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(54) Title: COMPOSITIONS AND METHODS

(57) Abstract: The invention relates to a method for alleviating the inhibition of neurite outgrowth from a neurone, wherein said neurone comprises a Nogo receptor, said method comprising contacting said neurone with a composition capable of causing phosphorylation of a Nogo receptor, wherein said composition comprises protein kinase A or casein kinase



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Compositions and Methods

Field of the Invention

The invention relates to the field of neurological injury or disease, in particular the invention relates to the alleviation of inhibition of neuronal regeneration, such as neurite outgrowth.

Background to the invention

Neurons extend neurites to communicate with other neurons or with their target tissues. This neuronal network in the adult central nervous system (CNS) regenerates only poorly after injury. This is a problem in the art, leading to poor patient outcomes following injury to the neuronal network.

Failure of the adult mammalian CNS to regenerate is due partly to the neurite outgrowth inhibitors associated with damaged myelin. Myelin-associated glycoprotein (MAG), Nogo-A (also known as Reticulon 4A) and oligodendrocyte myelin glycoprotein (OMgp) are myelin-associated inhibitors of neurite outgrowth that can bind to Nogo receptor 1 (NgR1). These myelin-associated proteins, Nogo-A, MAG and OMgp, transmit signals from oligodendrocytes into neurons through binding Nogo receptors. This Nogo signalling has critical roles in development and maintenance of the central nervous system (CNS). It can inhibit differentiation, migration, and neurite outgrowth of neurons, causing poor recovery of the adult CNS from damage.

Nogo-A binds to NgR1 through a domain called Nogo-66. The Nogo-66 domain is composed of 66 amino acids and the Nogo-66 domain alone, without other regions of Nogo-A, is sufficient to inhibit neurite outgrowth. MAG, but neither Nogo-A nor OMgp, can inhibit neurite outgrowth not only through NgR1 but also through NgR2, an NgR1 homologous protein (6).

NgR1 makes signalling complexes containing LINGO-1 and either p75^{NTR} or TAJ/TROY (McGee, **and** Strittmatter *Trends Neurosci* 26, 193-198 (2003); Schwab **et al.** *Trends Mol Med* 12, 293-297 (2006)). Both p75^{NTR} and TAJ/TROY belong to the TNFalpha receptor family and they are proposed to be the major components initiating intracellular signals for inhibition of neurite outgrowth. It is uncertain whether NgR2 makes complexes containing LINGO-1 and either p75^{NTR} or TAJ/TROY.

35

While information on the roles of Nogo signalling is expanding, information on the mechanisms controlling this signalling is limited. This is a problem in the art. Increased intracellular levels of cAMP are known to overcome the inhibitory effects of Nogo

signalling on neurite outgrowth (16). However, the detailed mechanism by which cAMP overcomes the effects of Nogo signalling is unknown.

BDNF, a member of the neurotrophin nerve growth factor family, not only stimulates
5 neurite outgrowth of several types of neural cells in vitro (17-19, 20), but also partially promotes the recovery from spinal cord injury (21-24). Pre-treatment with BDNF increases the levels of intracellular cAMP in cultured neurons, allowing neurons to extend neurites even in the presence of the myelin-associated inhibitors (25). Furthermore, BDNF is implicated in injury-induced neurite sprouting in the hippocampus
10 (26). These reports suggest that BDNF could potentially help regeneration of neuronal networks in the CNS even in the presence of the myelin-associated inhibitors of neurite outgrowth. However, the effect is limited and is not enough for complete regeneration of the neural networks.

15 A human neuroblastoma cell line SH-SY5Y shows BDNF-dependent neurite outgrowth after 5 days treatment with retinoic acid (RA) (18). SH-SY5Y cells initiate differentiation into neuron-like cells and start expression of neuron-specific proteins in response to RA. However, the neural cells differentiated from SH-SY5Y cells by RA show only limited morphological changes. BDNF treatment is required for efficient neurite outgrowth of
20 the SH-SY5Y-derived neural cells, otherwise longer treatment with RA is required (27). Caesin kinase II (CK2) has been studied in the context of neurones. Caesin kinase II has been implicated in the phosphorylation of two different surface proteins in neurones. Neither protein is related to Nogo receptors. Furthermore, the studies in this area have been entirely dependent on the use of inhibitors of caesin kinase II. Thus, the function of
25 intra-cellular CK2 has been studied in the art. Intra-cellular CK2 activity is known to be required for neurite outgrowth itself. Extra-cellular CK2 is known to exist. However, information on extra-cellular CK2 is largely unknown as noted above. In more detail, there are indications that amyloid beta precursor protein and neuroglycan C can be phosphorylated at the surfaces of neurones by endogenous extra-cellular
30 CK2. However, the effects of these phosphorylation(s) on neurite outgrowth (if any) are unknown.

Caesin kinase II has been used to treat collagen/laminin in certain *in vitro* preparations. These treatments have never involved cells. These treatments have only ever involved
35 *in vitro* preparations of matrix proteins such as collagen or laminin.

No caesin kinase II treatment of cells is known in the prior art. Application of exogenous caesin kinase II to cells is not known in the prior art.

Ulloa et al (1993 EMBO vol 12 pp 1633-1640) inhibited CK2 activity in N2A mouse neuroblastoma cell line with antisense oligos and with a specific inhibitor. N2A cells extend neurites with neither retinoic acid (RA) nor BDNF. Using an N2A cell line, they found that neurite outgrowth from N2A cells is inhibited by depletion of CK2, and that phosphorylation of a microtubule-associated protein, MAP1B, is changed by the depletion. MAP1B is required for rearrangement of cytoskeleton, which is required for neurite outgrowth. Thus, they concluded that the change of MAP1B phosphorylation causes inhibition of neurite outgrowth by CK2 depletion. MAP1B phosphorylation is intra-cellular. Other proteins associated with rearrangement of cytoskeleton have been known to be phosphorylated by CK2, intra-cellularly. These intra-cellular phosphorylation events are required for neurite outgrowth itself. Since normal neurones, as well as N2A cells, can extend neurites without any stimulation, the inference is that these intra-cellular phosphorylation events are catalysed by a basal level of intra-cellular CK2 in neurones. The paper is not related to Nogo signaling.

No relationship between phosphorylation and Nogo signaling is known to date. The mechanism controlling Nogo signalling is unknown to date.

The present invention seeks to overcome problem(s) associated with the prior art.

Summary of the Invention

It is a problem that adult nervous tissue regenerates only poorly, or not at all. This is a particular problem following damage caused by factors such as injury or disease. Various mechanisms governing inhibition of regeneration have been identified. One such mechanism is inhibition of neurite outgrowth by signalling through the Nogo receptors. Although various ligands and receptors in this pathway are well characterised, there has been no reliable way of alleviating this inhibition disclosed in the prior art.

The present inventors have addressed these problems. Through detailed studies of Nogo signalling, the inventors have identified ways in which said signalling can be inhibited. Moreover, the inventors have identified a specific molecular target within the Nogo receptor which is key to the regulation of Nogo signalling. This target is serine 281 of the Nogo receptor. Phosphorylation of this residue abolishes binding of inhibitors of neurite outgrowth to the receptor, thereby alleviating the inhibition of neuroregeneration. Furthermore, the inventors teach and demonstrate effective ways

in which this may be accomplished, such as by treatment with protein kinase A and/or caesin kinase II. The invention is based upon these surprising findings.

Thus, in one aspect the invention provides a method for alleviating the inhibition of
5 neurite outgrowth from a neurone,
wherein said neurone comprises a Nogo receptor,
said method comprising contacting said neurone with a composition capable of
causing phosphorylation of a Nogo receptor, wherein said composition comprises
protein kinase A or casein kinase II.

10 Suitably said composition comprises protein kinase A and casein kinase II.

Suitably said phosphorylation is phosphorylation of an amino acid residue
corresponding to serine 281 of said Nogo receptor.

15 Suitably said Nogo receptor is human NgR1.

In another aspect, the invention relates to use of a protein kinase A polypeptide for the
manufacture of a medicament for spinal cord injury.

20 In another aspect, the invention relates to protein kinase A polypeptide for use in the
treatment of spinal cord injury.

In another aspect, the invention relates to use of a caesin kinase II polypeptide for the
25 manufacture of a medicament for spinal cord injury.

In another aspect, the invention relates to caesin kinase II polypeptide for use in the
treatment of spinal cord injury.

30 In another aspect, the invention relates to a composition comprising protein kinase A
and casein kinase II, for use as a medicament.

In another aspect, the invention relates to use of a composition as described above for
the manufacture of a medicament for spinal cord injury.

35 In another aspect, the invention relates to a composition as described above for use in
the treatment of spinal cord injury.

In another aspect, the invention relates to use of a protein kinase A polypeptide or a caesin kinase II polypeptide for the manufacture of a medicament for causing neurite outgrowth.

- 5 In another aspect, the invention relates to a protein kinase A polypeptide or a caesin kinase II polypeptide for use in causing neurite outgrowth.

In another aspect, the invention relates to a method of treating spinal cord injury in a subject, said method comprising administering to said subject an effective amount of
10 a composition capable of causing phosphorylation of a Nogo receptor, wherein said composition comprises protein kinase A or casein kinase II. Suitably said administration is localised to the site of the injury.

Detailed Description of the Invention

15

Nogo signalling can inhibit neurite outgrowth (3, 4), differentiation (9, 10), migration (12) and synapse formation (11) of neurons in the CNS. Thus, Nogo signalling has been identified as a major inhibitor of the regeneration of the CNS, which does not occur under physiological condition.

20 We disclose that phosphorylation of Nogo receptors, e.g. by casein kinase II (CK2), inhibits binding of the myelin-associated proteins which inhibit neuronal regeneration such as neurite outgrowth. We demonstrate that brain-derived neurotrophic factor (BDNF) may optionally be used to induce the phosphorylation of Nogo receptor, which suppresses Nogo-dependent inhibition of neurite outgrowth from neuroblastoma-
25 derived neurons. In further embodiments, extra-cellular CK2 treatment overcomes inhibition of neurite outgrowth by the myelin-associated proteins. This is demonstrated for example in rat adult neurons. Thus the invention provides new strategies to control Nogo signalling and hence neuronal regeneration.

30 We disclose for the first time a relationship between phosphorylation and Nogo signalling. We disclose for the first time the effect of ecto-domain phosphorylation on Nogo-dependent inhibition of neurite outgrowth. Phosphorylation of Nogo receptors may not be required for neurite outgrowth itself, but it is required for overcoming inhibition of neurite outgrowth. The inhibition of neurite outgrowth occurs under *in vivo*
35 conditions, eg. after traumatic injury of the central nervous system.

Moreover, we show that Nogo signalling inhibits neurite outgrowth from mammalian cells such as SH-SY5Y-derived neural cells, and that the inhibition is suppressed by extra-

cellular treatment with CK2 without BDNF. CK2 treatment inhibits binding of Nogo-66, MAG and OMgp to their receptors, allowing neurite outgrowth in the presence of these neurite outgrowth inhibitors. Thus we advantageously show BDNF-independent effects of the invention. Suitably BDNF is not used in the methods of the invention. Suitably
5 BDNF is specifically omitted from the methods of the invention. Suitably the compositions of the invention do not comprise BDNF.

Definitions

The term 'comprises' (comprise, comprising) should be understood to have its normal
10 meaning in the art, i.e. that the stated feature or group of features is included, but that the term does not exclude any other stated feature or group of features from also being present.

Nogo Receptors

15 In a broad sense, "Nogo Receptors" may refer to any protein mediating Nogo-dependent inhibition of neurite outgrowth, and need not specifically refer only to receptors (NgRs) in the classic membrane-localised sense, but may also refer to any Nogo-binding protein mediating the Nogo signalling e.g. via proteins other than NgR
20 family members; indeed, our results with normal neurones show that CK2 treatment can block such NgR-independent Nogo signalling as well as NgR-dependent signalling. However, suitably the term "Nogo Receptors" may be given its conventional meaning in the art herein unless the context indicates otherwise.

25 NgR1 and its homologous proteins, NgR2 and NgR3, belong to a family of glycosylphosphatidylinositol (GPI)-linked proteins with eight leucine-rich repeat regions, which do not have intracellular domains. While NgR1 can interact with Nogo-A, MAG and OMgp, NgR2 interacts with only MAG in a sialic acid-dependent manner. This interaction can inhibit neurite outgrowth, too. The ligand for NgR3 is unknown.

30 NgR1 makes a complex involving LINGO-1 and neurotrophin receptor p75^{NTR}. Alternatively, TAJ/TROY, an orphan tumour necrosis factor receptor family member broadly expressed in neurones, is involved in the complex, instead of p75^{NTR}. It is unknown whether NgR2 can make a complex with LINGO-1, p75^{NTR} or TAJ/TROY, as seen for NgR1.

35 Thus, Nogo signalling can be initiated by at least three ligands in oligodendrocytes, and it can transduce signals into neurones through at least two receptors. Relative contributions of specific ligand-receptor systems in Nogo signalling to inhibition of neurite outgrowth may vary among different neuronal cell types.

The present invention is concerned with Nogo receptors present on the cell surface. Indeed, it is a specific teaching of the invention that exogenous substances are used to provoke phosphorylation of Nogo receptors on the cell surface of the target cell. Thus, the term "Nogo receptor" as used herein suitably refers to a Nogo receptor protein present at the cell surface of a target cell. Suitably the target cell is a vertebrate target cell, more suitably a mammalian target cell, most suitably a human target cell. In some embodiments the target cell is most suitably a human cell comprised by the subject to be treated, such as a human neurone, such as an adult human neurone.

10

In a broad aspect, the term "Nogo receptor" may refer to the polypeptide of any of the known Nogo receptors, such as NgR1, NgR2 or NgR3. Moreover, in targeting the Nogo receptor, it may be that more than one type of Nogo receptor is phosphorylated. This may bring advantages to the invention, such as alleviation of inhibition of neurite outgrowth independent of the particular Nogo receptor type or types which happen to be expressed on the particular target cell of choice. Moreover, it may be that by targeting a multiplicity of Nogo receptor proteins, that a stronger and/or faster effect is achieved.

20 It is important to note that each of the Nogo receptors possesses the conserved serine at amino acid residue 281. Thus, references to Nogo receptor mutants according to the invention may equally embrace any of the known NgR polypeptides, provided of course they have the specific mutation or substitution being discussed.

25 Suitably, a Nogo receptor according to the invention is one or more of NgR1, NgR2 or NgR3 or NgR3. More suitably, a Nogo receptor according to the invention is one or more of NgR1 or NgR2. An advantage of this is that these Nogo receptors are better characterised, and are therefore amenable to the production of more specific or defined effects *in vivo*. Most preferably, a Nogo receptor according to the present invention is NgR1. This has numerous advantages, some of which are set out in the examples section.

35 The invention is primarily concerned with vertebrate, such as mammalian applications. Therefore, suitably the Nogo receptor of the invention is a vertebrate, such as a mammalian Nogo receptor. Most suitably, the Nogo receptor of the invention is a human Nogo receptor. A human Nogo receptor has a polypeptide sequence corresponding to the human Nogo receptor amino acid sequence. Naturally, a Nogo receptor polypeptide according to the invention may be produced by any suitable

means, such as recombinant production from a non-human host cell. However, for ease of understanding, a Nogo receptor polypeptide produced from a non-human cell will be regarded as a human Nogo receptor if the amino acid sequence corresponds to the human amino acid sequence.

5

Nogo receptor mutants are disclosed and discussed herein. It will be appreciated from the above discussion that such mutants may comprise one of a number of individual Nogo receptor subtypes such as NgR1, NgR2, NgR3, etc. For ease of understanding, particular amino acids or particular mutations are discussed in the context of a Nogo receptor reference sequence.

