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Chapter 7

Ecto-Phosphorylation and Regeneration of the Adult Central Nervous System

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Abstract

Phosphorylation of ecto-domains of membrane proteins and extracellular matrix proteins, which is termed ecto-phosphorylation, activates intracellular signalling and has roles in several physiological processes including cell adhesion, fertilisation and fibrinolysis. We demonstrated that ecto-phosphorylation can promote endogenous neurogenesis in the damaged central nervous system (CNS), augmenting its functional recovery. Thus, regulation of ecto-phosphorylation could be a platform for development of therapeutic methods against CNS injury. Regeneration of the damaged CNS is long-awaited. While transplantation of neuronal progenitor cells is expected to be the first platform to develop the therapy, the potential of endogenous neurogenesis as a source of new neurons has been expected to be an inexpensive and non-invasive regenerative medicine for CNS injury. In this review, we focused on the spinal cord as a model of CNS recovery from traumatic injury. The spinal cord is the simplest part of the CNS and its function is well known. Therefore, estimation of recovery is easier than other part of the CNS. Firstly, we introduce endogenous neural stem cells (NSCs) in the adult spinal cord and their behaviour after injury and then discuss effects of ecto-phosphorylation, which induces regeneration of the adult spinal cord.

Keywords: adult neurogenesis, adult neural stem cells, activation of quiescent stem cells, spinal cord injury

1. Introduction

The adult mammalian central nervous system (CNS) loses its self-regeneration ability, whereas it contains neural stem cells (NSCs) that can differentiate into both neurons and glial

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cells [1, 2]. Under physiological conditions, neurogenesis in the adult CNS can be observed at the dentate gyrus of the hippocampus and the subventricular zone [3]. Neurogenesis is strictly inhibited in other regions of the adult CNS. After injury of the adult CNS, endogenous NSCs produce glial cells that are involved in formation of the glial scar. However, the stem cells rarely produce neuronal cells, which can contribute to regeneration of the neuronal network damaged by the injury. Thus, when the adult CNS is damaged, its neuronal network is not regenerated and incurable paralysis can be occurred. If cell fate decision of endogenous NSCs could be controlled in the injured CNS, the cells might be a source of new neurons and remyelinating oligodendrocytes to repair the damaged neuronal network. A recent report demonstrated that application of adenosine triphosphate (ATP) and a protein kinase promoted differentiation of neuronal cells in the damaged spinal cord, and diminished paralysis caused by the injury [4]. Thus, ecto-phosphorylation may provide a novel platform for regenerative medicine of the damaged CNS, in which endogenous NSCs are used for a source of new neurons.

2. Neural stem cells in the adult spinal cord

NSCs can be characterised by the ability of self-proliferation and of differentiation into both neuronal and glial cells [5]. Proliferating cells in the intact adult spinal cord are mostly oligodendrocyte precursors [6]. Other than that, ependymal cells existing around the central canal proliferate moderately, and radial glial cells existing throughout the spinal cord are also proliferative. These three types of cells are known to have potential to produce neurons at least *in vitro* and are proposed to be NSCs in the adult spinal cord [7–10].

When cultured NSCs derived from the spinal cord are transplanted into the adult spinal cord again, the differentiated cells from the transplanted NSCs are mainly astrocytes [11]. However, when the same NSCs are transplanted into a part of the hippocampus where neurons are generated through life, they produce neurons. This report demonstrates that NSCs derived from the adult spinal cord have ability to produce neurons, but the ability is inhibited by the microenvironment around NSCs in the spinal cord.

3. NSCs after spinal cord injury

The group of Frisen made transgenic mice, in which each type of stem cells was genetically labelled [6]. They observed cell fates of those stem cells after spinal cord injury. Radial glial cells produced only astrocytes after traumatic injury of the spinal cord, and oligodendrocyte precursors produced oligodendrocytes. Proliferation of ependymal cells was enhanced after spinal cord injury, and the cells moved to the injury site, producing astrocytes and very few oligodendrocytes (**Table 1**). Thus, endogenous neurogenesis is not activated after spinal cord injury.

