination can lead to axon degeneration
12 if remyelination does not happen. Therefore, remyelination of axons has been targeted in approaches to
13 enhance regeneration of the nervous system. Myelin sheaths on axons are produced and maintained by
14 oligodendrocytes (OLs) and SCs in the CNS and PNS respectively. Hence, understanding how OLs and
15 SCs recognize and initiate myelination or remyelination on axons is an important aspect in neural
16 regeneration.

17
18 Within the CNS, neurons and OLs communicate to coordinate myelination [41]. Myelination of
19 axons primarily occurs when oligodendrocyte precursor cells (OPCs) differentiate into OLs. OL
20 myelination is regulated by growth factors and neurotrophic factors, such as platelet-derived growth
21 factor- α (PDGF- α), which are produced by astrocytes and neurons. In addition to that, neurons also
22 control OL differentiation and myelination through neuregulins (NRG) and Notch signaling. Both
23 signaling pathways involve specific interactions between cell surface receptors and ligands of axons and
24 OLs. Specifically, NRG signaling is mediated by adhesion of axons to integrins of OLs. NRG also
25 promotes OPC survival, maturation and proliferation. In contrast, Notch-1 of OLs interacts with Jagged-1

1 on axons, but inhibits OPC differentiation into OL instead. Similarly, within the PNS, NRG1 on axonal
2 surface is also required for myelination by SCs.

3
4 Myelination typically starts from OPC recognizing and initiating adhesion to an axon. This
5 process involves adhesion molecules. For instance, integrins of OPCs/OLs and SCs were suggested to
6 bind to axon-derived laminin and initiate axon-glial contact for myelination [101-103]. A recent review
7 on the signaling pathways that are involved in axon remyelination after injury suggested the involvement
8 of CAMs in starting myelination [104]. In particular, the involvement of CAMs in myelination starts from
9 the selection of axon through cell-cell contact [105] and is similar to synapse formation, which involves
10 cadherins [106]. Therefore, enhanced myelination along with improved locomotor recovery were
11 observed when CAMs were used to modulate axon-glial contact (Table 3). However, such approaches are
12 not common, as there are not a lot of evidences that demonstrate that CAMs play an essential role in
13 remyelination. Since myelination requires direct axon-glial contact, exogenic CAMs may only work as
14 soluble factors. To stimulate remyelination for nerve regeneration, soluble CAMs can be used to enhance
15 OLs and SCs myelination on axons.

Table 3: Overview of CAMs that were utilized to promote myelination

Type of CAM	Species / Cell type / Model	Therapeutic format and delivery method	Therapeutic effect	Reference
PSA-NCAM	<i>in vivo</i> : mouse (compressed lesion at spinal cord)	Transfected Schwann Cells	- 2 weeks: enhanced total remyelination (9.5-fold) - 4 weeks: enhanced total remyelination (2.6-fold) - Enhanced locomotor recovery	[107]
L1	<i>in vivo</i> : mouse (compressed lesion at spinal cord)	Adeno-viral vector	- 3% of transduced cells were OLs - Upregulation in one of the MBP isoform (21.5 kDa) protein expression - Enhanced locomotor recovery	[97]
	<i>in vivo</i> : mouse (compressed lesion at spinal cord)	Transfected Schwann Cells	- 2 weeks: enhanced total remyelination (8-fold) - 4 weeks: enhanced total remyelination (3-fold) - Enhanced locomotor recovery	[108]
	Oligodendrocytes -DRG neurons co-culture	Soluble L1-Fc (0.01-1µg/ml) added to culture medium	-Enhanced OL survivability -Enhanced myelination in a dose-dependent way in terms of number of MBP+ cells and % myelinating MBP+ cells	[109]
	<i>in vivo</i> : rat (1 mm full transaction at optical nerve)	L1-Fc adsorbed onto chitosan coated PGA nerve conduits	- Enhanced remyelination (17.9-fold increase in myelinated fibers compared to without L1-Fc) - Enhanced locomotor recovery	[62]

4.1 Cadherins

Although NCAD has not been used as a biomolecule to enhance myelination/remyelination, the importance of NCAD in CNS myelination was demonstrated by using NCAD-blocking antibody and calcium withdrawal [110]. When the function of NCAD was blocked, the contact duration of OPCs and DRG neurons was shortened and myelination was significantly reduced. β -catenin and Wnt signaling pathways were suggested to be involved in NCAD function and myelination. This shows that NCAD is essential for the formation of initial contact between OPCs and axons for myelination to occur. Similarly, NCAD expression was observed in an inflammatory demyelination model. In this rat model, NCAD was expressed in remyelinating OPCs in the CNS but was downregulated after remyelination was completed [111].

Despite the fact that earlier studies showed that NCAD is involved in SC-axon interactions, recent results suggested that ECAD might play a more significant role in PNS myelination as compared to NCAD [112-115]. Specifically, functional studies on the role of cadherins in SC-DRG cultures showed that while NCAD knockdown delayed the initiation of myelination, the knockdown or overexpression of neuronal NCAD did not affect myelination. Therefore, the role of NCAD in SC-DRG myelination may be non-essential [114]. In contrast, the deletion of ECAD gene delayed myelination in the PNS and reduced the length of myelin segments. When ECAD was over-expressed, myelination was enhanced. Additionally, ECAD expression by SCs may be induced by Type III NRG1 of axons. Hence, the function of ECAD in SC-DRG myelination may be associated with the NRG signaling pathway [115]. These studies demonstrate that NCAD and ECAD play important roles in CNS and PNS myelination respectively. However, there is currently no attempt in applying NCAD and ECAD as biological stimuli for remyelination and therefore, their effectiveness remains unknown. Although myelination on NCAD or ECAD coated surfaces can be explored for *in vitro* studies, using NCAD or ECAD to enhance remyelination *in vivo* would likely require soluble forms of these CAMs or the overexpression of these CAMs in glial cells by genetic transfection.

4.2 NCAM

The involvement of NCAM in myelination appears to be limited and investigations have, thus far, only been done on the PNS. The expression of NCAM and L1 were found at the premyelinating stage when cells express L1 first, followed by NCAM. L1 was suggested to interact with NCAM on axons for SC recognition and adhesion when initiating myelination [116]. In addition, NCAM may be important in maintaining myelin but not in initiating myelin formation [117, 118]. Due to the lack of definitive effect of exogenic NCAM on myelination, the effectiveness of applying NCAM to promote remyelination in neural tissue engineering has not been explored.