10

Reference Sequence

It will be appreciated that particular amino acid residues will be discussed using their numeric address on the polypeptide, as is conventional in the art. When particular amino acid residues are referred to using numeric addresses, the numbering is taken using the amino acid sequence of human NgR1 as the reference sequence. Most suitably, the Nogo receptor reference sequence is human NgR1 amino acid sequence of NM023004:

20 MKRASAGGSRL LAWVLWLQAWQVAAPCPGACVCYNEPKVTTSCPQQGLQAVPVGIPAASQR
IFLHGNRISHVPAASFRACRNLTILWLHSNVLARIDAAFTGLALLEQLDLSDNAQLRSVDPATF
HGLGRLHTLHLDRCGLQELGPGLFRGLAALQYLYLQDNALQALPDDTFRDLGNLTHLFLHGNRI
SSVPERAFRGLHSLDRLLLHQNRVAHVHPPHAFRDLGRLMTLYLFANNLSALPTEALAPLRALQY
LRLNDNPWWCDCRARPLWAWLQKFRGSSSEVPCSLPQRLAGRDLKRLAANDLQGCATATGP
25 YHPIWTGRATDEEPLGLPKCCQPDAAADKASVLEPGRPASAGNALKGRVPPGDSPPGNGSGPR
HINDSPFGTLPGSAEPPLTAVRPEGSEPPGFPTSGPRRRPGCSRKNRTRSHCRLGQAGSGGG
GTGDSEGGSGALPSLTCSLTPLGLALVLWTVLGPC

This is to be used as is well understood in the art to locate the residue of interest. This is not always a strict counting exercise – attention must be paid to the context. For example, if the protein of interest such as human EHD2 is of a slightly different length, then location of the correct residue in the human sequence corresponding to (for example) S281 may require the sequences to be aligned and the equivalent or corresponding residue picked, rather than simply taking the 281st residue of the sequence of interest. This is well within the ambit of the skilled reader. Exemplary alignments are provided in the accompanying figures.

35

It will be apparent to the skilled reader that the invention is exemplified predominantly by reference to NgR1. It should be noted that NgR1 exhibits high sequence homology with other NgR family polypeptides. Thus, in some aspects the invention relates to the use of NgR1 in the development of therapeutics for application to other NgR family proteins.

In some aspects of the invention, it may be desirable to employ a functional test as to whether or not a particular polypeptide is to be considered an NgR family polypeptide. In addition to, or instead of, the sequence based criteria set out above, the following functional criterion may also be used: binding to ligands such as those noted herein. Moreover, capacity to function in a neurite outgrowth assay may also be used, such as by overexpression in SH-SY5Y cells with RA treatment as described in the examples. Thus, in order to determine whether or not a particular polypeptide is indeed to be considered an NgR family polypeptide, it may be tested whether or not that polypeptide functions in a neurite outgrowth assay. If the NgR protein supports Nogo signalling in this context, the polypeptide may be regarded as a Nogo receptor (NgR family polypeptide). Of course the aim of the invention is to alleviate inhibition of neurite outgrowth and this should be borne in mind when assessing NgR protein(s) – it is the wild-type proteins which inhibit neurite outgrowth in this assay, in particular when not treated with kinase according to the invention.

Nogo Receptor Mutants of the Invention

The invention relates to Nogo receptor polypeptides characterised in that serine 281 is substituted for any other amino acid other than serine. Suitably S281 is substituted for a non-phosphoacceptor amino acid, so that suitably S281 is not S, T, or Y.

Suitably the invention provides a NogoR having S281A. This has the advantage of being unphosphorylatable at this site. This means that the receptor has the biological property of being resistant to CKII / PKA inhibition of ligand binding. In other words, such a mutant possesses the novel function of signalling in response to Nogo ligand(s) even in the presence of CKII/PKA.

Suitably the invention provides a NogoR having S281D. This has the advantage of simulating phosphorylation at S281. This means that the receptor has the biological property of being permanently switched 'off' so that the inhibition of neurite outgrowth is permanently alleviated using this receptor. More specifically, this receptor does not bind Nogo ligand(s). In other words, such a mutant possesses the novel function of NOT

binding (or signalling) in response to Nogo ligand(s), regardless of the presence of CKII/PKA.

Suitably the Nogo receptor polypeptides (NogoR's) of the invention comprise the
 5 sequence of NM023004 except where indicated, e.g. at the residue corresponding to S281.

The invention also relates to NogoR's having at least 60% identity to NM023004, suitably
 at least 70% identity to NM023004, suitably at least 75% identity to NM023004, suitably at
 10 least 80% identity to NM023004, suitably at least 85% identity to NM023004, suitably at
 least 90% identity to NM023004, suitably at least 95% identity to NM023004, suitably at
 least 97% identity to NM023004, suitably at least 98% identity to NM023004, suitably at
 least 99% identity to NM023004, always with the feature that residue S281 is other than
 serine.

15 Truncated forms suitably correspond to this sequence across the length of such a
 truncated form. Suitably a NogoR polypeptide according to the present invention
 comprises at least 30 amino acids, suitably at least 80 amino acids, suitably at least 130
 amino acids, suitably at least 180 amino acids, suitably at least 230 amino acids,
 20 suitably at least 280 amino acids, suitably at least 330 amino acids, suitably at least 380
 amino acids, suitably at least 382 amino acids (e.g. full length NgR2), suitably at least
 402 amino acids (e.g. full length NgR3), suitably at least 417 amino acids (e.g. NgR1).

Polypeptides and Mutants

25 Nogo receptor sequences may be modified for use in the present invention. Typically,
 modifications are made that maintain the region of the sequence comprising serine
 281 (or the substitution at said address). Amino acid substitutions may be made, for
 example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence
 30 retains the required S281 residue. Amino acid substitutions may include the use of non-
 naturally occurring analogues, for example to increase half-life of a therapeutically
 administered polypeptide. The same applied to PKA or CKII polypeptides of the
 invention, in which case it is always required that the PKA/CKII kinase activity is retained
 following any mutants or substitutions introduced.

35 Conservative substitutions may be made, for example according to the Table below.
 Amino acids in the same block in the second column and preferably in the same line in
 the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

Proteins of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences.

Proteins of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A protein of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a protein of the invention.

Applications of the Invention

It will be noted by the skilled reader that the invention relates to a novel method of inhibiting Nogo signalling. This novel method involves the phosphorylation of the Nogo receptor. Techniques disclosed herein also involve Nogo receptor mutants. Clearly, each of these slightly different technical variations is a part of the same common invention concerned with inhibition of Nogo signalling. For convenience and ease of understanding, the invention has been principally described in connection with alleviating or reducing the inhibition of neurite outgrowth. This finds particular

application in fields such as spinal cord injury. However, broader aspects of the invention may involve manipulation of other properties of neurones such as their migration or differentiation. Indeed, in principle any process being controlled or affected by Nogo signalling (such as inhibition of neurite outgrowth, such as migration
5 of neurones, or such as any other biological phenomenon influenced by Nogo signalling) may be modulated or controlled using techniques disclosed herein such as phosphorylation of the Nogo receptor.

Without wishing to be bound by theory, the precise biomechanics of neurite outgrowth
10 are still a subject of active research. It may be that the Nogo signalling techniques disclosed herein are affecting the regulation of neurite outgrowth in the sense of affecting controlling signals, or it may be that they are affecting the neurite outgrowth machinery such as proteins involved in fibre formation which may be intimately involved in producing the effect of neurite outgrowth. The present invention does not
15 concern itself with the precise biomechanics of neurite outgrowth. It is a key teaching of the invention that manipulation of the phosphorylation state of the Nogo receptor, or indeed the use of specific Nogo receptor mutants disclosed herein, are useful in modulation of neurite outgrowth. This teaching, and ways in which the invention is put into practice, are typically independent of the precise molecular mechanism which
20 takes place at the point of physical neurite outgrowth itself.

A key application of the invention is in the treatment of spinal cord injury. In particular, the invention finds application in promotion of regeneration following spinal cord injury. Specifically, the invention is useful if alleviating or reducing the inhibition of neurite
25 outgrowth which is caused by signalling via the Nogo receptor. By suppressing or reducing signalling via the Nogo receptor, inhibition of neurite outgrowth is usefully diminished. This provides conditions which are advantageously permissive of neurite outgrowth and therefore of regeneration.

30 Numerous examples presented herein involve canulation of the subject for application of the compositions of the invention. It should be noted that when the subject being treated is a human, canulation is advantageously avoided. Rather than canulation, a topical injection or series of injections of the compositions of the invention would be suitably employed.

35 In vivo applications are demonstrated in the examples section. In overview, the invention is generally applicable as follows:

-add ng to μg of kinase such as CK2, optionally with 100 μM ATP, after injury eg. of the spinal cord

5 In an experimental system, an injury may be made in the spinal cord and a canule put at the lesion. Through this canule, kinase such as CK2 may be applied on the area.

It is also possible to apply kinase such as CK2 with injection, without canulation. This is preferred for human subjects. However, for experimental systems, canulation makes it easier to add kinase such as CK2 from time to time.

10 It may then be examined whether neurite sprouting is observed or not, for example with immunofluorescence of tissue section.

Following observation of sprouting, behaviour tests may be conducted to assess functional recovery.

15

Suitably CK2 alone may be used, or CK2 in combination with chondroitinase ABC, which can digest chondroitin sulphate, another inhibitor of CNS regeneration.

Suitably PKA may be used, or PKA in combination with chondroitinase ABC.

20

Suitably the methods of the invention may be *in vitro*.

Suitably the methods of the invention may be *in vivo* for example in treatment of a subject such as a human subject.

25 **Phosphorylation of the Nogo Receptor**

It is envisaged that the majority of practical applications or embodiments of the invention will affect signalling of the Nogo receptor via inducing its phosphorylation. Embodiments of the invention involving Nogo receptor mutants are discussed herein
30 below. However, turning to the consideration of phosphorylation of the Nogo receptor, the inventors have surprisingly identified a particular site within the Nogo receptor which mediates the advantageous effects of the invention. This site is serine 281 of the Nogo receptor.

35 The inventors turned their attention to the study of serine 281 and the surrounding amino acid residues. A range of experimental techniques, including sequence analysis as well as direct experimentation as presented in the accompanying examples, were used in order to validate this surprising finding.

One of the key insights disclosed by the present inventors is that serine 281 of the Nogo receptor is of paramount importance as a target for the present invention. Moreover, the inventors have disclosed a number of ways in which serine 281 of the Nogo receptor can be targeted and phosphorylated. Two of the most suitable ways of accomplishing this are the use of protein kinase A and/or caesin kinase II to directly phosphorylate serine 281 of the Nogo receptor. Thus, although protein kinase A and caesin kinase II are not necessarily physically related to one another, in the context of the present invention they form a single cohesive technical group of alternative ways in which the invention can be implemented. In other words, caesin kinase II and protein kinase A represent two equally valid ways of implementing the same single invention. Thus, the presence of these two structurally distinct alternatives in the appended claims is entirely consistent with a single inventive concept. Moreover, these two enzymes share the same technical benefit in the context of the invention, which is namely to catalyse the phosphorylation of serine 281 of the Nogo receptor. For at least these reasons, the application can be clearly seen to relate to a single inventive concept. Diverse ways of achieving the technical benefits of the invention are described in order to assist understanding, and in order to ensure that the skilled reader has no difficulty in the practice of the invention.

20

Serine 281

As noted above, it is an important part of the core inventive concept that the different technical ways in which the invention may be implemented are in fact each directed at producing the same technical effect, ie, phosphorylation of serine 281 of the Nogo receptor. In the course of their studies, the inventors found that this residue is part of a predicted phosphate acceptor site for both CK2 and PKA. Furthermore, this key finding has been backed up by experimental research which demonstrates that both CK2 and PKA both catalyse the phosphorylation of this important residue, and therefore produce the alleviation of inhibition of neurite outgrowth.

30

Caesin kinase II

Suitably caesin kinase II is vertebrate, more suitably mammalian caesin kinase II. More suitably, caesin kinase II is human caesin kinase II.

35

Caesin kinase II most commonly occurs as a hetero-tetramer. This hetero-tetramer typically comprises a catalytic sub-unit consisting of two alpha polypeptides, and a

regulatory sub-unit consisting of two beta polypeptides. Such a complex is typically constitutively active in the sense of having kinase activity. Furthermore, the catalytic sub-unit on its own (ie, the homo-dimer of two alpha polypeptides) has catalytic activity even in the absence of the regulatory sub-unit.

5

Therefore, either the hetero-tetramer form of CK2 or the homo-dimer form of CK2 may be applied in the present invention, specifically the alpha-beta hetero-tetramer CK2 or alpha-homo dimer CK2.

10 Most suitably, CK2 is used in the form of a commercial preparation, such as from New England Bio Labs. This is suitably the holo enzyme form of CK2.

Most suitably the CK2 used is composed of two polypeptide chains, each polypeptide chain having the sequence of a human CK2 alpha subunit such as: gene name;
15 CSNK2A1, accession number NM001895.3, NM177559.2, NM177560.2 (3 subtypes) gene name; CSNK2A2, accession number NM001896.2.

With regard to CSNK2A1, NM001895 and NM177559 can produce the same proteins, although they have different 5' non-coding regions. NM177560 encodes isoform b,
20 which has shorter N terminal region. Most suitably protein sequence of NM001895 and NM177559 is used, which is isoform a:

MSGPVPSRARVYTDVNTHRPREYWDYESHVVEWGNQDDYQLVRKLGRGKYSEVFEAINI
TNNEKVVVKILKPVKKKKIKREIKILENLRGGPNIIITLADIVKDPVSRTPALVFEHVNN
25 TDFKQLYQTLTDYDIRFYMYEILKALDYCHSMGIMHRDVKPHNVMIDHEHRKLRLIDWG
LAEFYHPGQEQYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMI FRKEPFFHGH
DNYDQLVRIAKVLGTEDLYDYIDKYNIELDPRFNDILGRHSRKRWERFVHSENQHLVSP
EALDFLDKLLRYDHQSRLTAREAMEHPYFYTVVKDQARMGSSSMPPGGSTPVSSANMSG
ISSVPTPSPLGPLAGSPVIAAANPLGMPVPAAAGAQQ

30

With regard to CSNK2A2, most suitably the following sequence is used:

MPGPAAGSRARVYAEVNSLRSREYWDYEAHVPSWGNQDDYQLVRKLGRGKYSEVFEAIN
ITNNERVVVVKILKPVKKKKIKREVKILENLRGGTNI IKLIDTVKDPVSKTPALVFEYIN
NTDFKQLYQILTDFDIRFYMYELLKALDYCHSKGIMHRDVKPHNVMIDHQQKKLRLIDW
35 GLAEFYHPAQEQYNVRVASRYFKGPELLVDYQMYDYSLDMWSLGCMLASMI FRREPFFHGH
QDNYDQLVRIAKVLGTEELYGYLKKYHIDLDPHFNDILGQHSRKRWENFIHSENRLVLS
PEALDLLDKLLRYDHQQRLTAKEAMEHPYFYPVVKESQPCADNAVLSSGLTAAR

Protein Kinase A

Suitably, protein kinase A is vertebrate, more suitably mammalian protein kinase A.

5 More suitably, protein kinase A is human protein kinase A.

The protein kinase A used may be composed of 2 alpha subunits and 2 beta subunits. The beta subunit homo-dimer is the inhibitory domain and cAMP is required for dissociation of the beta homo-dimer from alpha homo-dimer.