NSCs in the intact spinal cord	Genetic labelling	Protein markers	Increase at the lesion (fold)	Descendant cells (contribution)
Ependymal cells	FoxJ1 promoter	Sox9	4–5	Astrocytes (53%)
		Vimentin		Oligodendrocytes (3.2%)
Astrocytes	Connexin 30 promoter	Sox9	2	Astrocytes (47%)
		GFAP		
Oligodendrocytes	Olig2 promoter	Olig2	2	Oligodendrocytes (97%)
		Sox10		
		APC (mature oligodendrocytes)		

Table 1. Cell fate of NSCs after spinal cord injury (based on data from Ref. [6]).

4. Regulation of NSCs in the spinal cord

Whilst transplantation of cultured neuronal precursor cells is expected to provide a new therapy for spinal cord injury, attempts to use endogenous NSCs as a source of new neurons have been examined, as summarised in the sections.

4.1. Inflammatory cytokines

After spinal cord injury, pro-inflammatory cytokines including TNF-alpha, IL-6, and IL-1beta are secreted in the injured site, inducing inflammation. IL-6 promotes differentiation of NSCs into astrocytes. Suppression of IL-6 signalling decreases the production of astrocytes induced by spinal cord injury, promoting functional recovery of damaged spinal cord [12, 13].

4.2. Growth factor

Growth factors can modulate not only proliferation and differentiation of NSCs, but also survival, neurite outgrowth, and synapse plasticity of differentiated neurons. Both FGF2 and EGF suppress differentiation of NSCs, which in turn promoting proliferation. Administration of FGF2 and EGF into the lateral ventricle accelerates growth of not only subventricular zone NSCs but also NSCs around the central canal of spinal cord [14]. IGF-1 induces the production of oligodendrocytes by inhibiting bone morphogenetic protein (BMP) signals through induction of bone morphogenetic protein such as Smad6, Smad7 and Noggin [15]. When fibroblasts that can secrete Brain-derived neurotrophic factor (BDNF) are transplanted into the spinal cord injury lesion, oligodendrocytes production is induced [16]. After spinal cord injury, the exogenous delivery of nerve growth factor (NGF) can induce growth of corticospinal axons in rats [17, 18], whereas NT3 elicits growth of corticospinal axons [19, 20]. Glial cell-derived neurotrophic factor (GDNF) induces growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination [21]. These reports

suggest that control of growth factors in the injured area of the spinal cord may promote regeneration of the injured spinal cord. However, clinical trials using systemic delivery of growth factors for various disorders have failed either as a result of lack of efficiency or unacceptable side effects, or both [22, 23].

4.3. Transcription factor and growth factors

Neurogenin2 and Mash-1 are transcription factors required for neuronal differentiation. Production of neurons and oligodendrocytes at the lesion of spinal cord is induced by infection of retroviruses that express those transcription factors followed by application of BDNF, FGF2, and EGF [9]. However, it is unknown whether the treatment with transfection and growth factors can promote recovery of the function, or not.

5. Extracellular phosphorylation

Protein phosphorylation occurs not only in the intracellular space but also at the extracellular space (**Figure 1**). Phosphorylation of proteins located at the cell surface has been reported in many types of cells, including platelets [24, 25], monocytes [26], osteoblasts [27], vascular smooth