However, polysialylated-NCAM (PSA-NCAM), the post-translational modified form of NCAM (by adding polysialic acid), appears to play important roles in myelination and remyelination. PSA-NCAM is transiently expressed on OPCs and SCs during myelination and remyelination [119]. Therefore, transplanting SCs that have been transfected to over-express PSA-NCAM appears as an interesting approach for nerve regeneration. In a mouse SCI model, this method enhanced axon regeneration and remyelination by the transplanted SCs and ultimately, locomotor recovery. Interestingly, myelination of the regenerated axons by the migrated host SCs and endogenous OPCs were also enhanced by the transplanted SCs. Since PSA-NCAM is known to enhance cell migration by creating steric hindrance to the cell-cell contacts, the authors speculated that the transient expression of PSA-NCAM rendered the lesion site to be more permissive to host SCs and OPCs [107]. Taken together, delivering PSA-NCAM to the lesion site during the initial stage of nerve injury may be beneficial in enhancing neural regeneration and recovery. Hence, PSA-NCAM may be incorporated into scaffolds to enhance OPC and SC migration. Conversely, as cell adhesion and neurite outgrowth are also needed on the same scaffold, scaffolds may be designed to deliver PSA-NCAM and other CAMs in different ways. For example, a scaffold may be designed to sequentially deliver PSA-NCAM during the initial stage to enhance cell migration and present other CAMs for neuron adhesion and neurite outgrowth in the later stages of recovery.

4.3 L1

As compared to NCAD and NCAM, soluble L1 appears to be more effective in enhancing myelination. This makes L1 an ideal candidate for neural tissue engineering, given that it also plays an important role in enhancing neurite outgrowth. Within the CNS, L1 is found on neurites before myelination but not on OLs. Thereafter, the expression of L1 on myelinated axons is down-regulated after myelination but its expression remains on unmyelinated axons. Since myelination could be inhibited by treating axons with antibody against L1 or L1-Fc, this suggests that L1 is important in myelination [120]. In a separate study, L1-Fc promoted the survivability and myelination of OLs in a dose-dependent way (about 60-70% myelination rate at 1 μ g/ml). Findings from this study suggest that L1 is likely to act through β 1 integrin-contactin complex and Fyn activation, which integrate both β 1 integrin and contactin signals [109]. Thus, L1 may play a therapeutic role in remyelination. Indeed, SCs engineered to express L1 could enhance locomotor recovery and remyelination after compression injury on the spinal cord [97, 108]. To introduce soluble L1 directly to the injured nerve tissues in a more controlled manner, soluble L1 could be designed to be released from the scaffolds or conduits to stimulate remyelination. This was shown to be possible in a transected optic nerve model. In this study, L1-Fc coated nerve conduit promoted remyelination in addition to enhancing axon regeneration [62]. To deliver L1-Fc, these molecules were adsorbed onto chitosan-coated poly(lactic-co-glycolic acid) nerve conduits. As proteins adhere to chitosan reversibly, the increased number of myelinated axons is likely due to the soluble L1-Fc that were released from the nerve conduits [121]. More importantly, the dual role of L1-Fc in stimulating remyelination and axon regeneration demonstrate the potential of L1-Fc to be applied in regeneration of other nerve tissues.

Within the PNS, L1 is essential at different stages of SC myelination. In the presence of antibodies against L1, SCs were prevented from ensheathing [122] and myelinating neurons [123]. In addition, L1-deficient mice showed that axonal L1 regulates SC-axon adhesion of unmyelinated fibers

[124]. However, while axonal L1 appears to be essential for the adhesion and survival of SCs, different L1 knockout mouse models showed normal myelination [124-126]. This suggests that the function of L1 may be replaced by other CAMs *in vivo* during myelination.

4.4 Summary

As compared to neurite outgrowth, the roles of CAMs in myelination are less studied although it appears that CAMs may act as the initial recognition sites for OLs and SCs to adhere to axons. In terms of therapeutics, cells engineered to overexpress PSA-NCAM and L1 demonstrated positive results in promoting myelination and remyelination *in vivo*. Since cell transplantation is usually accompanied with cell survivability issues, combining these transfected cells or CAMs with scaffolds may be an effective alternative to promote remyelination. Indeed, increased myelination was observed with L1-Fc coated nerve conduits that appeared to stimulate remyelination through the delivery of soluble L1-Fc. Therefore, local delivery of soluble L1-Fc could be an effective starting point to utilize CAMs in stimulating remyelination *in vivo* (Figure 2). In neural tissue engineering approaches, scaffolds may be designed to deliver soluble CAMs to enhance myelination/remyelination of OLs/SCs on axons.

Figure 2. The use of CAMs to promote remyelination, which is usually assessed by myelin basic protein (MBP) staining. To promote myelination, CAMs are likely needed to be soluble or directly overexpressed within the myelinating glial cells. Question marks depict unexplored approaches. Other than L1, both soluble cadherins and NCAM have not been utilized to promote myelination. In neural tissue engineering approaches, scaffolds may be designed to deliver the soluble CAMs to enhance myelination/remyelination of oligodendrocytes/Schwann cells on axons.

5. The roles of cell adhesion molecules in neuronal differentiation

Stem cell transplantation and modulation of the fate of endogenous stem cells have great potential in repairing nerve tissues due to the ability of stem cells to differentiate into the desired neural cell types [127-129]. To control the fate of stem cells, majority of the approaches have focused on neuronal differentiation as oppose to glial differentiation, with the aim to regenerate neurons that were lost in the damaged nerve tissues. Although damaged OLs and SCs also need to be replaced, most works studied the differentiation of these cells from their progenitor as part of the process of remyelination and, therefore, was discussed above.

The role of CAMs, such as cadherins, NCAM and L1, in neural development has been well documented. [18, 130-132]. Since CAMs also play important roles in controlling stem cell fate, CAMs were utilized to induce and enhance neuronal differentiation (Table 4). In contrast, none of these approaches utilized CAMs to induce glial differentiation.