10

More suitably the protein kinase A used is composed of 2 alpha polypeptide chains having the sequence of a human PKA alpha subunit (i.e. a PKA alpha subunit homo-dimer): gene name PRKACA, accession number NM002730.3, NM207518.1 (2 subtypes). This has the advantage of avoiding use of the beta subunit (which requires cAMP to activate the PKA alpha-beta hetero-tetramer) and therefore has the further advantage of avoiding use of cAMP. With regard to PRKACA, NM 002730 encodes isoform 1, which is ubiquitously expressed. NM207518 encodes spermatogenic cell-specific PKA, isoform 2. Most suitably the isoform 1 sequence is used such as:

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20

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MGNAAAANKGSEQESVKEFLAKAKEDFLKKWESPAQNTAHLDDQFERIKTLGTGSFGRVM
LVKHKETGNHYAMKILDKQKVVKLKQIEHTLNEKRILQAVNFPFLVKLEFSFKDNSNLY
MVMEYVPGGEMFSLRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDQ
QGYIQVTDGFGAKRVKGRWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGY
PPFFADQPIQIYEKIVSGKVRFP SHFSSDLKDLLRNLLQVDLTKRFGNLKNGVNDIKNH
KWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNFDDEEEEEIRVSINEKCGKEFSEF

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25

High specific activity PKA is available from New England Biolabs.

Suitably alpha-homodimer PKA is used.

30

Without wishing to be bound by theory, it appears that protein kinase A may not have a physiological role in neurite outgrowth. In other words, it may be that *in vivo* in a naturally occurring biological system, PKA may not come into contact with a Nogo receptor and/or may not phosphorylate it. However, it is clearly demonstrated herein that PKA does in fact catalyse this key phosphorylation event. For these reasons, it may be that treatment using PKA offers a further advantage in avoiding disturbance of any natural biological processes.

35

From a comparison of the enzymes which might be used to target serine 281, without wishing to be bound by theory, it appears that CK2 may be a more likely biological candidate for "natural" phosphorylation of the Nogo receptor. For this reason, it may be that using CK2 for the treatments of the invention might offer a further advantage over the use of other possible enzymes such as PKA, since there may be a greater biological fidelity or specificity achieved by use of the most likely natural or biological kinase-substrate pairing. Nevertheless, as demonstrated by the present inventors, CK2 is an effective catalyst for bringing about the phosphorylation of serine 281 of the Nogo receptor.

It should be noted that sub-units of the particular kinases of interest described herein may be equally applied in the present invention. A sub-unit may be a subset of the collection of proteins which typically make up the active enzyme *in vivo*, or a sub-unit may refer to a fragment or a truncated form of one of the polypeptides of the kinase of interest. In particular, it may be advantageous to use only the catalytic sub-unit of the enzyme of interest. This may provide advantages such as a more active form of the enzyme, and/or may avoid immunogenicity or other such complications which might arise from using larger molecules or complexes. Indeed, use of a polypeptide which happens to be truncated, or mutated, is intended to be within the scope of the invention. Mutations may be made for example to enhance activity or provide independence from known cofactors of the enzyme being used. If a skilled reader wishes to determine whether or not a particular fragment or truncation of an enzyme of interest falls within the scope of the invention, it is essential that the enzyme used retains its functional catalytic activity. More specifically, a kinase used in the present invention is of interest only if it is capable of catalysing incorporation of phosphate into the Nogo receptor, such as at residue 281 of the Nogo receptor. This may be easily tested, for example by combining the kinase or fragment of interest with Nogo receptor in conditions permissive of phosphorylation, and assaying the Nogo receptor thereafter in order to determine if phosphorylation did indeed take place. Moreover, the activity of the kinase of interest may be assessed functionally, for example by applying it to cells and testing its alleviation of the inhibition of neurite outgrowth. Clearly, only those enzymes or fragments capable of producing this functional alleviation of inhibition of neurite outgrowth are of interest or application in the invention. The exemplary ways in which this functional assay may be carried out are presented in the examples section.

Thus, unless the context suggests otherwise, then references to kinases of interest in the invention such as "protein kinase A" or "caesin kinase II" should be taken to include any sub-units, fragments, mutants or truncations of those kinases, always provided that they

retain kinase catalytic activity for the Nogo receptor as explained above, most suitably in the functional assay for alleviation of inhibition of neurite outgrowth.

5 Suitably the protein kinase A or caesin kinase II used in the invention comprises full length polypeptide. More suitably the protein kinase A or caesin kinase II used in the invention comprises polypeptide corresponding to the human wild type enzyme/s. Most suitably the protein kinase A or caesin kinase II used in the invention is as disclosed in the examples section.

10 Suitably the protein kinase A or caesin kinase II used in the invention comprises E-coli expressed recombinant protein.

Suitably the protein kinase A or caesin kinase II used in the invention is exogenous i.e. is not made in the cells local to the site of administration but is rather supplied externally
15 or exogenously such as in a composition of the invention.

Compositions

Compositions of the invention comprise one or more catalysts such as enzymes
20 capable of bringing about phosphorylation of NogoR serine 281. Suitably such an enzyme is PKA. Suitably such an enzyme is CKII.

Compositions of the invention may comprise PKA and CKII.

25 CK2 is suitably present at 500 –1200 U/ml CK2 final concentration, or an equivalent amount if the composition is dried/lyophilised or concentrated e.g. for storage.

In one embodiment the invention provides a composition comprising 10-100 μ M ATP, 5-0.5 μ g CK2, 1-10 mM MgCl₂ or Mg-acetate and 10-50 mM KCl or K-acetate.
30

When the enzyme used is CK2 alpha subunit homo-dimer; K ion is optional (in fact K ion is not required); thus suitably compositions containing only CK2 alpha subunit homo-dimer as the enzyme comprise only Mg and ATP as further components.

Using a CK2 alpha and beta hetero-tetramer has the advantage that the beta subunit
35 enhances substrate-specificity of CK2 phoshorylation and K ion promotes this effect of beta subunit. Thus, when using a CK2 alpha and beta hetero-tetramer as the enzyme, suitably K ion is included in order to obtain this advantage.

In one embodiment the invention provides a composition comprising 10-100 μ M ATP, 5-0.05 μ g PKA and 1-10 mM $MgCl_2$ or Mg-acetate.

5 Polypeptides of the invention may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline.

10 The composition of the invention may be administered by direct injection.

The composition may be formulated for administration to sites of nervous tissue, such as spinal cord, and may therefore be formulated to be compatible with cerebrospinal fluid or other such tissue.

15 Typically, each protein may be administered at a particular activity dose rather than a specific mg/kg body weight amount. Exemplary activity levels for administration are given in the examples section. By way of example regarding volumes, for mice, typically we inject around 2 μ l in the CNS. Thus, for human, we typically inject 50-200 μ l of kinase+ATP solution. Doses of active ingredients are as discussed herein.

Formulation/Administration

25 Suitably injection is used to deliver a composition of the invention such as CKII and/or PKA.

Compositions of the invention (eg. comprising kinase(s), ATP and/or ion(s)), may be administered topically eg. during spinal decompression surgery.

30 If surgery is not required, injection may be used as an alternative method for the administration.

After surgery, the compositions of the invention can be administered by periodic injection, over an effective period, such as up to 1 month or longer such as up to 3 months.

Matrix, such as matrigel (BD Bioscience), may advantageously be used to keep the reagent at the administrated region. Instead of matrix, any slow release substances and/or devices could be used to control the dosage/release periods.

5 ATP

Since some neurones can secrete ATP, it may not be an essential element of the composition of the invention. Under *in vitro* conditions, we have demonstrated the effects of exogenous kinase such as CK2 without addition of ATP, since the culture
10 medium contains ATP.

Suitably compositions of the invention comprise ATP. This has the advantage of supplying ATP to help promote phosphorylation of the Nogo receptor.

Suitably the composition comprises 0.1-1 mM ATP for compositions comprising either CK2 or PKA.

15

Magnesium

Suitably the composition comprises a source of 5 mM Mg²⁺ ion.

Mg²⁺ ion may be supplied as MgCl₂, or MgAc (Magnesium acetate) such as (CH₃COO)Mg₂.

20

Potassium

When a composition of the invention comprises CK2, suitably said composition further comprises potassium ion. Potassium ion is not typically needed for compositions
25 comprising PKA, but may optionally be included for example when the composition comprising PKA also comprises CKII.

Potassium ion is suitably supplied as 25-50 mM K ion, such as KCl or potassium acetate (CH₃COOK).

30 Potassium ion such as KCl is not essential, but advantageously has promoting effects on kinase activity of alpha-beta hetero-tetramer CK2.

cAMP

Cyclic AMP (cAMP) may optionally be included in compositions of the invention, particularly when said compositions comprise PKA. Specifically, when only the alpha
35 form of PKA is used, cAMP is optional. However, when the beta form of PKA is used, cAMP is desirable to include in the compositions of the invention and has the advantage of enhancing/enabling PKA activity.

cAMP is suitably supplied as di-butylic cAMP or as conventional cAMP. Either compound is available commercially such as via Sigma Inc. or Merck Inc. Use of di-butylic cAMP has the advantage of being cell permeable. Moreover, conventional
5 cAMP does not penetrate plasma membranes. Since the compositions of the invention are suitably applied extracellularly, suitably conventional cAMP is used as cAMP according to the present invention since this offers the advantage of minimising or eliminating any side-effects on intracellular signalling, and offers the advantage of confining the cAMP added to the extracellular environment thereby helping to retain it
10 localised with the enzyme(s) of compositions of the invention.

Advantages

It is an advantage of the invention that phosphorylation of Nogo receptors suppresses Nogo signalling, allowing neurite regeneration.
15

It is an advantage of the invention that phosphorylation of Nogo receptors blocks binding of their agonists.

The invention finds particular application in spinal cord injury.
20

It is an advantage of the invention that the Nogo receptor is targeted. This has the advantage of providing methods and compositions which act in a ligand-independent manner.

25 Combinations

It may be advantageous to combine the invention with inhibition of chondroitin signalling. One way of accomplishing this is that chondroitinase, such as chondroitinase ABC, can be applied to promote conditions permissive of neurite outgrowth. It is an
30 advantage that chondroitinase targets a pathway distinct from the Nogo pathway. Therefore it is advantageous to combine chondroitinase with treatments or compositions of the invention in order to provide a dual targeted approach to promotion of neurite outgrowth.

35 It may be advantageous to combine the invention with another way of targeting the Nogo pathway. For example, it may be advantageous to combine the invention with the application of an anti-Nogo-receptor antibody, or with antibodies against ligands of the Nogo receptor. It is an advantage of these embodiments that multiple

interventions are being used to target the same point in signalling pathway. Therefore, the possibility of unwanted side-effects or crossover signalling into other pathways is advantageously avoided.

- 5 It may also be advantageous to combine the invention with an anti-LINGO antibody. An exemplary anti-LINGO antibody may be as supplied by Biogen Inc. Such a combination has the advantage of targeting a different physical molecule, yet still within the same overall signalling complex. Therefore, combinations such as this offer the advantage of signalling fidelity, ie, targeting only a single pathway, combined with
10 the advantage of targeting multiple different molecules, and thereby aiming to achieve a more robust blockade of signal.

- It may also be advantageous to combine the invention with rehabilitation, which can be effective to promote proper connection of neurones. Such combination may also
15 advantageously decrease aberrant connection.

- The compositions of the invention may find application in administration during or after surgery such as neurosurgery. In this embodiment surgery is an example of injury to adult nervous tissue. Thus, the kinase(s), ATP and any necessary metal ions, may be
20 applied during surgery.

Optionally Cethrin may also be comprised in a composition of the invention. Typically this is used at 0.3, 1, 3, 6 or 9 mg per dose.

- 25 Application of matrix, or inclusion of matrix in the composition(s) of the invention, has the advantage of helping the added reagents to remain or persist in the lesion.

- Inhibitors of inflammation may be comprised in a composition of the invention, and/or may be applied as soon as possible after the injury/surgery.
30

- Transplantation of stem cells may be carried out, suitably after inflammation has passed (otherwise transplanted cells might be killed by the host immune system). By way of example, for a mouse, cells are transplanted after 5 days from the injury, i.e. transplanting the cells after inflammation.
35

Thus, inflammation inhibitors, Rho inhibitors (such as C3 toxin) and/or ES cells may usefully be combined with the invention. Concerning ES cells, forced activation of Nogo signalling can inhibit neuronal differentiation but promote glial differentiation of

neuronal stem cells. So, if Nogo is active in the injured area, differentiation of transplanted cells may be advantageously promoted by the inhibition of Nogo signalling.

5

Alleviation of Inhibition of Neurite Outgrowth

It has been a persistent problem in the art that re-growth or regeneration of neurones happens only poorly, or not at all, in damaged adult nervous tissue. In this context, damaged may infer a physical damage by injury, or may infer damage by a neurological disorder such as a degenerative disease. Clearly, in any such setting, regeneration or re-growth of the nervous tissue is desirable.

It is well established that neurite outgrowth is inhibited in healthy adult nervous tissue. It is an aim of the invention to remove or to alleviate this inhibition. The net result of this is a promotion or enhancement of regeneration such as via neurite outgrowth. This might be referred to as stimulation of neurite outgrowth. Indeed, for the majority of applications, there may be no substantial difference between stimulation of neurite outgrowth and alleviation of inhibition of neurite outgrowth. However, due to the insights and understandings gained by the inventors of this cryptic system, the invention is consistently described in terms of alleviation of inhibition of neurite outgrowth. This is because it is established that certain Nogo family ligands are inhibitors of neurite outgrowth. Thus, it is alleviation of this inhibition produced by signalling via the Nogo receptor which is the subject of the invention. If it is helpful to regard removal of this inhibition as "stimulation" of neurite outgrowth, then this may be noted or referred to from time to time. It does not detract from the overall aim of the invention being to remove or ameliorate inhibition of neurite outgrowth, thereby permitting or promoting (or indeed stimulating) neural regeneration such as via neurite outgrowth.

30

Moreover, the invention may advantageously be combined with one or more stimulator(s) of neurite outgrowth, thereby simultaneously provoking outgrowth as well as removing or alleviating the inhibition, and leading to a more pronounced or enhanced regrowth or regeneration.

35

Further Applications

In a broad aspect, the invention relates to PKA for use as a medicament.

In a broad aspect, the invention relates to CK2 for use as a medicament.

In a broad aspect, the invention relates to a new extracellular use for CK2.

It is disclosed that protein kinase A (PKA), as well as CK2, can inhibit Nogo signaling.

5 Most suitably the kinase used in the invention is PKA.

The invention relates to alleviation of inhibition of neurite outgrowth after spinal cord injury, in particular by inhibition of Nogo signalling.

10 Applications of the invention are not only limited to alleviation of inhibition of neurite outgrowth. Nogo signalling is implicated in differentiation (9, 10), synapse formation (11), and migration (12) of neurons during development. It is also implicated in Alzheimer's disease, a neurodegenerative disorder causing dementia (13-15). Thus the invention may advantageously be applied in these areas as well as in especially
15 suitable areas such as alleviation of inhibition of neurite outgrowth.

In particular, the invention may be applied to neurodegenerative disease generally, such as Alzheimer's disease and/or Parkinson's disease. This is a particularly suitable application of the invention since NogoR can bind to amyloid B and to its precursor
20 APP.

The invention may also be applied to more acute neurological disorders such as stroke.

Inhibition of Nogo leads to increased myelination. Therefore the invention finds
25 application in any disorder of myelination, such as multiple sclerosis. Thus the invention may be applied to multiple sclerosis. Schizophrenia is another example of a nervous disorder connected with a defect of myelination. Thus the invention may be applied to schizophrenia. Moreover, the invention relates to a method for increasing myelination in a system, said method comprising phosphorylating a Nogo receptor in said system,
30 suitably by addition of PKA and/or CKII to said system.

The invention also relates to a method of inhibiting binding of inhibitors of neurite outgrowth such as Nogo ligand(s) to the Nogo receptor, said method comprising inducing phosphorylation of said Nogo receptor, such as at serine 281.