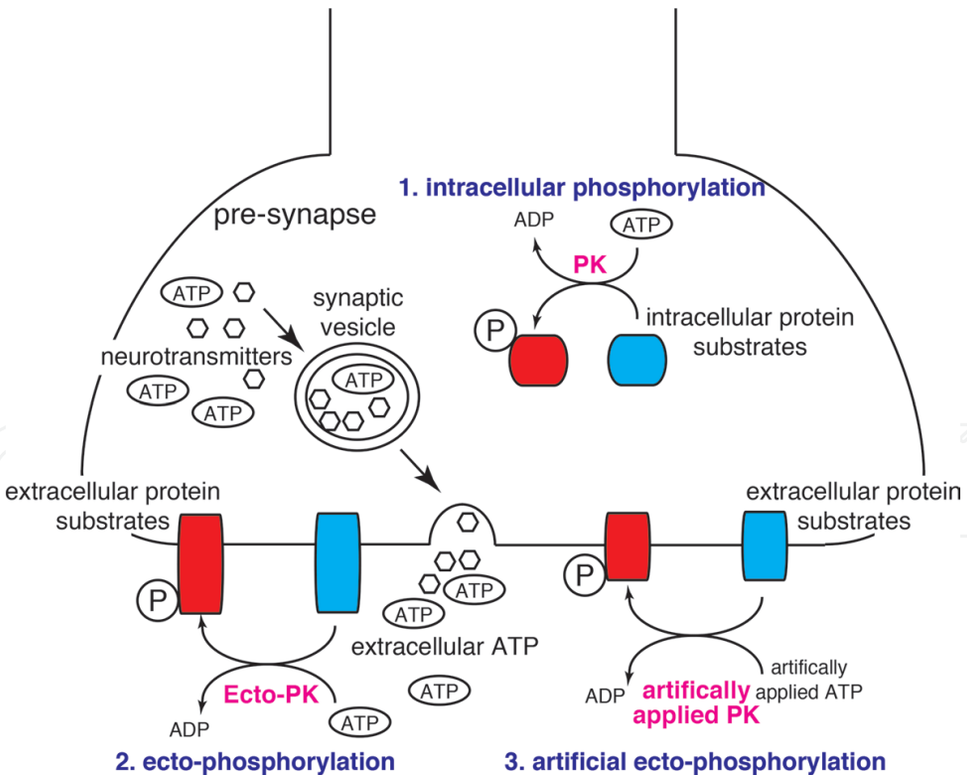

Figure 1. Three types of phosphorylation. (1) Intracellular phosphorylation by intracellular PKs. This type of phosphorylation has been studied extensively and intensively. (2) Endogenous ecto-phosphorylation by ecto-PKs. Some PKs can be secreted from cells through typical exsocytosis. Those ecto-PKs use extracellular ATP, which are secreted from many types of cells, including neurons and glial cells, as the donor of phosphorus residue for protein phosphorylation. (3) Artificial ecto-phosphorylation. In this case, both ATP and PKs are artificially applied. Any kinases that do not exist in the extracellular space can be applied, and they are applicable at any places even where endogenous PKs are not functional. Therefore, responses that endogenous ecto-PKs do not evoke can be expected.

muscle cells [28] and neurons [29]. Furthermore, some of extracellular matrix proteins are known to be phosphorylated [26, 30–32]. Yalak and Vogel identified 770 different phosphorylation sites in 66 extracellular proteins or in proteins with extracellular domain by annotation of secreted phosphorylated protein data available in public repositories [33]. Extracellular protein phosphorylation, which is termed ecto-phosphorylation, has been implicated in several physiological processes, including adhesion and migration of leukocyte and macrophage, fertilisation and fibrinolysis. Ecto-protein kinases (ecto-PKs) acting on the outer surface of the plasma membrane are reported to catalyse those phosphorylation. Ecto-PKs use extracellular ATP as a source of the phosphate group [28]. Thus far, FAM20C, protein kinase C (PKC), protein kinase A (PKA), casein kinase 1 (CK1) and casein kinase 2 (CK2) are reported as ecto-PKs [34, 35]. In addition to phosphorylation by ecto-PKs, ecto-domain of membrane proteins and proteins secreted from cells can be phosphorylated by kinases in the Golgi apparatus, which proteins pass through for secretion and for location to the plasma membrane. Ecto-domains of neuroglican C [36] and of amyloid beta precursor protein [37] can be phosphorylated by both ecto-PKs and kinases in the Golgi apparatus. Endogenous ecto-phosphorylations described in this chapter are summarised in **Table 2**.