1 **Table 4: Overview of CAMs that were utilized to promote neuronal differentiation**

Type of CAM	Species / Cell type	Format	Procedure	Concentration	Outcome	Reference
Cadherins	mESC, miPSC	NCAD-Fc, ECAD-Fc	Adsorbed onto untreated polystyrene culture dishes	- 10 µg/ml (NCAD or ECAD alone) - 5 µg/ml (co-immobilization)	- Nestin+ on NCAD/ECAD > gelatin - Expressed Pax6, MAP2, TH - Undetected GFAP	[26]
NCAD	mESC, miPSC	NCAD-Fc	Adsorbed onto untreated polystyrene culture dishes	10 µg/ml	- At 48 h, longer neurites on NCAD compared to PLL (2-fold) and gelatin (40-fold). - More neurites than PLL (4-fold) and gelatin (60-fold). - Spontaneous differentiation: no difference between NCAD and PLL but higher neuronal marker expression than gelatin.	[83]
	mESC	NCAD peptide (HAVDI)	Mixed with PEGDM hydrogel and photopolymerized	0-600 µM in gradient across hydrogel	- 66.7% TUJ1 ⁺ after 6 days, with no significant difference across NCAD concentration. - Longer neurite outgrowth with increased NCAD concentration - Higher TUJ1 mRNA at 292 µM and 467 µM NCAD.	[133]
	hiPSC-derived NSC	NCAD peptide (HAVDI)	Mixed with PEGDM hydrogel and photopolymerized	0-1 mM in gradient across hydrogel	- More TUJ1 ⁺ at 779 µM (~80%) than 578 µM after 10 days - Longer neurites at 445 µM - Higher TUJ1 mRNA at 577 µM	[134]
NCAM	rat hippocampal progenitor, mouse hippocampal progenitor	Purified NCAM	Added as soluble factor for 3 days	10 µg/ml	- Rat with bFGF: 85% MAP2 ⁺ (2.3 fold increased from no treatment), 2% GFAP (from 12%). - Mouse with bFGF: >90% MAP2 ⁺ (40% in control), 0.3% GFAP ⁺ (= control) - Rat without bFGF: similar %MAP2 ⁺ and %GFAP ⁺ compared to BDNF treated.	[135]

	rat hippocampal progenitor	Purified NCAM	Added as soluble factor for 3 days	10 µg/ml	- 60% MAP2 ⁺ compared to 40% in untreated - 2-fold increase in glutamatergic genes and proteins compared to control	[136]
L1	mNPC	Purified L1	Adsorbed onto PLL precoated coverslips	6 µg/ml	- 2-fold more β-tubulin ⁺ neurons from PLL - GFAP ⁺ reduced by 33% - 61% fewer cholinergic neurons on L1 than on PLL substrate after 15 days - 1.7-fold more GABAergic phenotypes on L1 than on PLL substrate after 5 days	[137]
	hESC-derived NSC	L1-Fc	- Adsorbed onto PDL - Affinity captured by Protein A (PA) or PA/PDL coated polycarbonate film (2D) or microfibrinous scaffold (3D)	10 µg/ml	-2D: 1.5-fold increase in % TUJ1 ⁺ on L1/PA/PDL and L1/PDL; increased neurite length on L1 -3D: 65.9% TUJ1 ⁺ on L1/PA/PDL compared 48.1% on L1/PDL and PDL (<40%), increased neurite length on L1	[59]
NCAD, L1	hESC-derived NSC	NCAD-Fc, L1-Fc	Affinity captured by PA coated polycarbonate film (2D) or microfibrinous scaffold (3D)	- L1: 10 µg/ml - NCAD: 1.375 µg/ml (low), 2.75 µg/ml (mid), 5.5 µg/ml (high)	- 2D: Low NCAD with L1 has the highest % cells with neurites and neurite length compared to other groups after 7 days. Higher % MAP2 ⁺ (49%) and NF (58%) in low NCAD/L1 among all combinations. - 3D: Similar to 2D, low NCAD with L1 has the highest neurite length but highest % MAP2 ⁺ with low NCAD (44%) and low NCAD/L1 (36%)	[84]

5.1 Cadherins

Cadherins play important roles in regulating cell sorting and differentiation, through several subtypes of cadherins [138]. The individual cadherin subtypes may support or suppress the differentiation of specific tissues. At the same time, these subtypes are able to convert from one to another. During development, the expression of ECAD was suggested to convert into NCAD during neural tube formation [139]. Thereafter, the down-regulation of NCAD is thought to be related to neuronal differentiation from neural progenitor cells (NPCs), by regulating the expression of β -catenin and AKT signaling pathways [140, 141]. For instance, the down-regulation NCAD and hence, its downstream target, β -catenin, induced premature neuronal differentiation *in vivo* [142]. Moreover, NCAD was also found to enhance the recruitment and migration of NPCs to the lesion site of a mouse model of demyelination,[143]. These evidences demonstrate that cadherins can direct cell fate *in vivo*.

In regenerative medicine, ECAD coatings supported NPC self-renewal while NCAD was utilized to promote neural differentiation [26, 144]. Similar to development, it appears that there is a transition in expression from ECAD to NCAD when cells differentiate. By exploiting this characteristic of the NPCs, a combination of NCAD and ECAD coatings can be useful for stem cell adhesion, promotion of neuronal differentiation and purification of neurons from undifferentiated pluripotent stem cells. This approach can be utilized as scaffold coatings for NPC delivery, although existing literature only demonstrates the effects of ECAD and NCAD coatings on culture dishes. Specifically, mouse embryonic stem cells (mESCs) and mouse induced pluripotent stem cells (miPSCs) were maintained on ECAD-Fc substrates and differentiated on NCAD-Fc coated surfaces [26]. Furthermore, the combination of NCAD-Fc and ECAD-Fc coatings on culture dishes allowed pluripotent stem cells to be cultured as monolayers instead of the conventional embryoid bodies format. In addition, NPCs could be generated from mESC or miPSC on this coating combination, with more Nestin⁺ NPCs on NCAD/ECAD-Fc than gelatin coated surfaces. When the NPCs were further differentiated on NCAD/ECAD-Fc surfaces, the gene expressions of neuronal markers, PAX6, MAP2, and TH were also higher; while NPC markers and GFAP⁺ glia were not

1 detected. [26] Since ESCs and iPSCs have low affinity towards NCAD coatings, the undifferentiated
2 ESCs and iPSCs may be further removed to generate pure neuronal populations [83].