35

Brief Description of the Figures

Fig. 1. BDNF promotes neurite outgrowth of RA-treated SH-SY5Y, and induces phosphorylation of NgR1. (A) SH-SY5Y cells were incubated with indicated reagents.

Cell images were taken with phase contrast microscopy. (B) Number of cells extending neurite-like structures of more than 40 μm and total cell number were counted from images taken in (A). More than 300 cells were examined in each sample. The proportion of cells extending neurite-like structures of more than 40 μm was indicated as a percentage of total cell number. An average of three experiments is shown. Error bars indicate S. E. between experiments. Data were analysed with student's *t* test. (C) Whole cell extracts were prepared from RA-treated SH-SY5Y cells after incubation with or without BDNF for 24 hours, and were analysed by SDS-PAGE. Immunoblotting was performed with the indicated antibodies. (D) Whole cell extract prepared from cells treated with both RA and BDNF was incubated with the indicated reagents for 1 hour at 37°C. Immunoblotting was performed with anti-NgR1 antibody. (E) RA-treated SH-SY5Y cells were incubated with BDNF in the presence or absence of one of the following: PKC inhibitor - 500 nM Gö6983 or Gö6976; PKA inhibitor - 2 μM KT5720 or a PKA inhibitor peptide 14-22; or either 500 nM or 1 μM of a casein kinase inhibitor, for 24 hours. Whole cell extracts prepared from these cells were analysed by SDS-PAGE. Immunoblotting was performed with anti-NgR1 antibody.

Fig. 2. CK2 promotes neurite outgrowth of RA-treated SH-SY5Y cells without BDNF. (A) RA-treated SH-SY5Y cells were incubated with the indicated reagents for 24 hours. Cell images were taken with phase contrast microscopy. (B) Number of cells extending neurite-like structures of more than 40 μm and total cell number were counted from images taken in (A). More than 300 cells were examined in each sample. Calculation was done as Fig. 1B. Result of student's *t* test between RA alone and RA+CK II+ATP was $p=0.0014$. (C) Myc-tagged wild type and mutant versions of NgR1 were over-expressed in SH-SY5Y cells. The cells were treated with RA, CK2 and ATP. Over-expressed NgR1-Myc was detected with anti-Myc antibody. (D) SH-SY5Y cells over-expressing 281S/A mutant NgR1-Myc were incubated with RA and NEP1-40. (E) SH-SY5Y cells over-expressing 281S/D mutant NgR1-Myc were treated as (C).

Fig. 3. CK2 phosphorylates NgR1, and inhibits binding of the myelin-associated proteins to NgR1. (A) Myc-tagged wild type, 281S/A or 281S/D mutant NgR1 were over-expressed in COS7 cells. The over-expressed cells were incubated with either the combination of ATP and CK2, or NEP1-40 for 30 min at 37°C. After washing with PBS, cells were incubated with Hisx6-tagged Nogo-GFP for 3 hours at 4°C. The cells were fixed and immunofluorescence was performed with anti-Myc and anti-GFP antibodies. (B) COS7 cells over-expressing Myc-tagged wild type NgR1 were treated as described in (A). Proteins were extracted and the Myc-tagged NgR1 was immunoprecipitated with anti-Myc antibody. Co-precipitated Hisx6-tagged Nogo-GFP was detected by

anti-GFP antibody. (C) Cell surface proteins in NgR1-overexpressing COS7 cells were labelled with biotin. The biotinylated proteins were fractionated and analysed by SDS-PAGE. Biotinylated NgR1-Myc was detected with anti-Myc. antibody. (D) COS7 cells over-expressing either wild type NgR1-Myc or 281S/A mutant NgR1-Myc were incubated with α -³²P-ATP in the presence or absence of CK2. Myc-tagged proteins were immunoprecipitated and analysed with SDS-PAGE. Proteins were blotted onto a PVDF membrane and the membrane was exposed to X-ray film to detect incorporated ³²P. After autoradiography, the membrane was used for immunoblotting with anti-Myc antibody. (E and F) COS7 cells over-expressing Myc-tagged wild type, 281S/A or 281S/D mutant NgR1, were treated as described in (A). After washing with PBS, cells were incubated with either His-tagged OMgp (E) or HA-tagged MAG (F) for 3 hours at 4°C. The cells were fixed and immunofluorescence was performed with either anti-Myc and anti-His antibodies (E) or anti-Myc and anti-HA antibodies (F).

Fig. 4. The CK2 target motif containing serine²⁸¹ in human NgR1 is conserved in vertebrate NgR1 and NgR2. (A) Amino acid sequences of C-terminal flanking region in NgR1 of human (GenBank accession number NM 023004), mouse (NM 022982), rat (AF462390), danio (NM 203478) and chicken (XM415292) were compared. Amino acids identical to human NgR1 are indicated in red. (B) Amino acid sequences of C-terminal flanking regions in human NgR1 (GenBank accession number NM 023004), NgR2 (NM 178570) and NgR3 (NM 178568) and mouse NgR1 (NM 022982), NgR2 (NM 199223) and NgR3 (NM 177708) were compared. (C) Whole cell extract was prepared from COS7 cells over-expressing Myc-tagged NgR2 after incubation with α -³²P-ATP in the presence or absence of CK2. Myc-tagged NgR2 was immunoprecipitated from the whole cell extract and analysed by SDS-PAGE. Proteins were blotted onto a PVDF membrane and the membrane was treated as described in Fig. 3c. (D) COS7 cells over-expressing Myc-tagged human NgR2 were incubated with or without the combination of ATP and CK2 for 30 min at 37°C. After washing with PBS, cells were incubated with HA-tagged MAG for 3 hours at 4°C. The cells were fixed and immunofluorescence was performed with anti-Myc and anti-HA antibodies. (E) After incubation with CK2 and ATP, NgR2-Myc was digested with trypsin in gel and the tryptic peptides were analysed with mass spectrometry. The peptide sequence detected as a phospho-peptide is described.

Fig. 5. CK2 rescues postnatal rat neurones from inhibition of neurite outgrowth by the myelin-associated inhibitors, Nogo, MAG and OMgp. (A) DRG neurones from postnatal day 5 rats were cultured with or without the Nogo-66 fragment in the presence of the indicated reagents. After 24 hours from addition of the indicated reagents, cells were fixed and α -tubulin III was detected by immunofluorescence. (B) The number of cells

extending neurites of more than 20 μ m and total α -tubulin III positive cell number were counted from images taken in (A). The population of cells extending neurite-like structures of more than 20 μ m was indicated as a percentage of total cell number. An average of three experiments is shown. Error bars indicate S. E. between experiments.

5 Data were analysed with student's t test. (C and D) DRG neurones from postnatal day 5 rats were seeded on an 8 well chamber slide coated with poly-D-Lysine and either MAG (C) or OMgp (D), and incubated with or without CK2 for 24 hours. Cells were fixed and immunofluorescence was performed with anti- α tubulin III. (E) The number of cells extending neurites of more than 20 μ m and total α -tubulin III positive cell number were

10 counted from images taken in (C and D). The population of cells extending neurite-like structures of more than 20 μ m was indicated as a percentage of total cell number. Calculation was done as (B). (F) CG neurones from postnatal day 8 rats were cultured with or without the indicated myelin associated inhibitors for 24 hours in the presence or absence of CK2. Cells were fixed and α -tubulin III was detected by

15 immunofluorescence. (G) CG neurones over-expressing either wild type NgR1-Myc or 281S/A mutant NgR1-Myc were cultured with or without the Nogo fragment in the presence or absence of CK2 for 24 hours. Over-expressed NgR1-Myc was detected by immunofluorescence. (H) The number of Myc-positive cells extending neurites of more than 20 μ m and total Myc-positive cell number were counted from images taken in (G).

20 Calculation was done as (B).

Figure 6 shows Amino acid sequences of vertebrate NgR1s. NCBI accession numbers of NgR1s are rat; AF 462390, human; NM 023004, mouse; NM 022982, danio; NM 203478, chicken; XM 415292 . Red characters show homologous amino acids to human NgR1.

25 Green characters show similar amino acids to human NgR1. NF; N-terminal flanking region, LRR; leucine rich repeat motif, CF; C-terminal flanking region.

Figure 7 shows: A, The RA-treated SH-SY5Y cells were cultured with the indicated reagents for 24 hours. After fixation, beta-tubulin III was detected with anti-beta-tubulin

30 III antibody. B, Wild type or 281S/A mutant NgR1-Myc was over-expressed in SH-SY5Y cells. After 5 days treatment with RA, these cells were incubated with 500 U/ml PKA and 500 nM (74 kBq/ml) ATP. Then, NgR1-Myc was immuno-precipitated and analysed with SDS-PAGE. C, RA-treated SH-SY5Y cells over-expressing either wild type or 281S/A mutant NgR1-Myc were incubated with 500 U/ml PKA and 500 nM ATP for 24 hours, and

35 were fixed. NgR1-Myc was detected with anti-Myc antibody. PKA, as well as CK2, induced neurite outgrowth from the RA-treated SH-SY5Y cells (Fig. 7A). PKA phosphorylated wild type NgR1-Myc (Fig. 7B). Mutagenesis at serine281 strongly inhibited both the phosphorylation with PKA (Fig. 7B) and neurite outgrowth after PKA

treatment (Fig. 7C). Thus, both PKA and CK2 can phosphorylate NgR1 and can cancel the inhibitory effects of Nogo signalling on neurite outgrowth.

Figure 8 shows Amino acid sequences of human NgRs. NCBI accession numbers of NgRs are as for Figure 4B. Red characters show homologous amino acids to human NgR1. The closed triangle shows the position of serine281 in human NgR1.

Figure 9 shows photographs and a sequence alignment. (A) Full length of PirB was expressed in COS7 cells with streptoavidine-binding peptide (SBP) tag at the C terminal. The COS 7 cells were incubated with 2000 U/ml PKA, 5 mM MgSO₄ and 100 μ M 32P-ATP in OptiMEM (Invitrogen) at 37°C for 1 hour. After the incubation, the cells were washed and proteins were extracted with 0.5% Triton X-100, 20 mM Tris HCl (pH8.0) and 150 mM NaCl. SBP-tagged PirB was precipitated from the extract with streptoavidin-magnetic beads (Invitrogen). Proteins bound to the beads were analysed by SDS-PAGE, and incorporated 32P was detected by autoradiography. (B) COS7 cells over-expressing PirB were treated as described in (A), except for cold ATP. Proteins analysed by SDS-PAGE was stained with coomassie brilliant blue G-250. The band corresponding to PirB was cut out, and analysed by mass spectrometry. Amino acid sequence of phosphopeptide detected by mass spectrometry was boxed. Closed triangle indicates the phosphorylated amino acid, serine425. Amino acid sequences around the phosphorylation site of human LILRB1, 2, 3 and 5, and rat and mouse PirB were compared. Amino acid residues homologous to mouse PirB was indicated with red characters.

Figure 10 shows photographs and a bar chart. (A) Organotypic culture of Entorhinal-hippocampal slices was prepared from 129X1/SvJmsSlc (albino) mice postnatal day 5 and 6. Culture media were changed every 2 days. On the day in vitro (DIV) 10, On the day in vitro (DIV) 10, the entorhinal-hippocampal projection was transected with sterile scalpel blade. The lesioned slices were cultured for 8 days with or without 1250 U/ml PKA and 100 μ M ATP. On the DIV17, a small aliquot of Dil paste (Invitrogen) was applied on the entorhinal side of the transection. On the DIV 18, the slices were observed with confocal microscopy to examine regeneration of entorhinal-hippocampal projection. Location of lesion made at DIV 10 is indicated as a white dot line. (B) Intensity of Dil signal near the dentate gyrus was measured with ImageJ (version 1.42q, National institute of health, USA). Measured area (100 μ m square) was indicated in (A) as a open white square. Intensity was indicated as a percentage of Dil signal in control (not lesioned). Experiment was repeated for 5 time in triplicate. Typical

images are indicated in (A), and average of the 5 independent experiments was plotted in (B). A student t-test was used to assess statistical significance.

The invention is now described by way of example. These examples are intended to be illustrative, and are not intended to limit the appended claims.

Examples

Methods and Reagents

A human neuroblastoma cell line, SH-SY5Y was purchased from ATCC. Ham's F12 medium, Neurobasal-A medium, mouse anti-GFP monoclonal antibody and B27 supplement were purchased from Invitrogen. Retinoic acid, creatine and creatine phosphokinase, mouse anti-Myc monoclonal antibody and poly-D-Lysine were purchased from Sigma. Rat neurons and NSF-1 supplement were purchased from LONZA. BDNF, Gö6983, Gö6976, the CK2 inhibitor (4,5,6,7-Tetrabromo-2-azabenzimidazole), KT 5720, myristoylated PKA inhibitor peptide 14-22 amide and NEPI-40 peptide were purchased from Merck. Anti-LINGO-1 was purchased from Millipore. Anti-Nogo A and anti-NgR were purchased from Santa Cruz. ATP was purchased from Roche. CK2 and lambda protein phosphatase were purchased from New England Biolab. Collagen IV, OMgp-His and Nogo-Fc was purchased from R&D. Rabbit anti-Myc polyclonal antibody was purchased from Cell Signaling. α -³²P-ATP was purchased from GE healthcare.

Cell culture

SH-SY5Y cells were cultured in Ham's F12 medium with 10% foetal bovine serum at 37°C in 5% CO₂ and 95% air. Passage numbers between 15 and 21 were used in experiments described in this paper. Further passages induce spontaneous differentiation and cells tend to extend neurites without BDNF (data not shown). For RA treatment, the cells are seeded (20,000 cells/well) on collagen IV coated 4 well chamber slides. After 24 hours culture, medium was changed to Ham's F12 medium containing 10 μ M RA and 10% foetal bovine serum. Medium was changed to fresh medium on day 3 and cells were used for experiments after 5 days culture. Encinas et al. (18) reported that RA-treated SH-SY5Y cells initiate apoptosis after withdrawal of serum only when the cells are cultured at low density. The cell density used in this paper was higher than that in the paper by Encinas et al. (18) and cell death due to depletion of serum or BDNF was not significant. In this paper, the SH-SY5Y cells treated with RA for 5 days are called RA-treated SH-SY5Y. For BDNF treatment, the RA-treated SH-SY5Y cells were washed with serum-free Ham's F12 medium and incubated with 25 ng/ml BDNF in serum-free Ham's

F12 medium for 24 hours. As a control (RA alone), the RA-treated SH-SY5Y cells were incubated with serum-free Ham's F12 alone for 24 hours.

The DRG neurons from postnatal day 5 rats were resuspended in Neurobasal-A media containing 2 mM glutamine and 2% NSF-1 supplement, and seeded (5,000 cells/well) on poly-D-Lysine coated 8 well chamber slides. After 4 hours from seeding, medium was replaced with fresh medium.

The CG neurons from postnatal day 8 rats were resuspended in Neurobasal-A medium containing 25 mM KCl, 2 mM glutamine and 2% B27 supplement, and seeded (10,000 cells/well) on poly-D-Lysine coated 8 well chamber slides. After 4 hours from seeding, medium was replaced with fresh medium.

CK2 treatment of cells

For CK2 treatment of RA-treated SH-SY5Y cells, the cells were washed with serum-free Ham's F12 and incubated with either 100 nM ATP or 500 U/ml CK2 or both in serum-free Ham's F12 containing 25 mM KCl and 5 mM MgCl₂ for 24 hours.

For CK2 treatment of DRG neurons from postnatal day 5 rats, medium was changed to neurobasal-A medium containing 2 mM glutamine 10 mM KCl, 5 mM MgCl₂ and 2% NSF-1 supplement with 500 U/ml CK2, after 4 hours incubation from seeding. As control (no treatment), medium was changed to the medium without CK2. For CG neurons from postnatal day 8 rats, neurobasal-A medium containing 2 mM glutamine, 2% B27 supplement and 25 mM KCl was used.