These findings demonstrate that ecto-phosphorylation, as well as intracellular phosphorylation, can activate substrate proteins, inducing intracellular signalling.

Ecto-phosphorylation	Location	Effects	Cells
105, 39, 20 kDa proteins	Membrane	NGF-dependent neurite outgrowth	PC12 cells
12, 13 kDa proteins	Membrane	Correlation with neurite outgrowth	Primary embryonic chick neurons
48/50 kDa protein	Membrane	Long-term potentiation	Hippocampal pyramidal neurons
Laminin	Extracellular matrix	Cell adhesion and migration	–
Collagen XVII	Extracellular matrix	Unknown	–
Vitronectin	Extracellular matrix	Unknown	–
Fibronectin	Extracellular matrix	Unknown	–
NCAM	Membrane	Unknown	Neuronal cells
MAP1B	Post-synaptic area	Synapse formation	Cortical neurons
Beta-amyloid	Secreted	Promotion of aggregation	–

Table 2. Endogenous phosphorylation of ecto-domains of membrane proteins and extracellular matrix proteins described in the chapter.

6. Extracellular phosphorylation and neurite outgrowth machinery of neurons

In 1989, extracellular ATP was reported to stimulate uptake of noradrenaline into PC12 adrenal pheochromocytoma cells [38]. Addition of either ATP or ATPgammaS, but not adenosine diphosphate (ADP), guanosine tri-phosphate (GTP) or AppNHp, increased noradrenaline

uptake into PC12 cells. This suggests that added ATP was used as the donor of phosphate group in the phosphorylation reaction. Although protein responsible for the regulation of noradrenaline uptake is still unknown, 105, 39 and 20 kDa proteins at the surface of PC12 cells were identified as substrates for ecto-phosphorylation [39]. NGF, which can induce neuronal differentiation and neurite outgrowth of PC12 cells, enhances extracellular phosphorylation of PC12 cells [39]. A non-permeable and non-specific inhibitor of kinases, K252b, blocked both NGF-dependent neurite outgrowth and enhancement of extracellular phosphorylation [39, 40].

In 1995, primary embryonic chick neurons were incubated with radioactive ATP to show ecto-protein kinase activity [41]. Incorporation of radioactivity was detected with 116, 105, 67, 53, 17, 13 and 12 kDa proteins. Addition of either a pseudo-substrate peptide for PKC or a neutralising antibody against catalytic domain of PKC inhibited phosphorylation of 67, 13 and 12 kDa proteins, indicating that ecto-PKC catalysed phosphorylation of those proteins. Extracellular phosphorylation of 12 and 13 kDa proteins by PKC is regulated by development of the brain and is correlated with neurite outgrowth of neurons in the CNS. The 12 and 13 kDa proteins have not been identified.

7. Long-term potentiation and extracellular phosphorylation

Long-term potentiation is the long-lasting improvement in neuronal communication, which is the major cellular mechanism for learning and memory [42, 43]. Extracellular phosphorylation of neuronal surface proteins is implicated in long-term potentiation in the hippocampus [44–46]. Using mouse hippocampal slices, addition of either ATP or ATPgammaS, but not AppNHp, was reported to amplify permanently the magnitude of the population spike [44]. A 48/50 kDa protein at the surface of hippocampal pyramidal neurons becomes phosphorylated during the amplification [45]. Addition of a neutralising antibody against PKC inhibits the extracellular 48/50 kDa protein phosphorylation, and it blocks the stabilisation of long-term potentiation in hippocampal slices [45]. Thus, PKC-mediated extracellular phosphorylation is required for maintenance of hippocampal long-term potentiation. However, the 48/50 kDa protein has not been identified.