3
4 Alternatively, the NCAD-derived peptide, HAVDI, could also be utilized to enhance neuronal
5 differentiation. As compared to NCAD, HAVDI has the advantage of being smaller in size and more
6 metabolically stable. By crosslinking HAVDI to polyethylene glycol dimethacrylate hydrogels, neuronal
7 differentiation from mESCs was enhanced. Furthermore, this approach allowed the HAVDI to be
8 crosslinked within the hydrogels in a concentration gradient format. Consequently, when mESCs were
9 seeded onto the hydrogels with different HAVDI concentrations, the mESCs differentiated into 66.7%
10 TUJ1⁺ cells after 6 days in neural differentiation medium. Although there was no significant difference in
11 the percentage of TUJ1⁺ cells across different HAVDI concentrations, longer neurite outgrowth was
12 observed on the differentiated neurons at high HAVDI concentrations, which implied enhanced neuronal
13 differentiation. In addition, significantly higher TUJ1 mRNA expression level was also found in cells on
14 hydrogels with 292 μ M and 467 μ M of HAVDI [133]. The same approach of using HAVDI also
15 enhanced cell survival, neurite extension and neuronal differentiation in hNPCs [134]. However, the
16 optimal concentration of HAVDI within the matrix that supported hNPCs differentiation was 577 μ M,
17 which was different from mESCs. Altogether, these results show that cell fate is controlled by HAVDI,
18 and potentially, NCAD, on biomaterials is concentration dependent and needs optimization for different
19 cell types.

20
21 In these studies, both NCAD and HAVDI enhanced differentiation and neurite outgrowth,
22 although only gene expression changes were observed in HAVDI. The effectiveness of NCAD and
23 HAVDI in promoting neuronal differentiation was demonstrated in the presence of differentiation
24 induction media. The differentiation induction media included components such as brain-derived
25 neurotrophic factor, glial cell line-derived neurotrophic factor, cyclic adenosine monophosphate (for
26 hiPSC on HAVDI) [134], retinoic acid (for mESC on HAVDI) [133], CKI-7 and SB431542 (for mESC

and mIPSC on NCAD) [83]. Interestingly, enhanced neuronal differentiation of mESCs on NCAD as compared to gelatin-coated surface was also observed in the absence of CKI-7 and SB431542, small molecule inhibitors that promote neural differentiation [83]. This result indicated that NCAD may be sufficient in enhancing neuronal differentiation even without the addition of exogenic factors.

5.2 NCAM

NCAM promotes tissue differentiation and is involved in neuroectoderm neural differentiation and cellular communication [145]. Therefore, purified NCAM was used as a strategy to induce neuronal differentiation *in vitro* and may be useful in neural tissue engineering. Specifically, a sustained delivery of NCAM by encapsulation in a scaffold can be utilized to enhance neuronal differentiation of NPCs that have been seeded onto the scaffold and also the native NPCs that are found within the injured nerve tissues. This is supported by the positive outcomes with soluble NCAM on mouse NPC. When NCAM was added during NPC differentiation, the percentage of MAP2⁺ neurons increased 2.3-fold as compared to the control with no treatment. Concurrently, GFAP⁺ astrocytes were reduced from 12% to 2% in the presence of NCAM [135]. In another study, different concentrations of soluble NCAM were added during NPC differentiation. Correspondingly, the optimal concentration of soluble NCAM that enhanced neuronal differentiation of NPCs was suggested to be 4.2 µg/ml, where the maximum percentage of MAP2⁺ neurons (60%) was observed. Further investigation found that these differentiated neurons expressed markers of glutamatergic neuron [136]. Therefore, NCAM may be effective in differentiating NPCs along the glutamatergic lineage. For example, NCAM-incorporated biomaterials can be used to deliver NPCs to the damaged brain to replace glutamatergic neurons.

NCAM was demonstrated to be an effective soluble factor that enhances neuronal differentiation in the presence of either NPC maintenance medium (with bFGF) or differentiation medium (without bFGF) [135]. This suggests that NCAM-loaded scaffolds may work similarly well *in vivo* where the amount of bFGF in the microenvironment is not defined. Similar to NCAD coating, soluble NCAM could

enhance neuronal differentiation directly without additional neurogenic factors [135]. However, in this study, NCAM was delivered to the cells in soluble format, as opposed to NCAD coatings, as mentioned above. Thus, a direct comparison of the efficacy of NCAM vs. NCAD appears difficult, since the studies that utilized NCAM and NCAD used different delivery and evaluation methods. Nevertheless, soluble NCAM may be more promising than NCAD coatings since NCAM enhanced NPC differentiation into MAP2⁺ mature neurons (60%), whereas the less mature marker, TUJ1, was used to evaluate the efficacy of NCAD coatings after neuronal differentiation [83, 136].

5.3 L1

Similar to NCAD, L1 can be delivered by overexpression or as coating on scaffold. Here, the direct effect of L1 on neuronal differentiation was demonstrated with L1 overexpression. Specifically, murine ESCs transfected to overexpress L1 showed enhanced neuronal differentiation *in vitro* and *in vivo* and decreased astrocyte commitment (increase in β -tubullin⁺, NF⁺, GAD⁺, TH⁺ cells; decrease in nestin⁺ and GFAP⁺ cells). When the cells were transplanted into an excitotoxic lesion in the mouse striatum, these L1-overexpressing mESCs improved functional recovery as compared to controls without L1 overexpression [146]. Similarly with “substrate adherent embryonic stem cell-derived neural aggregates” (SENA), L1 overexpression increased the number of surviving cells, promoted neuronal differentiation and reduced unwanted glial differentiation after transplantation to a compression-lesioned spinal cord. It also resulted in better improvement in locomotor function in terms of Basso, Beattie and Bresnahan (BBB) scores [147]. While L1-overexpressing mESCs appears to be effective in improving recovery *in vivo*, there is a lack of flexibility in controlling the spatial distribution of L1. Manipulating the spatial distribution of L1 may be utilized in order to better control the types of cells that migrate to the lesion site, which can be achieved when L1 is incorporated with scaffold.

When introduced as a coating, L1 could also enhance the differentiation of NPCs into neurons, which makes L1-coated scaffolds potential cell carriers for NPC transplantation. In particular, purified L1

1 from homogenates of mice adult brain was coated onto PLL pre-coated coverslips (in 1-15 μ g/ml range)
2 [137]. These L1-coated substrates inhibited mNPC proliferation and promoted neuronal differentiation
3 better than laminin and PLL alone. Specifically, there were significantly more β -tubulin⁺ (or TUJ1⁺)
4 neurons and less GFAP⁺ astrocytes on L1 coating (6 μ g/ml) than laminin and PLL. Similarly, enhanced
5 neuronal differentiation was also observed with hNPCs seeded on L1-Fc that was presented on
6 polycarbonate films and fibrous scaffolds [59]. Interestingly, these L1-induced cellular differentiations
7 might have occurred through heterophilic instead of homophilic interactions [137]. This suggests that L1
8 coating may stimulate neuronal differentiation through binding with other molecules on the cell surface
9 that are not involved in cell adhesion, indicating the potential effectiveness of soluble L1 in stimulating
10 neuronal differentiation. Furthermore, neurotransmitter-specific neurons could potentially be obtained
11 selectively by using L1 coating. In particular, L1 appears to support the generation of specific subtypes of
12 neurons, with more GABAergic neurons and fewer cholinergic neurons being generated on L1 coatings as
13 compared to PLL [137]. Such neurotransmitter-specific differentiation depended on the homophilic
14 interaction of L1 coating and L1 that was expressed on the cell surfaces [137]. Hence, incorporation of L1
15 coating with neural tissue engineering strategy that targets nerve tissues with a loss of GABAergic
16 neurons may lead to more favorable results than the other CAMs. In comparison, L1 and NCAM are
17 favorable for the generation of GABAergic and glutamatergic neurons respectively. Therefore, the
18 generation of other types of neurons such as cholinergic neurons may consider using NCAD, which was
19 not shown to support the generation of specific subtypes of neurons.