For CK2 treatment of COS7 cells, cells were washed with serum-free Ham's F12 medium and incubated with or without both 500 μ M ATP and 1200 U/ml CK2 in serum-free Ham's F12 medium containing 25 mM KCl and 5 mM MgCl₂ for 30 min at 37°C.

Phosphorylation by CK2 with α ³²P-ATP

For treatment of COS7 cells over-expressing Myc-tagged NgR1 or NgR2, cells were scraped off from the culture dish. The cells were washed with PBS and resuspended in PBS containing 25 mM KCl, 5 mM MgCl₂ 1200 U/ml of CK2, 200 μ M ATP (3.7 kBq/ml). After incubation for 30 min at 30°C, phosphorylation was terminated and proteins were extracted with 0.1% Triton X 100, 250 mM NaCl and 25 mM EDTA. Myc-tagged proteins were immunoprecipitated with anti-Myc antibody and analysed by SDS-PAGE.

Expression and purification of myelin-associated proteins

First strand DNA was synthesised from mRNA purified from human foetal brain (TAKARA) with SuperScript II reverse transcriptase (Invitrogen) and oligo dT. The cDNA of human Nogo-A was amplified by PCR with the first strand DNA as template. The amplified Nogo-A (Genbank accession number NM 020532) fragment, Nt 3444-3709, was

integrated into pEGFP N2 (TAKARA). The Nogo-A fragment and EGFP region was cut out and integrated into pcDNA 3.1/His vector (Invitrogen). The Hisx6, Nogo-A and EGFP region of the vector was cut out from the vector and integrated into pBEn-SBP-SET vector (Stratagene), then transfected to ArcticExpress (DE3)RIL E. coli (Stratagene).
5 Expression of Hisx6-Nogo-A fragment-EGFP protein was induced by 1 mM IPTG overnight at 18°C. Hisx6 Nogo-A fragment-GFP was purified with a TALON column (TAKARA).

For expression of human MAG (NM_002361), cDNA of MAG (Nt 198-1655) was amplified by PCR with the first strand DNA as template and a termination codon was added at the 3' end of the MAG cDNA by PCR. The MAG cDNA was integrated into
10 pDisplay vector (Invitrogen). The plasmid was transfected into COS7 cells. The transfected cells were maintained with DMEM containing 10% serum and 600 µg/ml of geneticin (Invitrogen). For purification of MAG, the cells were cultured with VP-SFM medium (Invitrogen) with 2 mM L-glutamine for 3 days. HA-tagged MAG was purified from the conditioned medium with HA-tag protein purification kit (Sigma).

15

Expression of NgR1-Myc and NgR2-Myc

NgR2 cDNA was amplified by PCR with the first strand DNA as template. The PCR product was ligated into pCR Blunt vector (Invitrogen). Sequence of the NgR2 cDNA inserted in pCR Blunt was confirmed. IMAGE clone encoding full length NgR1 was
20 purchased from Gene Service Inc. The cDNAs of NgR1 (NM_023004, Nt. 184-1543) and NgR2 (NM_17857, Nt. 1-1200), respectively, were transferred into pDisplay vector with SalI and KpnI site. The region encoding NgR, Myc tag and transmembrane domain is cut out from the vector with KpnI and NotI. The KpnI-NotI fragments were integrated into pCEP4 (Invitrogen). Electroporation with Nucleofector (amaxa) was used for
25 transfection. The NgR/pDisplay were transfected into COS7 and NgR1-Myc-transmembrane domain/pCEP4 was transfected into SH-SY5Y cells. For transfection into rat neurons, NeuroMag kit (OZ Bioscience) was used. Neurons cultured for 24 hours on 8 well chamber slides coated with laminin and with or without the Nogo-66 fragment were used for transfection. After the transfection, neurons were cultured for another 24
30 hours and CK2 was added into culture media to a final concentration of 500 U/ml. Neurons were cultured with CK2 for 24 hours and fixed.

Neurite outgrowth assay

For inhibition of neurite outgrowth with myelin-associated inhibitors, 1 µg of Nogo-Fc,
35 500 ng of HA-tagged MAG or 500 ng of His-tagged OMgp were spotted on different wells of poly-D-Lysine coated 8 well chamber slides, and were left overnight on a clean bench without cover. After washing with PBS twice, neurons were seeded and treated as described in "cell culture". The neurons were cultured for 24 hours at 37°C and were

fixed with 2% paraformaldehyde and 0.1% triton X100 in PBS. Immunofluorescence was performed with anti- α -tubulin III monoclonal antibody.

Binding assay of myelin-associated proteins

- 5 After treatment with or without CK2, COS7 cells over-expressing wild type or mutant NgR were incubated with Hisx6 tagged Nogo-GFP (10 μ g/ml), HA-MAG (50 μ g/ml) or Hisx6 tagged OMgp (10 μ g/ml) in PBS for 3 hours at 4°C. Cells were washed twice and fixed with 2% paraformaldehyde in PBS for 20 min at 4°C then with 2% paraformaldehyde and 0.1% Triton X 100 in PBS. To examine the expression levels of NgR1 at the cell
- 10 surface, a cell surface protein isolation kit (Pierce) was used. Surface proteins of COS 7 cells over-expressing NgR1 were labelled with biotin and were extracted. Biotinylated proteins were precipitated with streptavidin beads and were analysed by SDS-PAGE. Each sample fractionated from 5x10⁶ cells was analysed by SDS-PAGE.

15 Immunoblotting

- Cells were scraped off from dishes and washed with PBS twice, and then resuspended in PBS containing 0.1% Triton X100 and phosphatase inhibitor cocktail (Roche) and EDTA-free protease inhibitor cocktail (Roche). After incubation for 15 min on ice, samples were centrifuged at 14,000 x g for 20 min at 4°C. The supernatants containing
- 20 30 μ g of proteins were analysed by SDS-PAGE and blotted to PVDF membrane. The PVDF membrane was incubated in blocking buffer (5% skimmed milk, 0.4% Triton X-100 in PBS) for 1 hour, first antibody diluted in the blocking buffer and appropriate secondary antibody labelled with horse radish peroxidase. Bound antibodies were visualised with ECL or ECL Plus kit (GE healthcare).

25

Immunofluorescence assay

- The fixed cells were washed with PBS and incubated with 3% BSA and 0.5% Triton X-100 in PBS at room temperature for 30 min and with first antibody, 3% BSA and 0.5% Triton X-100 in PBS at 37°C for 2 hours. After washing with PBS, cells were further incubated with
- 30 appropriate secondary antibody, 3% BSA and 0.5% Triton X-100 in PBS at 37°C for 30 min. After washing with PBS 3 times, cells were mounted with VECTASHIELD mounting medium with DAPI (VECTOR) and observed with confocal laser scanning microscopy.

Immunoprecipitation

- 35 After CK2 treatment (Fig. 3C) or incubation with Nogo-GFP (Fig. 3B), proteins were extracted with 0.1% Triton X100 in PBS containing phosphatase inhibitor cocktail and EDTA-free protease inhibitor cocktail. The extracts were incubated with 25 μ g of anti-Myc rabbit polyclonal antibody and protein A magnetic beads (New England Biolab)

for 2 hours at 4°C. After washing 5 times with 0.1% Triton X100 in PBS, beads were incubated with 1x SDS-PAGE loading buffer and heated for 3 min.

Mass spectrometry

- 5 The COS7 cells over-expressing NgR2-Myc were cultured with 500 U/ml of CK2 and 500 μ M ATP for 1 hour. Cell extraction and immunoprecipitation of NgR2-Myc with anti-Myc antibody were described above. Precipitated proteins were analysed by SDS-PAGE and the proteins were visualised with colloidal coomassie brilliant blue staining. The band corresponding to NgR2-Myc was cut out from the gel. Proteins in the gel piece
10 were digested in gel with trypsin. Mass spectrometry analysis was done by Cambridge Centre for Proteomics (Cambridge, UK).

Example 1: Nogo signalling inhibits neurite outgrowth from RA-treated SH-SY5Y cells

- SH-SY5Y cells showed limited morphological changes after RA treatment for 5 days, but
15 efficient neurite outgrowth was observed after sequential treatment with RA for 5 days and BDNF for 1 day (Fig. 1A), as previously reported by Encinas et al. (18). We found that NEP1-40, a competitive inhibitor of Nogo-66 binding to NgR1 (28), promoted neurite outgrowth from neural cells differentiated from SH-SY5Y cells by RA, without BDNF (Fig. 1A and B). This suggests that Nogo signalling inhibits neurite outgrowth from the SH-
20 SY5Y-derived neural cells, and that BDNF suppresses the effects of Nogo signalling.

- As shown in Fig. 1C, proteins involved in Nogo signalling, Nogo-A, NgR1 and LINGO-1 were expressed in RA-treated SH-SY5Y cells. While neither Nogo-A nor LINGO-1 showed significant changes after BDNF treatment, the upper band of NgR1 was increased by BDNF treatment (Fig. 1C, arrow). Treatment with lambda phosphatase
25 decreased the upper band, only in the absence of phosphatase inhibitors (Fig. 1D). These results indicate that BDNF promotes phosphorylation of endogenous NgR1 in RA-treated SH-SY5Y cells, contributing to the lower mobility on SDS-PAGE. To investigate which kinase is involved in the phosphorylation of NgR1, RA-treated SH-SY5Y cells were incubated with BDNF in the presence of kinase inhibitors. Neither PKC inhibitors Gö6983 and Gö6976, nor PKA inhibitors KT 5720 and a PKA inhibitor peptide, show effects on the
30 level of the upper band of NgR1. However, a CK2 inhibitor decreased the upper band (Fig. 1E). Consistent with this, BDNF is known to activate CK2 in neural cells (29). These results indicate that CK2-like activity is involved in the phosphorylation of NgR1. Thus, BDNF induces phosphorylation of NgR1 by CK2-like activity, which is coincident with
35 promotion of neurite outgrowth by BDNF.

Example 2: RA-treated SH-SY5Y cells extend neurites after extra-cellular treatment with CK2, without BDNF

NgR1 is a glycosylphosphatidylinositol-anchored membrane protein, which does not

have a cytoplasmic domain (30, 31). Thus, the phosphorylation sites in NgR1 after BDNF treatment are extra-cellular, and they could be phosphorylated by extra-cellular treatment with CK2. Neither addition of ATP alone nor CK2 alone to the culture medium induced neurite outgrowth from SH-SY5Y-derived neural cells. However, the cells showed significant neurite outgrowth after simultaneous incubation with both CK2 and ATP for 24 hours (Fig. 2A and B). Thus, extra-cellular treatment with CK2 suppresses inhibitory effects of Nogo signalling on neurite outgrowth from the SH-SY5Y-derived neural cells, without BDNF.

Example 3: Serine²⁸¹ in human NgR1 is a key target for CK2 mediated suppression of Nogo signalling

Human NgR1 contains 5 candidate sites for phosphorylation by CK2, threonine¹⁷³, serine¹⁹², serine²⁸¹, threonine³²⁵ and serine³⁴⁵, and these candidate sites were substituted to alanine. The kinase used in this example is CK2. When the RA-treated SH-SY5Y cells over-expressing these NgR1 mutants were treated with ATP and CK2 for 24 hours, cells carrying the serine²⁸¹ to alanine substitution (281S/A) in NgR1 failed to show significant neurite outgrowth (Fig. 2C). However, the cells carrying the 281S/A mutant NgR1 still had the potential to extend neurites, since they showed neurite outgrowth after treatment with NEP1-40 for 24 hours (Fig. 2D). These results indicate that the 281S/A mutant NgR1 is not constitutively active and binding of Nogo is required for inhibition of neurite outgrowth from RA-treated SH-SY5Y.

Serine²⁸¹ in NgR1 was substituted to aspartic acid (281S/D) and the mutant NgR1 was over-expressed in RA-treated SH-SY5Y cells (Fig. 2E). Although, aspartic acid is negatively charged, like a phosphorylated serine residue, the cells over-expressing 281S/D mutant NgR1 did not extend neurites after RA treatment without co-treatment with CK2 and ATP (Fig. 2E). This indicates that 281S/D mutant NgR1 is not a constitutively negative mutant of NgR1.

These results indicate that serine²⁸¹ in human NgR1 is essential for CK2 to suppress the inhibitory effects of Nogo signalling, even though other membrane proteins can be phosphorylated by extra-cellular treatment with CK2 (32, 33).

Example 4: CK2 inhibits binding of the myelin-associated inhibitors to wild type NgR1, but not to the mutant NgR1 carrying a serine²⁸¹ substitution

The Nogo-66 fragment bound to COS7 cells over-expressing wild type NgR1 (Fig.3A). However, the Nogo-66 fragment failed to bind to the cells after treatment with CK2 and ATP. When 281S/A mutant NgR1 was over-expressed, instead of wild type NgR1, CK2 treatment failed to block the binding of the Nogo-66 fragment. NEP1-40 inhibited binding of the Nogo-66 fragment to both wild type and the 281S/A mutant NgR1.

When 281S/D mutant NgR1 is over-expressed, binding of the Nogo-66 fragment was not observed even without CK2 treatment (Fig. 3A right). Taken together with the results in Fig. 2E, 281S/D mutant NgR1 can neither bind to Nogo-66 nor inhibit signalling through endogenous NgR1. The interaction between the Nogo-66 fragment and over-expressed NgR1 was also observed in immunoprecipitation assays (Fig. 3B). While treatment with either ATP or CK2 alone failed to inhibit the interaction, co-treatment with ATP and CK2 inhibited binding of the Nogo-66 fragment to the over-expressed NgR1. To examine the expression levels of mutant and wild type NgR1-Myc at the cell surface, cell surface proteins were biotinylated. Comparable levels of biotinylated NgR1-Myc, wild type, 281S/A and 281S/D, were detected by western blotting (Fig. 3C).

Furthermore, over-expressed NgR1 is phosphorylated after the extra-cellular treatment with CK2. COS7 cells over-expressing either wild type or 281S/A mutant NgR1 were incubated with CK2 in the presence of α -³²P-ATP and over-expressed NgR1s were immunoprecipitated. Although both wild type and 281S/A mutant NgR1 were phosphorylated with CK2 treatment, phosphorylation of the 281S/A mutant NgR1 was much weaker than that of wild type NgR1 (Fig. 3D). These results indicate that serine²⁸¹ of NgR1 is required for both efficient phosphorylation of NgR1 by CK2 and inhibition of interaction between Nogo-66 and NgR1.

Fig. 3E and F show that CK2 treatment inhibited binding of both OMgp and MAG to wild type NgR1, but not to 281S/A mutant NgR1. Neither OMgp nor MAG bound to COS7 over-expressing 281S/D mutant NgR1. These results indicate that CK2 treatment can inhibit binding of Nogo-66, MAG and OMgp to NgR1 and that serine²⁸¹ in NgR1 is essential for the effect of CK2.

Example 5: Serine²⁸¹ of human NgR1 is conserved in both vertebrate NgR1 and NgR2

Fig. 4A shows that serine²⁸¹ is in the C-terminal flanking region of the leucine-rich repeats in human NgR1 (30, 31), and that the target motif for CK2 including serine²⁸¹ is conserved in human, mouse, rat, danio and chicken. NgR1 of mouse, rat and chicken has another candidate target motif for CK2 in the C-terminal flanking region, at serine³⁰⁴. However, the latter serine is not conserved in human and danio NgR1.

In addition to the CK2 target motif, serine²⁸¹ is involved in the PKA target motif, and the PKA target motif is also conserved in other species (Fig. 4A). This suggests that PKA, as well as CK2, might phosphorylate NgR1, thereby abrogating inhibitory effects of Nogo signalling on neurite outgrowth. However, we could not detect a contribution of PKA to the phosphorylation of NgR1 in RA-treated SH-SY5Y cells after BDNF treatment (Fig. 1E).