8. Extracellular phosphorylation and synapse

Synapse formation is an essential step to make neuronal network. K-252b, which is a non-permeable and non-specific inhibitor of kinases, inhibits synapse formation between cortical neurons *in vitro* [47]. This suggests that ecto-kinase sensitive to K-252b has a role in synapse formation. Lately, the same group has indicated that MAP1B, which is a tubulin-binding protein distributed in axon, especially in growth corn, is a substrate of ecto-protein kinases, and K-252b inhibits phosphorylation of MAP1B [48]. Originally, MAP1B was thought to be located in the cytoplasm. However, at least some splice valiant forms of MAP1B can be located at the

plasma membrane [49]. The membrane-bound MAP1B is located in the post-synaptic area, but not in pre-synaptic area [50]. The membrane-bound MAP1B can interact with myelin-associated glycoprotein (MAG) and the binding enhances phosphorylation of MAP1B [51]. Neither the kinase catalysing MAP1B phosphorylation nor the phosphorylation site in MAP1B has been elucidated. These reports suggest a possibility that ecto-phosphorylation of membrane-bound MAP1B has a role in regulation of synapse formation.

These findings suggest that ecto-phosphorylation can regulate neurite outgrowth, long-term potentiation and synapse formation of neurons. This gives an idea that ecto-phosphorylation may promote regeneration of the damaged neuronal network.

9. Ecto-domain phosphorylation of NgR

9.1. Effects on terminally differentiated neurons

NgR is a receptor of myelin-associated glycoproteins, Nogo-A, MAG and oligodendrocyte-myelin glycoprotein (OMgp) [52, 53]. Binding of those glycoproteins to NgR inhibits axonal outgrowth of neurons, at least *in vitro*. NgR signals activate intracellular Rho protein regulating rearrangement of cytoskeleton, which suppresses axonal outgrowth and synapse formation. Paralysis by spinal cord injury can be reduced by administration of an antibody against Nogo-A protein [54]. Genetic depletion of NgR shows NgR is partially responsible for limiting the regeneration of certain fibre systems in the adult CNS [53, 55].

We found that the extracellular domain of NgR can be phosphorylated by PKA and CK2, and that the phosphorylation inhibits binding of NgR agonists [56]. Interestingly, both kinases phosphorylate the same amino acid residue of the ecto-domain of NgR (**Figure 2**). *In vitro* study indicated that ecto-domain phosphorylation of NgR by either PKA or CK2 can overcome the inhibition of axonal outgrowth by NgR agonists Nogo-A, MAG and OMgp.