20
21 As compared to PLL and laminin, the established coatings for NPC adhesion, L1 was more
22 effective in enhancing neuronal differentiation by serum removal. Therefore, L1 may be used as a more
23 effective coating over PLL and laminin for neural tissue engineering. The effect of L1 coating was similar
24 to soluble NCAM, which stimulated lineage selection of neurons from NPCs over astroglial
25 differentiation. Such outcomes are advantageous in assuring high purity and yield in neuronal
26 differentiation [135, 137]. However, the efficiency of L1 in promoting neuronal commitment in mNPCs

(~15% TUJ1⁺) appeared to be lower than NCAM (~60% MAP2⁺), although the mNPCs were derived from different regions of the brain [135, 137].

With the positive results of CAMs in inducing neuronal differentiation, it would be interesting to investigate whether these CAMs work synergistically. Indeed, a combination of NCAD and L1 coatings synergistically enhanced neuronal differentiation. Therefore, coating NCAD/L1 onto 3D scaffolds presented a feasible and better approach to deliver CAMs and NPCs for neural tissue engineering *in vivo*. As compared to NCAD or L1 alone, a combination of NCAD and L1 coating on 2D films and 3D microfibrous scaffolds enhanced NPC survivability, neurite outgrowth and expression of neuronal markers [84]. Here, neuronal differentiation of mNPCs was observed on different amounts of NCAD that were coated together with L1. On 2D, higher percentage of neurons with MAP2⁺ (49%) and NF⁺ (58%) stainings and longer neurite length were observed on L1 coatings that were coupled with a low concentration of NCAD (1.37 µg/ml) as compared to higher concentrations of NCAD (2.75 and 5.5 µg/ml). The results on 3D microfibrous scaffolds were also similar to 2D, where cells on L1 coatings that were coupled with a low concentration of NCAD had the longest neurite length [84]. These results suggest that the synergism between NCAD and L1 is concentration dependent, where low concentration of NCAD had better synergistic effects with L1 in promoting neuronal differentiation. Thus, similar optimization could also be effective in identifying the optimal concentrations for CAMs when they are applied in combination.

5.4 Summary

CAMs can be effective coating materials for neuronal differentiation, while ECAD is an effective substrate for stem cell maintenance (Figure 3). On the other hand, consistent with studies on neurite outgrowth, cadherins and L1 are generally utilized as coatings for cell adhesion, while NCAM is typically added into the medium in soluble form. As compared to laminin and PLL, CAMs appear to be advantageous as CAMs better support neuronal differentiation and inhibit astrocyte differentiation.

Figure 3. Existing use of CAMs in promoting neuronal differentiation. Some CAMs were used as soluble factors while others were substrate-bound, where cells adhered directly to the CAMs. Most CAMs were shown to inhibit astrocytic differentiation. Both soluble and substrate-bound CAMs can be delivered to enhance neuronal differentiation. In addition, a mixture of CAMs (Eg: NCAD/ECAD and NCAD/L1) can work synergistically for specific purposes.

6. Methods and design considerations for modulating cell-cell interactions in neural tissue engineering

6.1 Major challenges of therapeutic implementation

CAMs hold great potential in neural tissue engineering and nerve regeneration as CAMs can target all three aspects of neurite outgrowth, myelination and neuronal differentiation. However, most of

1 these outcomes were derived from *in vitro* studies and there are only a limited number of translational
2 approaches that aim at delivering these CAMs *in vivo*. Therefore, strategies with practical considerations
3 must be made to introduce CAMs to cells and damaged nerve tissues efficiently. Other than direct bolus
4 injection, CAMs may also be delivered using functionalized biomaterials to achieve long-term and
5 localized delivery.

6
7 CAMs delivered using biomaterial scaffolds are advantageous as these substrates can also deliver
8 cells, multiple regenerative cues (such as biochemical, mechanical and topographical) and act as a
9 mechanical support for neural tissue regeneration [1]. Furthermore, CAMs on scaffolds can also act as
10 adhesion substrates for cell attachment. Currently, there are only few studies that utilized scaffolds to
11 deliver CAMs. Majority of these studies applied CAMs as coatings on biomaterials to enhance neuronal
12 differentiation, neuronal adhesion and neurite outgrowth.

13
14 To realize the translational potential of CAMs-functionalized biomaterials for nerve regeneration,
15 several challenges need to be addressed. For instance, the bioactivity of CAMs after incorporation into
16 biomaterials needs to be maintained *in vivo*. Furthermore, the results on NCAM and L1 also showed that
17 soluble form of these CAMs might be even more effective than substrate bound format in some cases.
18 This is because the soluble CAMs can bind to a different receptor or binding site of the cells, in addition
19 to the adhesion site of the same CAM. This mechanism that is stimulated by soluble CAMs has been
20 shown to be important in promoting axon regrowth, remyelination and neuronal differentiation [24, 33, 56,
21 58, 63, 109, 135, 136]. Therefore, a controlled release system is also needed to deliver soluble CAMs. To
22 design such a system, the format and dosage of the CAMs need to be considered for maximal desired
23 effects on nerve regeneration. For example, to promote neuronal differentiation *in vivo*, the level of
24 soluble CAMs that is delivered from a scaffold needs to be maintained for a prolonged time period.
25 Finally, different types of scaffolds and materials may be required in the CNS and PNS. For example, a
26 softer material is usually used in CNS regeneration to match the stiffness of the CNS tissue while a stiffer

conduit is usually more effective after nerve injuries in the PNS to prevent nerve compression. Hence, the types of scaffold and material will also determine how CAMs can be delivered by the scaffold. Different types of scaffold such as hydrogels, nanofibers or conduits require different methods to deliver the CAMs. Even with the same methods of delivery, like encapsulation within the material, the different scaffold designs would also have distinctive release profiles.