Moreover, the CK2 target motif containing the serine²⁸¹ of human NgR1 is conserved in human and mouse NgR2 (Fig. 4B). Serine²⁸¹ in human NgR1 corresponds

to serine²⁸² in human NgR2. Although NgR3 has a candidate target site for CK2 phosphorylation in the C-terminal flanking region, similar to NgR1 and NgR2, the site is 11 amino acids upstream of the serine²⁸¹ of NgR1. Conversely, the PKA target motif containing serine²⁸¹ in NgR1 is conserved in the three NgRs.

5 Consistent with the conserved CK2 target motif in NgR2, CK2 phosphorylates NgR2 (Fig. 4C) and CK2 treatment inhibited binding of MAG to COS7 cells over-expressing NgR2 (Fig. 4D). Although human NgR2 contains two CK2 target sites, serine²⁸² and threonine³⁶⁶, only the CK2 target site containing serine²⁸² is conserved in human NgR1. Mass spectrometry detected phosphorylation of the peptide containing
10 serine²⁸² of human NgR2, but not the peptide containing threonine³⁶⁶, after CK2 treatment (Fig. 4E).

Example 6: CK2 rescues rat neurons from inhibition of neurite outgrowth by Nogo, MAG or OMgp.

15 We examined whether phosphorylation of NgRs can rescue rat neurons from inhibition of neurite outgrowth by the myelin-associated inhibitors. When postnatal rat DRG neurons were treated with CK2 for 24 hours, the neurons overcame the inhibition of neurite outgrowth by the Nogo-66 fragment (Fig. 5A and B). ATP alone did not suppress
20 the inhibitory effects of Nogo-66 fragment (data not shown). Although BDNF can induce neurite outgrowth from RA-treated SH-SY5Y cells (Fig. 1A and B), BDNF fails to block the effect of the Nogo-66 fragment on DRG neurons (Fig. 5A and B). This suggests a difference between neuroblastoma cells and normal neurons in BDNF signalling, and it is consistent with the limited effects of BDNF on neurite outgrowth in vivo (21-24, 26).

25 CK2 treatment can rescue neurons from inhibition of neurite outgrowth not only by the Nogo-66 fragment but also by MAG or OMgp (Fig. 5C-E). Fig. 5F shows that not only DRG neurons from postnatal day 5 rats, but also cerebellar granule (CG) neurons from postnatal day 8 rats can extend neurites in the presence of Nogo-66, MAG or OMgp, when the neurons are treated with CK2 for 24 hours.

30

Example 7: CK2 fails to rescue neurons expressing 281S/A mutant NgR1 from inhibition of neurite outgrowth by Nogo-66

To assess the requirement of the serine²⁸¹ in NgR1, wild type and 281S/A mutant NgR1 were over-expressed in CG neurons from postnatal day 8 rats. Both CG neurons over-expressing wild type and 281S/A mutant NgR1 extended neurites, which was blocked
35 by the Nogo-66 fragment. After CK2 treatment for 24 hours, neurons over-expressing wild type NgR1 extended neurites in the presence of the Nogo-66 fragment. Contrary, neurons over-expressing the 281S/A mutant NgR1 failed to extend neurites even after

treatment with CK2 for 24 hours (Fig. 5G and H). These results indicate that CK2 can inhibit Nogo signalling through wild type NgR1 but not through 281S/A mutant NgR1, which is consistent with Figs. 2 and 3. Thus, Nogo signalling through NgR1 can block neurite outgrowth from postnatal rat neurons as previously reported (3, 4), and serine²⁸¹ of NgR1 is indispensable for CK2 to suppress the inhibitory effects of Nogo signalling on neurite outgrowth.

Example 8: Method for alleviating inhibition of neurite outgrowth in a subject

A method for alleviating the inhibition of neurite outgrowth from a neurone is demonstrated. In this example the subject is a mouse. Mouse neurones comprise a Nogo receptor.

A spinal cord injury model is used. Following induction of the experimental injury, said neurone is contacted with a composition capable of causing phosphorylation of a Nogo receptor. In this step, one of the following two compositions is applied, typically by injection or via a canula introduced at the time of the experimental injury:

A) (PKA) :

10-100 μ M ATP, 5-0.05 μ g PKA and 1-10 mM MgCl₂ or Mg-acetate.

or

B) (CKII):

10-100 μ M ATP, 5-0.5 μ g CK2, 1-10 mM MgCl₂ or Mg-acetate and 10-50 mM KCl or K-acetate.

For human applications, higher levels of kinases are used, typically 0.05-5 mg kinase, either PKA or CK2. Concentration of ions and ATP for human use are typically as above.

Example 9 - PirB/LILRB

Nogo-A, MAG and OMgp are myelin-associated proteins that can bind to NgR (Gonzenbach, R. R., and Schwab, M. E. (2008). Disinhibition of neurite growth to repair the injured adult CNS: focusing on Nogo. Cell Mol Life Sci 65, 161-176.). Recently, mouse PirB (paired-Ig-like receptor B, NM011095) and its human homologues LILRBs (leukocyte immunoglobulin-like receptor Bs) have been reported to be the second group of receptors for the myelin-associated proteins (Atwal, J. K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C., and Tessier-Lavigne, M. (2008).

PirB is a functional receptor for myelin inhibitors of axonal regeneration. Science 322, 967-970.). Although expression of PirB/LILRB appears to be limited in some specific regions of the brain, it can cooperate with NgRs to inhibit neurite outgrowth (Atwal et al., *ibid*).

5

In figure 9, PirB was over-expressed in COS7 cells, and the cells were incubated with catalytic subunit of protein kinase A (PKA) and ATP. PirB on the cell surface can be a substrate of PKA (Figure 9A).

10 Mass spectrometry indicates that the phosphorylation site is serine⁴²⁵ in the extracellular domain of PirB.

The PKA target site is conserved in human, rat and mouse (Figure 9B). These results suggest that extracellular treatment with PKA could have advantageous effects on
15 signalling from PirB/LILRB, in addition to signalling from NgRs.

Thus in one embodiment the invention relates to a method for alleviating the inhibition of neurite outgrowth from a neurone, wherein said neurone comprises a PirB/LILRB receptor, said method comprising contacting said neurone with a composition
20 capable of causing phosphorylation of PirB/LILRB, wherein said composition comprises protein kinase A or casein kinase II. Suitably said composition comprises protein kinase A and casein kinase II. Suitably said phosphorylation is phosphorylation of an amino acid residue corresponding to serine 425 of said PirB/LILRB.

25 It may be advantageous to target both Nogo and PirB/LILRB; in this embodiment the invention provides a method for alleviating the inhibition of neurite outgrowth from a neurone, wherein said neurone comprises a Nogo receptor and a PirB/LILRB receptor, said method comprising contacting said neurone with a composition capable of causing phosphorylation of a Nogo receptor and a PirB/LILRB receptor, wherein said
30 composition comprises protein kinase A or casein kinase II. Suitably said composition comprises protein kinase A and casein kinase II. Suitably said phosphorylation is phosphorylation of an amino acid residue corresponding to serine 281 of said Nogo receptor and phosphorylation of an amino acid residue corresponding to serine 425 of said PirB/LILRB.

35

Example 10 – Regeneration Of Mammalian CNS According To The Present Invention

In this example we demonstrate the action of the invention on mammalian CNS.

Neurones in the entorhinal cortex form a strong projection to the dentate gyrus of the hippocampus during postnatal period. The entorhinal cortex-hippocampus system is one of the first regions to be affected in Alzheimer's disease, and problems in the system is thought to be result in both impaired sense of direction and disorder of memory.

Organotypic slice culture of the brain preserves many features of the *in vivo* system, and it is widely used as a model of intact brain.

Entorhinal-hippocampal projection in the intact brain can be conserved in the organotypic slice culture *in vitro*. Entorhinal-hippocampal slices prepared from mice postnatal day 5-7 show poor regeneration from lesion, after 6 days culture *in vitro* (Kluge, A., Hailer, N. P., Horvath, T. L., Bechmann, I., and Nitsch, R. (1998). Tracing of the entorhinal-hippocampal pathway *in vitro*. *Hippocampus* 8, 57-68. Prang, P., Del Turco, D., and Kapfhammer, J. P. (2001). Regeneration of entorhinal fibers in mouse slice cultures is age dependent and can be stimulated by NT-4, GDNF, and modulators of G-proteins and protein kinase C. *Exp Neurol* 169, 135-147.).

Chondroitin sulfate proteoglycans and myelin associated proteins are known to be the major inhibitors of the regeneration (Mingorance, A., Sole, M., Muneton, V., Martinez, A., Nieto-Sampedro, M., Soriano, E., and del Rio, J. A. (2006). Regeneration of lesioned entorhino-hippocampal axons *in vitro* by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NgR signaling. *FASEB J* 20, 491-493.).

Migration of microglia, which is resident macrophage in the central nervous system (CNS), can be observed, and glial scar-like structures can be formed by glial cells in the organotypic culture after lesion (Mingorance et al., *ibid*). All these responses of entorhinal-hippocampal slice culture to lesion is similar to that of normal brain *in vivo*. Thus the validity of demonstrating the invention in this model system as an illustration of its applicability in mammals generally is well established.

In Figure 10, entorhinal-hippocampal slices from mice postnatal day 5 or 6 were transected on day *in vitro* (DIV) 10, and they were incubated with or without PKA and ATP for 8 days.

Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) is a lipophilic membrane stain that diffuses laterally to stain the entire cell, but it cannot move

through cell-to-cell interaction. Therefore, projection of neurones can be traced with Dil.

5 In slices without treatment or slices treated with either PKA or ATP, location of Dil was limited in the entorhinal side of the transection (Figure 10), indicating that the brain slices can regenerate the neural network, as poor as that in the brain *in vivo*. Only when the slices were incubated with both ATP and PKA, Dil was observed in the hippocampal side beyond the transection (Figure 10).

10 These results indicate that treatment with combination of PKA and ATP can overcome endogenous inhibitors for CNS regeneration, promoting regeneration of the neuronal network according to the present invention.

15 However, Dil was not detected beyond the dentate gyrus of the hippocampus, even after treatment with PKA and ATP. This indicates that the treatment does not change the intrinsic layer structures in the normal hippocampus (Figure 10). This is an important feature for promotion of CNS regeneration, to avoid unexpected regeneration that can result in disorders.

20 Thus, exogenous addition of PKA according to the present invention is demonstrated to be a useful method to promote regeneration of the CNS, which is strictly inhibited under normal conditions.

Summary of Examples Section

25 We disclose that Nogo signalling can be suppressed by phosphorylation of Nogo receptors. The phosphorylation sites in Nogo receptors are extra-cellular (Figs. 3 and 4). BDNF induces the ecto-domain phosphorylation of NgR1 in neural cells differentiated from SH-SY5Y cells by RA (Fig. 1). Ecto-domain phosphorylation of NgRs inhibits binding of myelin-associated inhibitors of neurite outgrowth, Nogo-66, MAG and OMgp (Fig. 3
30 and 4).

Ecto-domain phosphorylation has been reported in many types of cells, including neurons, immune cells, epithelial cells and endothelial cells (34). Ecto-domain phosphorylation can be catalysed by both ecto-protein kinases after substrate proteins are sorted to the plasma membrane, and intracellular kinases before the sorting (33,
35 35). While intracellular kinases use intracellular ATP as a source of phosphate group, ecto-protein kinases use extra-cellular ATP. The concentration of extra-cellular ATP under physiological conditions has been estimated at micro-molar level (36). When tissues are damaged, ATP can be released from dying or injured cells through

damaged plasma membranes. Inflammation, which can be induced by tissue damage, can also induce release of ATP from a variety of cell types including neurons (37). Thus, the concentration of extra-cellular ATP could be increased by tissue damage. Although in vitro function of ecto-domain phosphorylation has been reported (34), its in vivo function is still unclear.

Recently it was shown that ecto-domain phosphorylation of collagen XVII can inhibit its shedding by metalloproteinases (38). Ecto-domain phosphorylation of collagen XVII is catalysed by CK2, and its phosphorylation site is located in the target site for metalloproteinases (38). While NgRs are also known to be shed by metalloproteinases (39, 40) and they can be phosphorylated by CK2 (Fig. 1-4), the phosphorylation site is not included in the shedding site. The shedding site in NgR1 is amino acid 358 (39), and the phosphorylation site is 281 (Figs. 3 and 4). We could not detect digested fragments of NgR1 when RA-treated SH-SY5Y cells were analysed by western blotting (Fig. 1). Ecto-domain phosphorylation of NgR1 appears not to regulate its shedding. Instead, we indicate that ecto-domain phosphorylation of NgRs blocks binding of myelin-associated inhibitors of neurite outgrowth, Nogo, MAG and OMgp (Fig. 3 and 4).

Although BDNF can induce phosphorylation of NgR1 and overcome Nogo signalling in RA-treated SH-SY5Y cells (Fig. 1), it fails to attenuate effects of Nogo signalling on neurite outgrowth from rat primary neurons (Fig. 5). Furthermore, although endogenous ecto-CK2 activity has been reported to be associated with neural cells (34), incubation of neural cells with ATP alone failed to inhibit Nogo signalling (Fig. 2). Thus, endogenous ecto-CK2 activity appears to be insufficient to inhibit Nogo signalling. These results suggest that phosphorylation of NgRs could not occur naturally in the CNS, which is consistent with the fact that Nogo signalling can inhibit neurite outgrowth in the CNS (3, 4).

We indicate that extra-cellular treatment with exogenous CK2, instead of BDNF, rescues neurons from inhibition of neurite outgrowth by Nogo signalling (Fig. 5). Extra-cellular treatment with exogenous CK2 phosphorylates serine²⁸¹ in NgR1, which can inhibit binding of Nogo, MAG and OMgp to NgR1 (Fig. 3). The CK2 phosphorylation site in NgR1, serine²⁸¹, is conserved in NgR2, and phosphorylation of NgR2 inhibits binding of MAG (Fig. 4). Although signalling through NgRs is thought to be a main pathway of Nogo signalling, a recent paper indicates that neurons from NgR1^{-/-} mice are still sensitive to inhibition of neurite outgrowth by Nogo signalling (8). This suggests that Nogo signalling could inhibit neurite outgrowth from neurons not only through NgRs but also through unknown Nogo receptors. While depletion of NgR1 fails to rescue neurons from the inhibitory effects of the Nogo-66 fragment (8), extra-cellular treatment with CK2 can (Fig. 5). Therefore, it appears that CK2 treatment must block both NgR-

dependent and any independent unknown pathways for inhibition of neurite outgrowth by the myelin-associated inhibitors, Nogo-66, MAG and OMgp. Since the target site for CK2, serine²⁸¹, is also a target site for PKA (Fig. 4), extra-cellular treatment with PKA, as well as CK2, is useful in blocking Nogo signalling.

5 In summary, we find that BDNF induces phosphorylation of NgR1 in RA-treated SH-SY5Y cells by a CK2-like activity, which overcomes inhibition of neurite outgrowth by Nogo signalling. However, this inhibition of Nogo signalling by BDNF does not occur in rat neurons. Instead of BDNF, extra-cellular treatment with exogenous CK2 rescues neurons from inhibition of neurite outgrowth by Nogo signalling. CK2 phosphorylates ecto-
10 domains of both NgR1 and NgR2, which blocks binding of both MAG to NgR2, and Nogo, MAG and OMgp to NgR1. These results indicate that phosphorylation of Nogo receptors can suppress Nogo signalling. Thus, phosphorylation of Nogo receptors is demonstrated as a novel target for manipulation of Nogo signalling.

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All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described aspects and embodiments of the present invention will be apparent to those skilled in the art without
20 departing from the scope of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the art are intended to be within
25 the scope of the following claims.