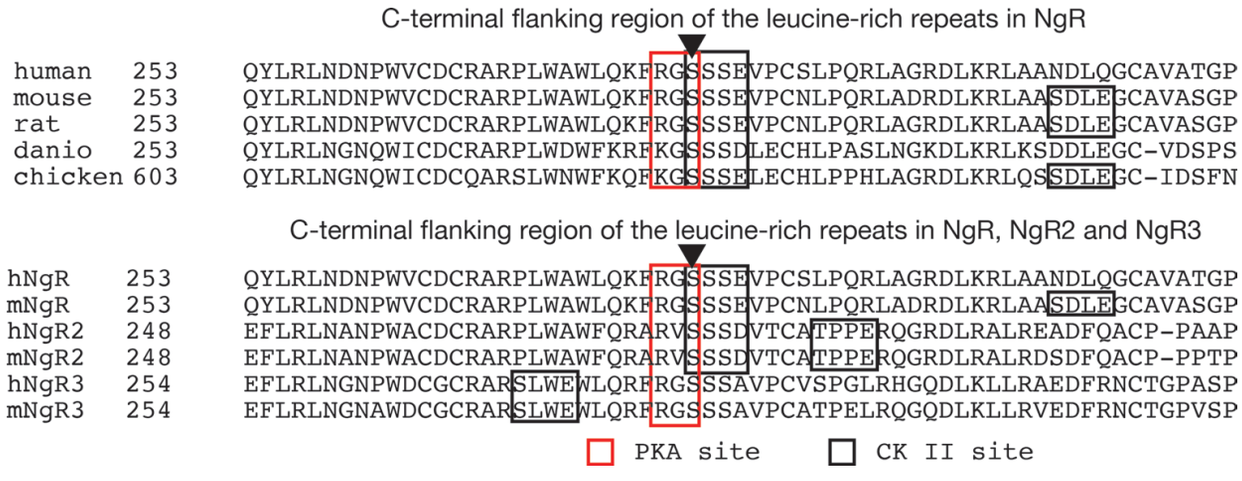

Figure 2. Phosphorylation sites in the ecto-domains of NgRs. (▼) indicates the phosphorylation site detected by mass spectrometer after the *in vitro* phosphorylation assay.

9.2. Effects on differentiation of NSCs

We reported that neurogenesis in the injured spinal cord can be augmented by artificial ecto-phosphorylation with continuous application of active PKA and ATP [4]. Since the treatment promoted functional recovery of the injured spinal cord, ecto-phosphorylation can be a novel target of therapies against paralysis caused by spinal cord injury.

In vitro assay indicated that NgR is expressed in ependymal cell-like NSCs derived from adult mouse spinal cord [4]. Noteworthy, proliferation of ependymal cells is enhanced after spinal cord injury, and the cells move to the injury site [6]. When differentiation of the NSCs was induced in the presence of NgR inhibitors, a transient increase of cells expressing a transcription factor Olig2 was observed on day 5 of *in vitro* differentiation. Olig2 can suppress differentiation into astrocytes but can promote differentiation into oligodendrocytes and motor neurons. Whereas oligodendrocytes were not observed in the descendant cells on day 14 of *in vitro* differentiation, increased proportion of neuronal cells was observed. When active PKA and ATP were applied on mice with spinal cord injury, NgR within the spinal cord was phosphorylated, and cells expressing neuronal precursor cell markers, such as doublecortin and neurogenin2, were increased. Significant improvement of hindlimbs' paralysis was also observed.

Our report proposed that myelin proteins released from damaged oligodendrocytes can suppress both axonal outgrowth from survived neurons and neurogenesis of NSCs, through

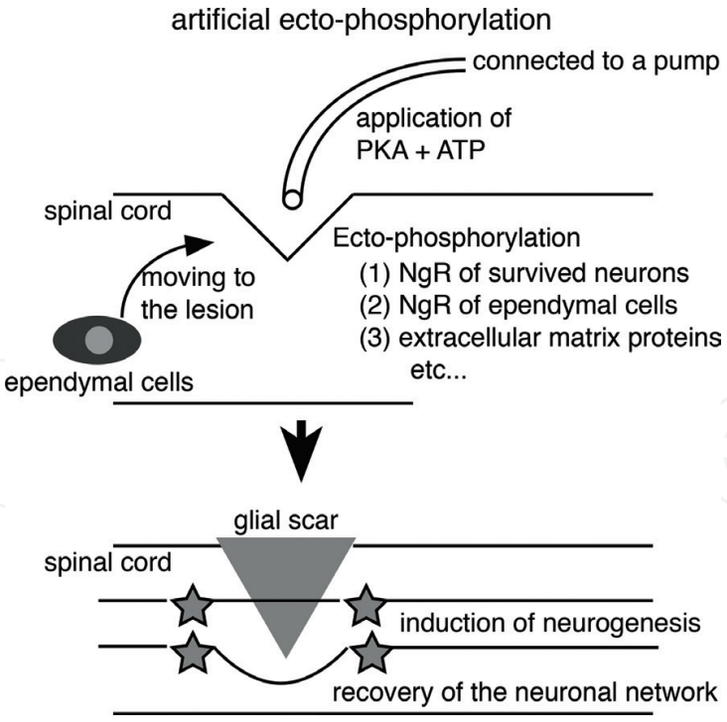

Figure 3. Possible effects of artificial ecto-phosphorylation induced by direct application of active kinases. Application of active PKA and ATP induces ecto-phosphorylation of NgR expressed in both differentiated neurons and NSCs and of other proteins. The phosphorylation can promote neurogenesis, enhancing functional recovery of the spinal cord from traumatic injury.