Here, we will discuss the existing methods of immobilizing CAMs, which are mainly surface-modification methods. We will also suggest other potential approaches for delivering CAMs and summarize the currently-available information on effective format and dosages of CAMs to regenerate damaged nerve tissues as references for future design strategies (Figure 4).

Figure 4: Approaches for scaffold mediated delivery of CAMs to injured nerve tissues using spinal cord injury as an example.

6.2 Delivery methods

As CAMs are also adhesion substrates for cells, CAMs were usually applied as coatings on culture plates or biomaterial surfaces in a similar way as ECM molecules. In earlier studies, CAMs were primarily coated by adsorption onto culture plates. These culture plates were pre-coated with polyornithine [54], nitrocellulose [22, 23], polyethylenimine [58] or PLL [137] to assist the adsorption of CAMs. However, although physical adsorption is simple and effective on culture dishes, the CAMs may be short-lived on these materials. Furthermore, the spatial mode of presentation of CAMs is important as adsorbed CAMs are expected to be randomly orientated on the surface and possess lower loading efficiencies and functional effects [59]. To improve the efficiency of delivering and presenting CAMs on biomaterial surfaces, CAMs could be immobilized onto the modified surfaces.

Currently, chemical crosslinking and biochemical conjugation are two main strategies to immobilize CAMs onto a material surface for long-term delivery. While direct chemical crosslinking is effective to covalently bind CAMs to biomaterial surfaces, the functionality of CAMs may be degraded through the process [55]. In contrast, biochemical conjugation is advantageous in presenting the correct orientation of CAMs, and therefore, ensures the accessibility of the binding sites of CAMs [55, 59]. For biochemical conjugation to work, IgG antibodies that bind specifically to the Fc fragments were crosslinked onto the surfaces of biomaterials [55, 67, 85]. Following that, chimeric CAMs such as NCAD-Fc and L1-Fc were immobilized by the IgG antibodies through affinity capture or antigen-antibody interactions. Similarly, these Fc-tagged chimeric proteins can also bind to protein A coating on microfibrinous surfaces [59, 84]. As compared to Fc-recognizing IgG antibodies, protein A is considerably cheaper and may have better loading efficiency due to its smaller size. Besides surface modification, CAMs could also be encapsulated and immobilized within hydrogels. This was demonstrated to be feasible by photo-polymerizing hydrogels that entrapped HAVDI, the NCAD peptide [133]. In doing so, the immobilized HAVDI enabled NPC to adhere, similar to the surface modification approaches. In short,

1 CAMs immobilized on biomaterials or scaffolds act as both adhesion and signaling sites for neural cells
2 seeding. Among the conjugation methods, biochemical conjugation with antibodies or protein A appears
3 to be most effective and common. In comparison, as demonstrated by NCAD-Fc, chemical conjugation
4 might not be effective in preserving the function of CAMs [55]. However, this approach might work well
5 for smaller CAM-derived peptides such as HAVDI, where the conformation of the peptide would not be
6 affected by chemical conjugation [133].

7
8 Besides these methods, there are also other potential surface functionalization techniques that
9 may be used to incorporate CAMs onto biomaterials. These include mussel-inspired bioadhesive coatings
10 and layer-by-layer polyelectrolyte coatings [148, 149]. The layer-by-layer technique allows deposition of
11 ultrathin layers of charged polymers, where the CAMs may be immobilized by electrostatic interactions.
12 For mussel inspired bioadhesive coatings, polydopamine can be deposited onto the surfaces of a variety of
13 materials. Thereafter, CAMs could be adsorbed onto these polydopamine-coated surfaces by electrostatic
14 interactions or reactions with the quinone function group of polydopamine via amine or thiol groups[150].
15 These surface modification techniques may help improve the loading efficiency of the CAMs onto the
16 biomaterials in addition to bioconjugation.

17
18 Since CAMs can also induce neurite outgrowth and neural differentiation in soluble form [24, 33,
19 56, 58, 135, 136], modifying biomaterials for local delivery and sustained release of soluble CAMs may
20 be a viable strategy for engineering nerve regeneration. Local and sustained delivery of soluble CAMs by
21 biomaterial scaffolds may also help improve functional recovery. This is supported by an earlier study,
22 where soluble L1 was delivered by direct bolus injection using an Alzet pump to the injured site and was
23 demonstrated to improve nerve repair [63]. In a similar manner, soluble L1-Fc was delivered in a
24 controlled release fashion after L1-Fc was adsorbed onto chitosan-coated nerve conduits. Although it was
25 not mentioned in that study, the L1-Fc is expected to be released from the chitosan-coated conduits based
26 on published results that used chitosan for protein delivery [62, 121]. Therefore, scaffolds that provide

sustained release of soluble CAMs to damaged nerve tissues presents a great opportunity to further enhance the existing works on using scaffolds for nerve repair. Scaffold-mediated protein delivery for neural tissue engineering is not new as this approach has been used to deliver other soluble protein factors through controlled release [1, 151, 152]. For example, neurotrophin-3 was released over 3 months from polymeric scaffolds and was able to direct regeneration *in vivo* [153, 154]. Similarly, growth factors released from scaffolds also promoted neuronal differentiation [155-157]. However, this approach remains underexplored for the delivery of soluble CAMs.

6.3 Format and dosage of CAMs

In majority of the published studies, purified CAMs and Fc-tagged CAMs were commonly used. While these two formats of CAMs have been effective in promoting neurite outgrowth and neuronal differentiation, there has not been any direct comparison between the effectiveness of these two formats. However, Fc-tagged CAMs may be advantageous in terms of the possibility of subsequent biochemical conjugation. Recently, more single-chain variable fragment (scFv) antibodies, peptides and small organic molecules were designed to mimic the functions of the CAMs that are involved in neural regeneration [158-161] (Table 5). These small peptides or organic molecules appear to be an exciting option as they are smaller in size and are more metabolically stable. In addition, majority of these mimetics have similar potency as compared to CAMs, where some were tested both *in vitro* and *in vivo* [162]. Besides confirming the effectiveness of CAMs in neural regeneration, these CAM mimetics are also more likely to become clinically relevant as regulatory approved drugs [163]. Therefore, localized and sustained delivery of these soluble mimetics by scaffolds appears to be a promising strategy for neural regeneration.