CLAIMS

1. A method for alleviating the inhibition of neurite outgrowth from a neurone,
wherein said neurone comprises a Nogo receptor,
5 said method comprising contacting said neurone with a composition capable of
causing phosphorylation of a Nogo receptor, wherein said composition comprises
protein kinase A or casein kinase II.
2. A method according to claim 1 wherein said composition comprises protein
10 kinase A and casein kinase II.
3. A method according to claim 1 or claim 2 wherein said phosphorylation is
phosphorylation of an amino acid residue corresponding to serine 281 of said Nogo
receptor.
15
4. A method according to any of claims 1 to 3 wherein said Nogo receptor is
human NgR1.
5. Use of a protein kinase A polypeptide for the manufacture of a medicament for
20 spinal cord injury.
6. Protein kinase A polypeptide for use in the treatment of spinal cord injury.
7. Use of a caesin kinase II polypeptide for the manufacture of a medicament for
25 spinal cord injury.
8. Caesin kinase II polypeptide for use in the treatment of spinal cord injury.
9. A composition comprising protein kinase A and casein kinase II, for use as a
30 medicament.
10. Use of a composition according to claim 9 for the manufacture of a
medicament for spinal cord injury.
- 35 11. A composition according to claim 9 for use in the treatment of spinal cord injury.
12. Use of a protein kinase A polypeptide or a caesin kinase II polypeptide for the
manufacture of a medicament for causing neurite outgrowth.

13. A protein kinase A polypeptide or a casein kinase II polypeptide for use in causing neurite outgrowth.
- 5 14. A method of treating spinal cord injury in a subject, said method comprising administering to said subject an effective amount of a composition capable of causing phosphorylation of a Nogo receptor, wherein said composition comprises protein kinase A or casein kinase II.
- 10 15. A method according to claim 14 wherein said administration is localised to the site of the injury.