NgR activation. However, our results do not eliminate corporation of ecto-phosphorylation of other proteins. Application of active PKA and ATP to the injured spinal cord should phosphorylate many ecto-domains of membrane proteins and extracellular matrix proteins, in addition to NgR. The favourable effects on recovery from spinal cord injury could result from cooperation of ecto-phosphorylation of those proteins (**Figure 3**).

10. Phosphorylation of cell adhesion molecules and extracellular matrix proteins

Ecto-phosphorylation of proteins described below was originally reported with non-neuronal cells or in the extracellular matrix. However, when active PKA and ATP were applied to the damaged spinal cord, phosphorylation of these extracellular matrix proteins can be expected and similar effects should be induced.

10.1. Laminin

Laminin is an extracellular matrix protein found in basement membranes. Laminin interacts with cell surface proteins, influencing not only cell attachment on the basement membrane but also cell function and differentiation. Laminin is phosphorylated by CK2 [26]. This phosphorylation enhances heparin binding to laminin, cell attachment and migration. Laminin can be phosphorylated by PKC, in addition to CK2. Phosphorylation by PKC enhances self-assembly, heparin binding and cell attachment [57].

Laminin has important roles in neuronal differentiation of human embryonic stem cells [58], proliferation of human neural stem cells [59], netrin-mediated axonal guidance [60] and NGF-mediated neurite outgrowth from both PC-12 cells [61] and primary cultured neurons derived from mouse dorsal root ganglia [62]. Thus, phosphorylation of laminin could contribute to regulation of neuronal differentiation, migration and neurite outgrowth.

10.2. Collagen XVII

Collagen XVII can bind to alpha6 integrin, contributing to cell adhesion and motility. The serine 544 in the extracellular domain of collagen XVII is phosphorylated by CK2 and the phosphorylation inhibits shedding of the extracellular domain by metalloproteases of the A disintegrin and metalloproteinase (ADAM) family [32]. Although the exact function of collagen XVII is unknown, collagen XVII is expressed in the CNS and its distribution is changed in neurodegenerative disorders [63].

10.3. Vitronectin

Vitronectin is a glycoprotein in blood and extracellular matrix. Threonine 50 and 57 of vitronectin can be phosphorylated by CK2 [31] and the phosphorylation enhances its binding to both alpha(v)beta3 integrin [64] and urokinase receptor [65]. Furthermore, the serine 378 of

vitronectin is phosphorylated by PKA, which induces a conformational change and enhances the phosphorylation by CK2 [66]. Vitronectin is known to regulate differentiation of cerebellar granule cell precursor cells [67].

In addition, fibronectin [30] and neuronal cell adhesion molecules [29] are known to be phosphorylated. However, effects of their phosphorylation are unknown.

11. Perspective

It has been expected to develop a CNS injury treatment via activation of endogenous stem cells, because it may provide simple and inexpensive therapy with minimal invasion. We demonstrated that application of PKA and ATP can induce neurogenesis of endogenous NSCs in the damaged spinal cord, diminishing paralysis caused by the damage. The ecto-domain of NgR in the spinal cord is phosphorylated by the PKA application. NgR expression is detected in ependymal cell-like NSCs derived from the spinal cord, and ecto-phosphorylation of NgR promotes neuronal differentiation. However, multiple sites of extracellular proteins and domains should be simultaneously phosphorylated by the application. The favourable effects of the application are possibly due to a cooperation of phosphorylation of those proteins, including ecto-phosphorylation of NgR. Artificial ecto-phosphorylation could be the platform for development of therapies for cure of paralysis caused by spinal cord injury. More study is required for revealing the precise mechanism of which artificial ecto-phosphorylation promotes regeneration of the damaged CNS.

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