Depending on the format of CAMs, the effective dosage and concentration of CAMs that are required to elicit biological effects can be very different. The effective dosage of CAMs that is needed also depends on the cell type, application and the type of CAMs (Table 2 -5). For example, the optimal amount of HAVDI to induce neuronal differentiation for mESCs (292 μ M, equivalent to 259 μ g/ml) was

1 much higher than the typical amount of NCAD-Fc that was used as a coating (10 µg/ml) [26, 133].
2 Although earlier studies on 2D showed that as low as 0.1 µg/ml could induce neurite outgrowth in
3 neurons [22], majority of the studies that coated or conjugated CAMs onto biomaterials appear to be in
4 the range of 5 µg/ml to 250 µg/ml (Table 3). This range of concentration could be a good starting point
5 for future applications in tissue engineering. Further optimization may be required when different cell
6 types, different culture conditions and different delivery systems are used. For instance, L1 supported the
7 differentiation and attachment of neurons but not astrocytes [95]. Similar considerations should also be
8 made when specific neuron subtypes are desired. For example, NCAM supported NPC differentiation into
9 glutamatergic neurons [136] while L1 promoted GABAergic neuron differentiation [137].

10
11 Besides individual CAMs, the synergism between different CAMs should be considered. For now,
12 only two combinations have been tested: ECAD/NCAD [26] and NCAD/L1 [84]. By presenting both
13 combinations as coatings, synergism in promoting neural differentiation and neurite outgrowth was
14 observed. For example, lower concentration of NCAD (1.375 µg/ml) was needed for mNPC
15 differentiation towards neuronal cells when it was co-coated with L1-Fc (10 µg/ml) [84]. Furthermore,
16 there remains a question on how CAMs synergize with other factors such as neurotrophic factors, ECMs,
17 and physical cues (topography, stiffness) in controlling neurite outgrowth, myelination, and neuronal
18 differentiation.

Table 5: Small peptide, antibodies and organic molecules as mimics of CAMs for neural regeneration

Type of CAM	Type of mimetics	Model / Cell type	Procedure / Concentration	Outcome	Reference
NCAD	Dimeric cyclic peptide mimetics - N-Ac- <i>CHAVDINGHAV</i> <i>DIC-NH₂</i> , N-Ac- <i>CINPISGINPISG</i> <i>C-NH₂</i>	Rat cerebellar neurons	0 - 100 µg/ml	- HAVDI: maximal ~70% increase in neurite length at 11 µg/ml (7.3 µM) - INPISG: maximal ~50% increase in neurite length at (~25 µM)	[164]
	Peptidomimetic small molecule agonists	Chick retinal explant	50 – 100 µM	- 8 molecules identified - Neurite length increased by 129 - 161 % - Neurite density increased by 142 - 217 % - Enhanced retinal cell migration	[165]
NCAM	Peptide – FGL	Rat cerebellar, dopaminergic, hippocampal neurons	0 - 200 µg/ml	FGL promotes survival of dopaminergic, hippocampal and cerebellar granule neurons - Dose-dependent neurite outgrowth - Promoted neuronal survival	[166]
	Peptide – BCL	Rat cerebellar neurons,	0 – 33.5 µM	- Dose-dependent neurite outgrowth	[167]
	Peptide – Plannexin	Rat cerebellar neurons	0 - 100 µg/ml	- Dose-dependent neurite outgrowth - Promoted neuronal survival	[168]
	Peptide – FGL	Rat NSC, <i>In vivo</i> : Rat	10 µg/ml, <i>In vivo</i> : subcutaneous injection 10 mg/kg	- In contrast to NCAM that promoted neuronal differentiation, FGL promoted oligodendroglial differentiation - Promoted NSC proliferation and mobilization <i>in vivo</i>	[169]

	Peptide – FGL	Rat spinal cord NSC	FGL-modified self-assembly peptides	- Supported proliferation and migration - No difference in MAP2 ⁺ and GFAP ⁺ differentiation	[170]
	Peptide – FGL	<i>In vivo</i> : Rat stroke model (Transient middle cerebral artery occlusion)	Subcutaneous injection 10 mg/kg	- Promoted NSC mobilization - Increased NG2 ⁺ cells - Modulated neuroinflammation (increased CD206 ⁺ cells)	[171]
	Peptide – C3	Rat hippocampal neurons	0 – 1 μ M	-Dose-dependent -Maximal response at 0.54 μ M (2-fold increase in neurite length)	[172]
	Peptide – P2	Rat hippocampal neurons	0 – 10 μ M	-Dose-dependent - Maximal response at 0.8 μ M (5-fold increase in neurite length) - More effective than C3 in stimulating neurite outgrowth by 7 to 15 times	[173]
L1	scFv antibody, peptides derived from scFv	Mouse cerebellar granule neurons, dorsal root ganglion neurons, motor neurons	1 – 100 μ M (coated on PLL)	- Promotion of neurite extension (10 μ g/ml, 32 nM) similar L1-Fc (2 μ g/ml, 5 nM) with an increase of ~4-fold over PLL - Soluble form less effective than substrate coated at the identical concentrations - Dose-dependent with the maximal effects seen at 10 μ g/ml approximately 2-fold increase in lengths of total and longest neurites. - Peptides derived from L1 scFv antibodies are not as potent as the antibodies	[174]

scFv antibody	U-87 MG cells	0.001 – 1 µg/ml	- Dose-dependent neurite outgrowth - Similar potency to extracellular domain of L1	[175]
Recombinant αL1 Fab fragment	Mouse cerebellar granule neurons <i>In vivo</i> : mouse spinal cord compression injury	0 – 200 µg/ml <i>In vivo</i> : Subdural delivery by osmotic pump (200 µg/ml for 14 days)	- Dose-dependent, similar to L1-Fc - <i>In vivo</i> : enhanced regeneration and reduced the glial scar	[176]
Small Molecule Agonists	- Mouse cerebellar, dorsal root ganglion neurons, - Schwann cells - <i>In vivo</i> : mouse spinal cord injury (compression)	0 – 1000 nM (coated on PLL) <i>In vivo</i> : Applied with micro-capillary to lesion site (200 nM)	- 8 small molecules identified - Dose-dependent neurite outgrowth - Similar potency at 100nM compared to L1-Fc (10ug/ml) - Stimulated neuronal survival, neuronal migration, and neurite outgrowth - Enhanced SC proliferation and migration and myelination of neurons - <i>In vivo</i> : improved recovery of motor functions, enhanced remyelination, neuronal survival, and monoaminergic innervation, reduced astrogliosis, and activation of microglia.	[162]
Small organic molecule (Phenelzine)	<i>In vivo</i> : mouse spinal cord injury (thoracic compression)	Intraperitoneal injection once daily for 6 weeks.	- Improved hind limb function, reduced astrogliosis and promoted axonal regrowth/sprouting - Decreased levels of pro-inflammatory cytokines	[177]

	Small organic molecule (Phenelzine)	Zebrafish spinal cord injury (complete transection)	500 nM in aquarium water	- Promoted locomotor recovery, axonal regrowth and remyelination in larval and adult zebrafish	[178]
	Small organic molecule (Tacrine)	Zebrafish spinal cord injury (complete transection)	250 nM (larva), 1 μ M (adult)	- Promoted locomotor recovery, regrowth of severed axons and myelination, and reduced astrogliosis in the spinal cord of both larval and adult zebrafish	[163]
CHL1	scFv antibody	Mouse cerebellar and hippocampal neurons	0.1 – 1000 nM (coated on PLL)	- Dose-dependent neurite outgrowth - 2-fold increase from control (PLL)	[179]

1 It should be noted that the concentrations reported in the literature were mainly used for surface
2 coatings. For CAMs usage as soluble factors to stimulate neurite outgrowth, dose dependency has been
3 observed within a range of concentration between 0 to 10 $\mu\text{g/ml}$ [24, 33, 56, 58]. On the other hand, only
4 NCAM has been reported to be used to induce neuronal differentiation after adding into cell cultures at 10
5 $\mu\text{g/ml}$ for 3 days [135, 136]. These dosages may be used as references when designing sustained release
6 systems for soluble CAMs.

7
8 Finally, additional considerations should also be made when delivering CAMs *in vivo*. So far,
9 only two studies have reported the direct delivery of CAMs to the lesioned sites. In a rat contusion SCI
10 model, L1-Fc solution was infused intrathecally at a concentration of 200 $\mu\text{g/ml}$ and at a rate of 0.5 $\mu\text{l/h}$
11 for 2 weeks after injury. The concentration was selected based on *in vitro* experiments using DRG
12 neurons. The rats showed functional recovery 12 weeks after injury [63]. In the other study, nerve
13 conduits were coated with chitosan and then in L1-Fc solution at a concentration of 10 $\mu\text{g/ml}$. Although
14 the loading efficacy and drug release kinetics of L1-Fc from the nerve conduits were not reported, axonal
15 regeneration and remyelination were observed [62]. Despite the encouraging results using L1-Fc, there
16 remains a lack of information in terms of the dosage that is required for other CAMs and other *in vivo*
17 models. The dosage of CAMs to be delivered *in vivo* may, thus, need to be further optimized especially
18 when a controlled release system is involved.

19 20 **6.4 Limitations and opportunities**

21 Although utilizing CAMs appears to an effective method to regulate cell-cell contact, it is not the
22 only way to control cell-cell interactions. For example, degradability of hydrogels can regulate NCAD
23 mediated cell-cell contact to facilitate the maintenance of neural progenitor cell stemness [180]. In this
24 study, elastic-line protein, poly(ethylene glycol) and alginate hydrogels were used but similar concept can
25 be applied to other hydrogels to regulate cell-cell contact by controlling the degradability of hydrogels.
26 However, the usage of CAMs has the potential to control cell-cell contact more precisely, through proper

1 selection of CAMs and spatial control of the distribution of CAMs. For instance, NCAD and L1 may be
2 used in the CNS to promote neuronal growth and prevent astrocyte proliferation to minimize glial scar
3 formation.

4
5 Since scaffolds are also used to deliver cells in addition to drugs and proteins, the effects of
6 scaffold properties and CAMs on regulating cell fate should also be considered. In this sense, scaffold
7 properties such as surface chemistry, topographical cues, substrate stiffness, porosity, and degradability
8 may affect cell fate and the delivery of CAMs. Ideally, the scaffold should be able to deliver CAMs to
9 cells that are seeded on the scaffold and cells in the native tissues, while supporting cell growth within the
10 scaffold. With optimization, some of these scaffold properties may work synergistically with CAMs to
11 further enhance nerve regeneration.

12
13 To optimize the delivery of CAMs to the injured nerve tissues, the timing of delivery is also
14 important in addition to the other factors mentioned above. Currently, this factor appears to be overlooked.
15 The timing of delivery is especially important for stimulating remyelination, as different CAMs appear to
16 play distinct roles at various stages of myelination. Furthermore, CAMs alone may not be sufficient to
17 fully regenerate injured nerve tissues. Other soluble and physical factors may be co-delivered with CAMs
18 for greater therapeutic outcomes. In particular, factors such as RGD ligand, glial cell-derived neurotrophic
19 factor and chondroitinase ABC, have been shown to work synergistic with CAMs for nerve regeneration
20 [181-183]. Therefore, future strategies that include multiple factors may be more promising in achieving
21 successful nerve regeneration and functional recovery.

22 23 **7. Future prospects and conclusions**

24 CAMs play important roles in nerve regeneration. Here, we have reviewed the effects of NCAD,
25 NCAM and L1 in three aspects of neural regeneration, namely neurite outgrowth, myelination, and

1 neuronal differentiation. Through *in vitro* and *in vivo* models, numerous studies have provided biological
2 understanding of the effects of CAMs in these aspects. We have then compared and identified the types
3 and formats of CAMs that are most efficacious in each aspect of neural regeneration. The cell adhesive
4 and nerve regenerative functions of CAMs make them good candidates as therapeutic targets.

5
6 With this information, we saw encouraging outcomes from applying CAMs in neural tissue
7 engineering and nerve regeneration approaches. This review attempted to identify the optimal methods of
8 incorporating CAMs within tissue scaffolds. These techniques, unfortunately, still constitutes a relatively
9 small number of studies, with more optimization and sustained delivery systems needed to utilize CAMs
10 more effectively as therapeutics. In addition, the synergism between CAMs in neurite outgrowth and
11 neuronal differentiation may also be exploited to further enhance nerve regrowth.

12
13 In conclusion, this review highlighted the potentiality of scaffold-delivered CAMs in neural tissue
14 engineering. When designing scaffolds to deliver cells and CAMs to the damaged nerve tissues, other
15 soluble and physical factors may be included for greater therapeutic outcomes. Future neural tissue
16 engineering designs to include CAMs and other neuro-regeneration factors will offer promising strategies
17 for nerve regeneration and functional recovery.

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