Figure 1

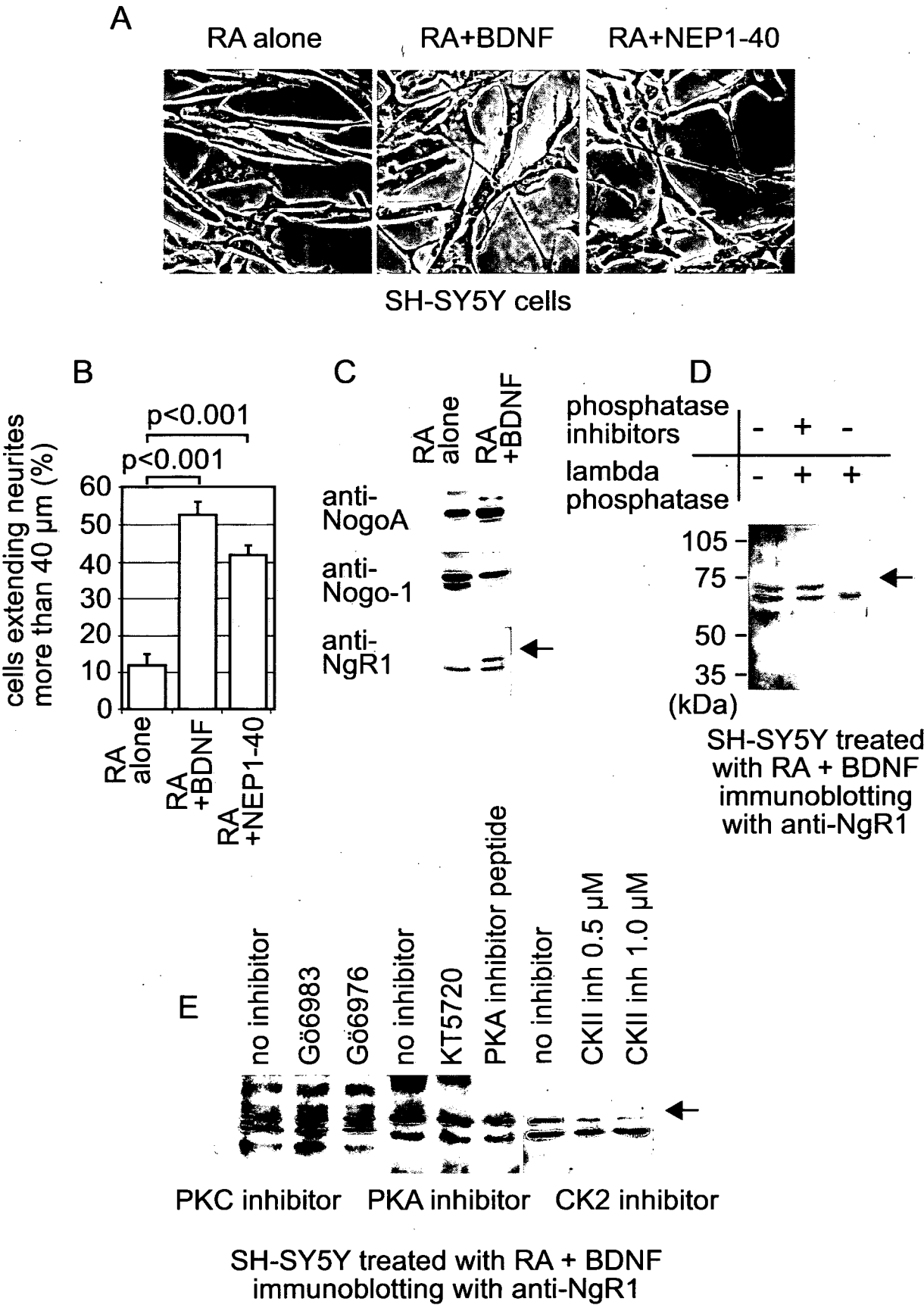


Figure 2

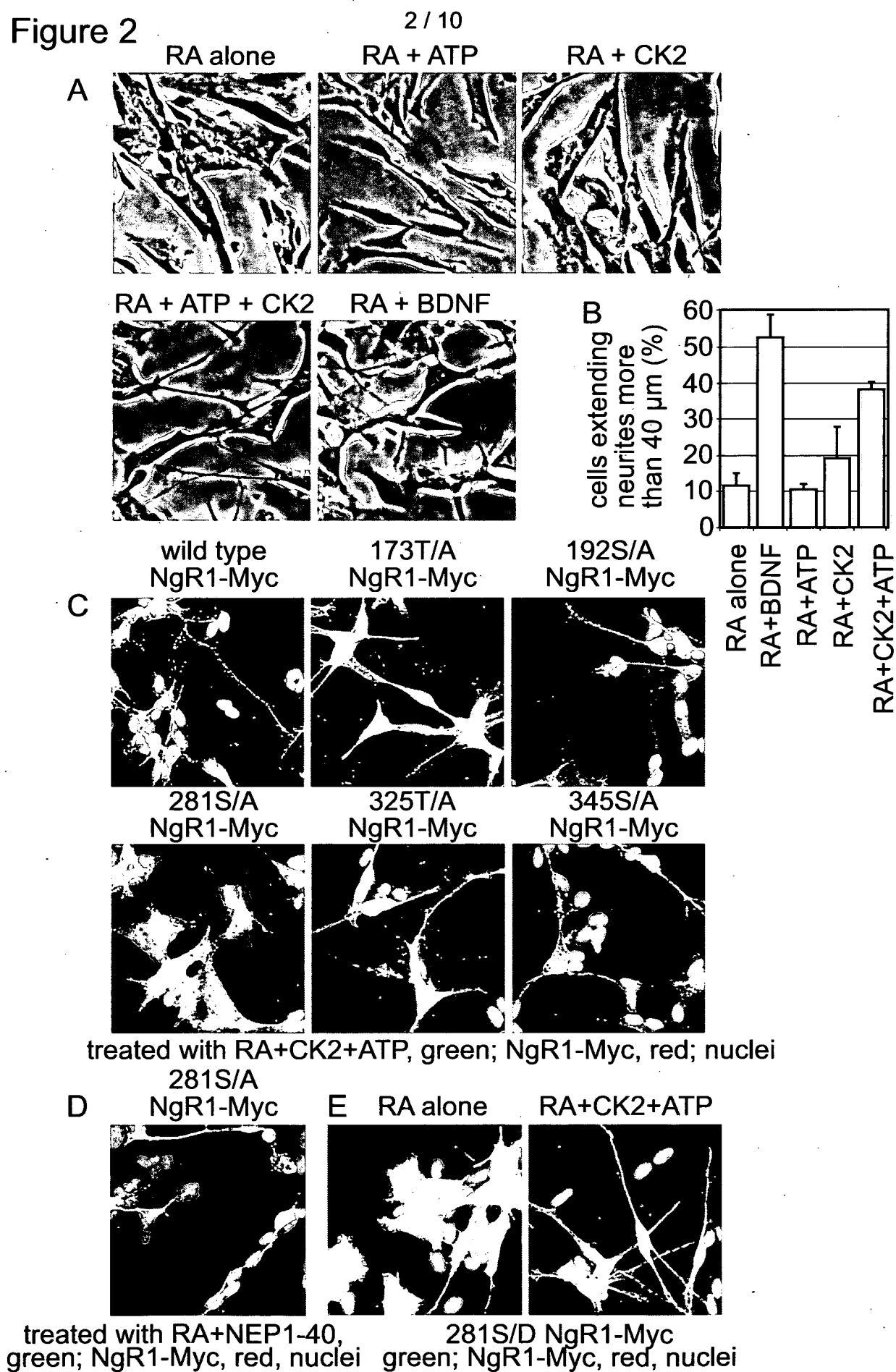


Figure 3

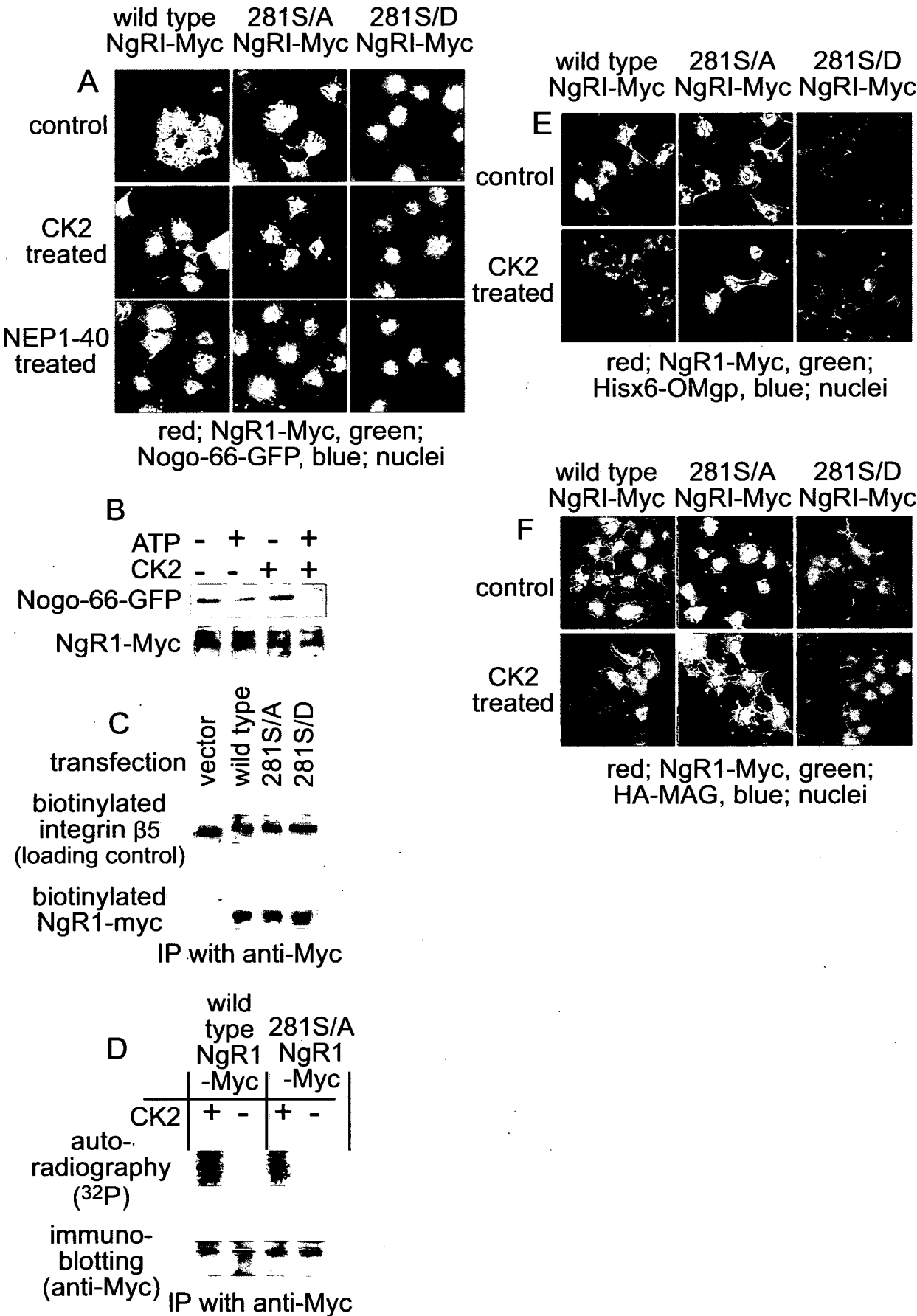


Figure 4

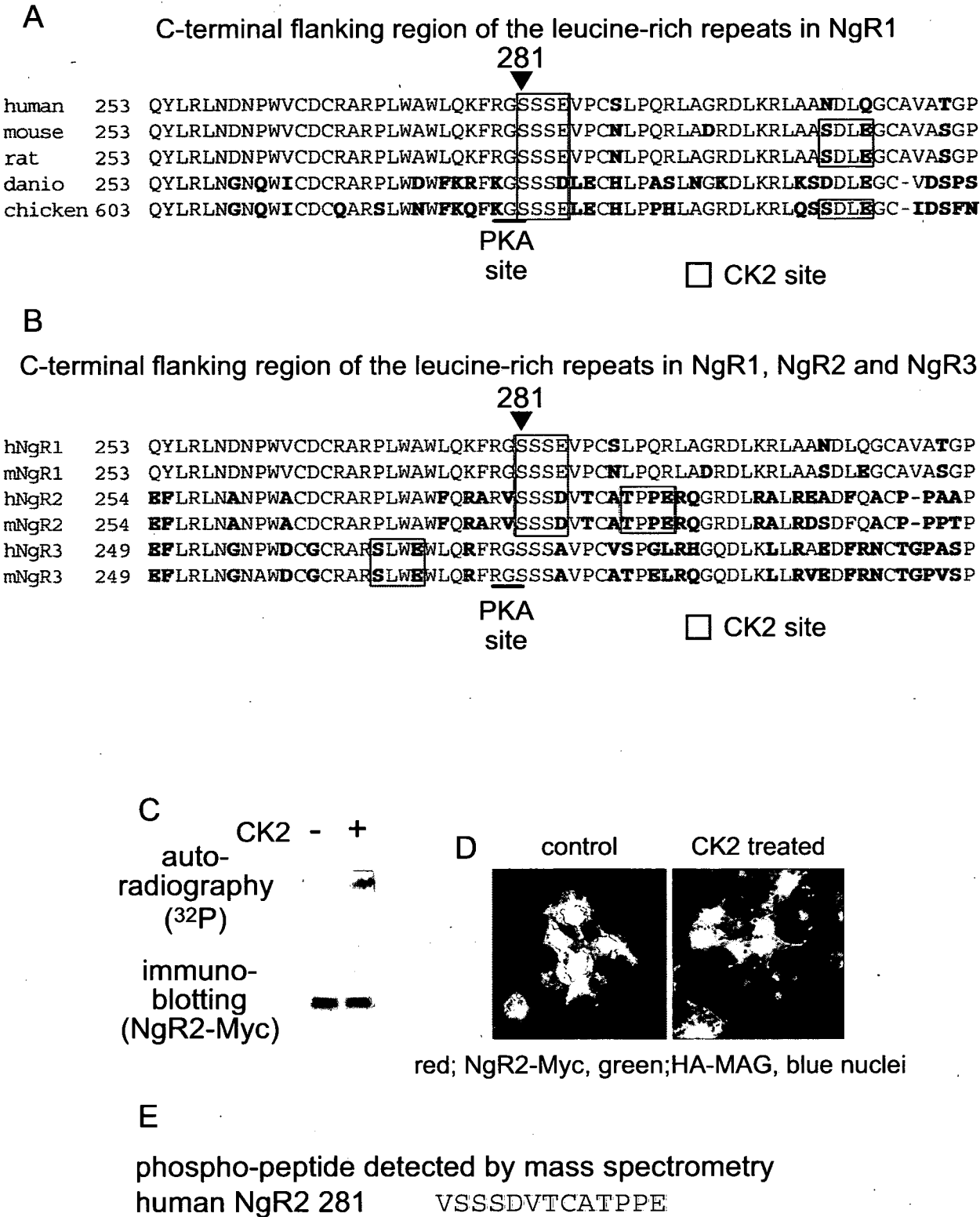


Figure 5

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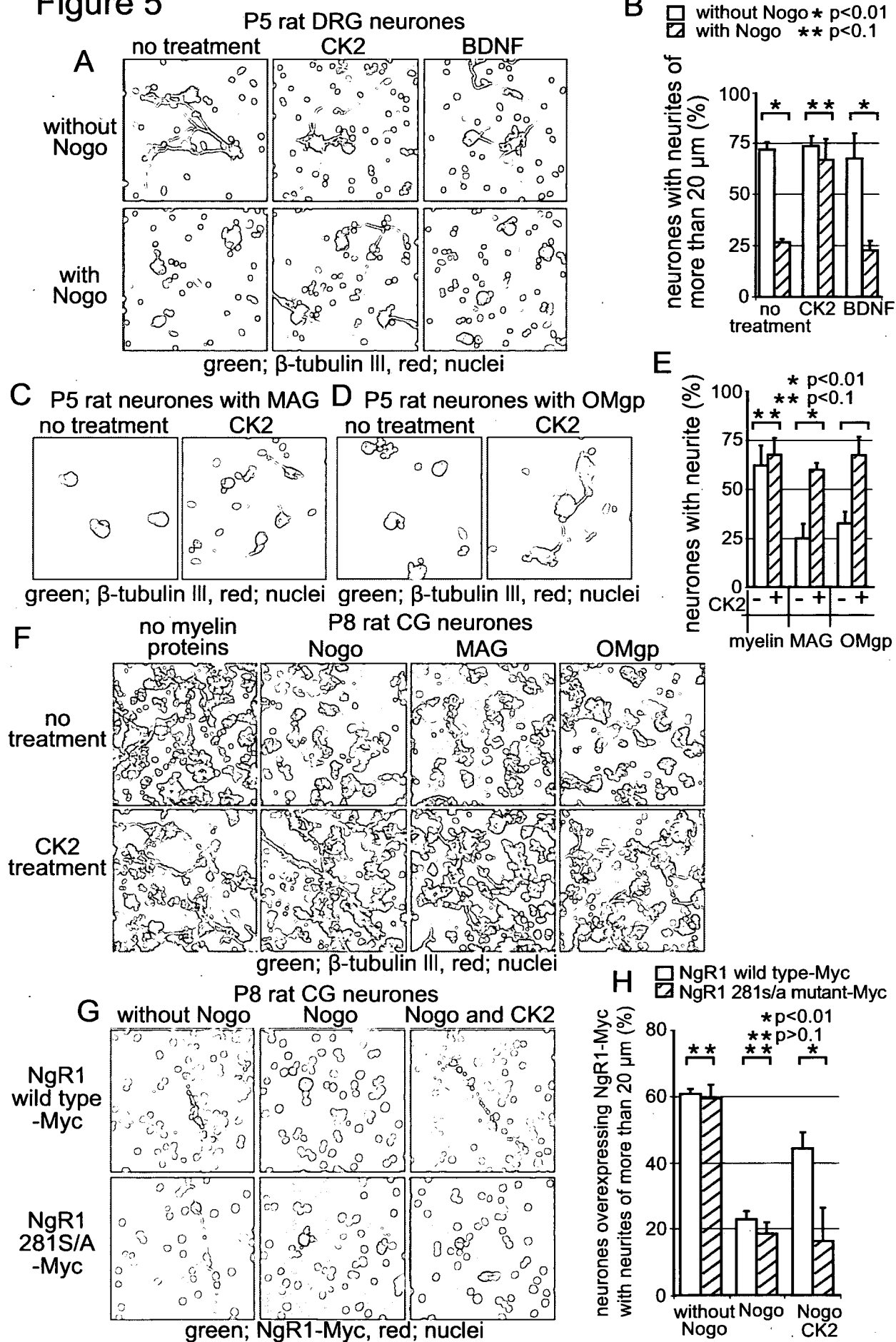


Figure 6

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		NF									
human	1	MKRASAGGSRL LAWVLWLQAWQVA APCPGACVCYNEPKVTTSCPQQGLQAVPVGIPAA SS									
mouse	1	MKRASSGGSRL LAWVLWLQAWRVAT PCPGACVCYNEPKVTTSCPQQGLQAVPTGIPAS SS									
rat	1	MKRASSGGSRL PTWVLWLQAWRVAT PCPGACVCYNEPKVTTSCPQQGLQAVPTGIPAS SS									
danio	1	MK TL LIVEGGRL LCLMF WNLV PV INS CPAK VCYSEPKAT VAC QQQGL FS I PTE IPVRS									
chicken	351	SARS LPEGS KLLILVL C LN IQSEVESCPGACVCYSEPKITIS CQQQGLTA I PTE IPIQS									
		LRR1			LRR2			LRR3			
human	60	QRIFLHG NR ISHVPAAS FRAC RLTILWLHSN V LARIDAAAF TGL AL LE QDLSDNAQL									
mouse	60	QRIFLHG NR ISHVPAAS FQ SCRNL TILWLHSN ALARIDAAAF TGL TL LE QDLSDNAQL									
rat	60	QRIFLHG NR ISHVPAAS FQ SCRNL TILWLHSN ALAGIDAAAF TGL TL LE QDLSDNAQL									
danio	60	QRIFL Q SN KL TV VRSTSF SSV HN LT VLW MY SNN SH IEAGAFYGLERLE E LDIGD NS NL									
chicken	410	QRIFL HN NKIT L VRSTSF TS CRN MTILWI HSNN SL IE PGAF YGLNKLE E LDLSDNTNL									
		LRR3			LRR2			LRR5			
human	119	RSVDP AT FFHGLGRLHTLHLDRCGLQELGPG LFR GLAALQYLYLQDNALQALPDD TFR DL									
mouse	119	HV VDP TT FFHGLG HL HTLHLDRCGL REL GPG LFR GLAALQYLYLQDN NL QALPD N TFRDL									
rat	116	RVVDP TT FRGLG HL HTLHLDRCGLQELGPG LFR GLAALQYLYLQDN NL QALPD N TFRDL									
danio	119	RI I SPTAFRGLTKLHTLHL ERC GLSEL PV G VFR GLFSLQYLYLQDN NL LALHED TFL DL									
chicken	469	KSI NP V TFR GLV HL HTLHLDRCGL MEL STGL FR GLFSLQYLYLQDN NL QNLDD TF IDL									
		LRR5			LRR6			LRR7			LRR8
human	178	GNLTHLFLHG NR IS SV PERAFRGLHSLDRLL LLH QNRVAHVHPAFRDLGRLMTLYLFAN									
mouse	178	GNLTHLFLHG NR IS SV PE HA FRGLHSLDRLL LLH QNHVARVHPAFRDLGRLMTLYLFAN									
rat	178	GNLTHLFLHG NR IS SV PE HA FRGLHSLDRLL LLH QNHVARVHPAFRDLGRLMTLYLFAN									
danio	178	ANLTYLFLHNN KIK V TD HMLRGLVNLDRL LLH QNRIVHV QQQ AFNDLS KL TTLFLF FN									
chicken	528	ANLTYLFLHG NKIK S SE NVFRGLINLDRL LLH QNRVSLVHRRSFHDLGKVM TLY LF FN									
		LRR8			CF						
human	237	NLSALPTEALAPL RAL QYLRLNDNPWVDCRARPLWAWLQKFRGSSSEVP CS LPQRLAG									
mouse	237	NLSM L PAEVL MPL RS LQ YLRLNDNPWVDCRARPLWAWLQKFRGSSSEVP CN LPQRLAD									
rat	237	NLSM L PAEVL VPL RS LQ YLRLNDNPWVDCRARPLWAWLQKFRGSSSEVP CN LPQRLAG									
danio	237	NL TM L G ESMN PLV S LQ YLRLNG NQ WICDCRARPL WD W FK R FK GSSSD LE CHLPAS LN G									
chicken	587	NL TV L G TM AP LV S LQYLRLNG NQ WICDC QARS L WN W FK Q FK GSSSE LE CHLP PH LAG									
		CF									
human	296	RDLKRLAANDLQGC AVAT G YP HP I WTGR-----ATDEEPL----GLPKCCQPDAAD									
mouse	296	RDLKRLAASDLEGC AVAS G FR PIQTSQ-----LTDEELL----SLPKCCQPDAAD									
rat	296	RDLKRLAASDLEGC AVAS G FR PFQTNQ-----LTDEELL----GLPKCCQPDAAD									
danio	296	KDLKRL KSD DL EGC -VD SP SQVQTSIFNSKVHSGKFLSLDDPL--VESIPRCCLSD-ND									
chicken	646	RDLKRL QSS DL EGC -ID S FNQIRTSVFS TK TRSGKLATGSPPLSSH DG SMKCCQPE-MD									
human	343	KASVLEPGRPASAGNALKGRVPPGD T PPGNGSGPRHINDSPFGTLP GS AEPL T AVRPE									
mouse	343	KASVLEPGRPASAGNALKGRVPPGD T PPGNGSGPRHINDSPFGTLP SS AEPL T ALRPG									
rat	343	KASVLEPGRPASVGNALKGRVPPGD T PPGNGSGPRHINDSPFGTLP GS AEPL T ALRPG									
danio	351	KSS II SSKSIPDPSSYNSRQITNNPLKEKENISKTKFREVERTKNETRNKQSLNDG PL G									
chicken	703	KSF I YEAKGKAGPSSSHSRPSSNNPLKDKENMSKTKYVETDPSKNG-SNKQ-INDSPFG									
human	402	GSEPPGF-----PTSGP-RRRPGCSRKNRTRSHCRLGQAGSGGGGTGDS									
mouse	402	GSEPPGL-----PTTGP-RRRPGCSRKNRTRSHCRLGQAGSGASGTGDA									
rat	402	GSEPPGL-----PTTGP-RRRPGCSRKNRTRSHCRLGQAGSGSSGTGDA									
danio	410	TMSNNLDQSLDRIDPELLGNLEPSTAPT KKKK CKSK PK SDQNC LK G-----									
chicken	764	FP I VD P PLTKLR--PEFLEPIEPSTVPTKKRQGC SK KNKSKA Q CRLTQQG-----									
human	445	EGSGALP SL TCSLTPLGLALVLWTVLGPC									
mouse	445	EGSGALP AL ACSLAPLGLALVLWTVLGPC									
rat	445	EGSGALP AL ACSLAPLGLALVLWTVLGPC									
danio	457	HGST-IQVLA----VIFLPLF-WLSLALS									
chicken	810	NSST-LQLSLTSL-LIS P PLV-W S LLLLC									

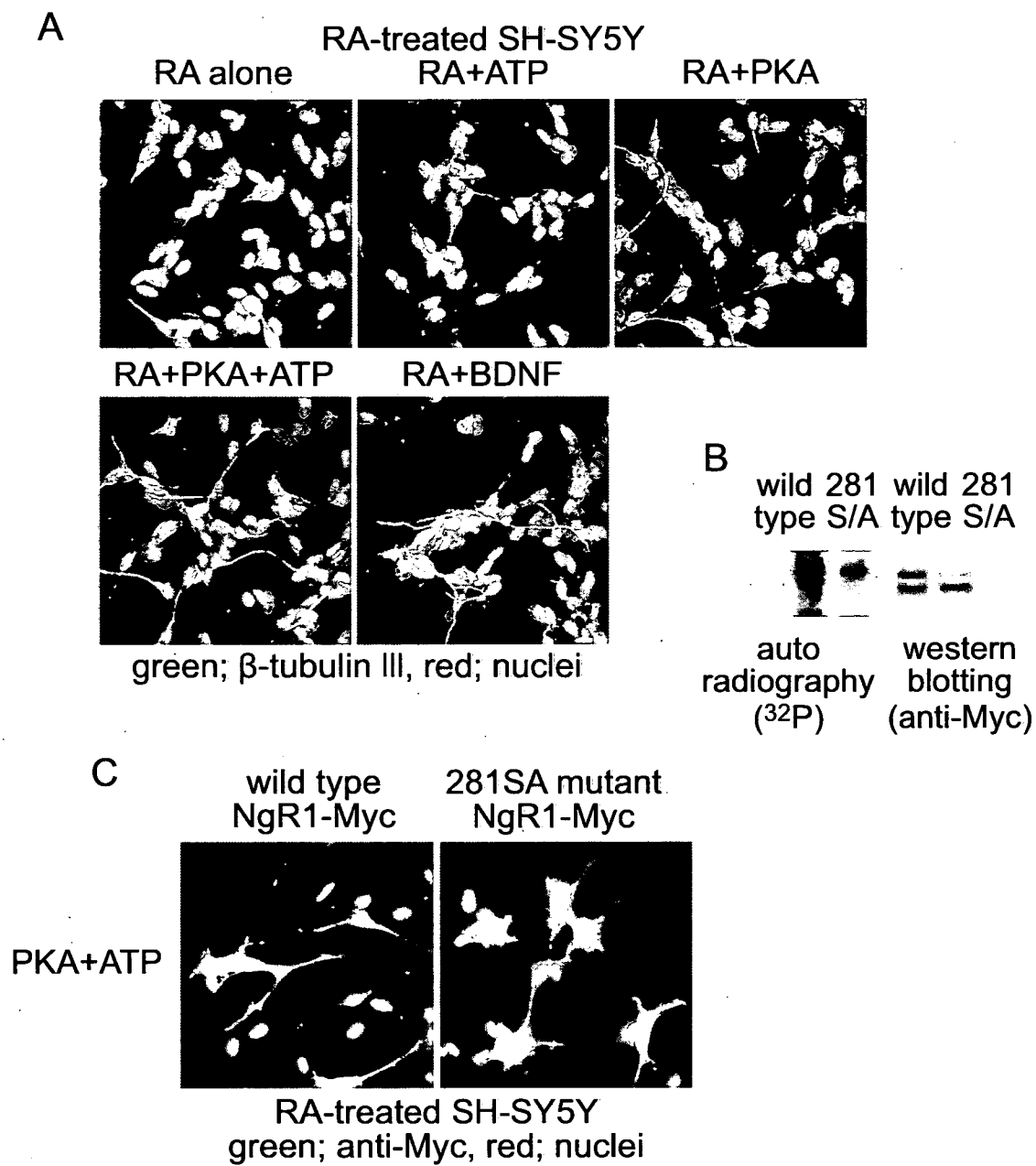
☐ CK2 target site (T/S X X D/E)

rat: AF 462390, human; NM 023004, mouse; NM 022982,
danio; NM 203478, chicken; XM 415292

□ CK2 target site (T/S X X D/E)

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Figure 7



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Figure 8

Ngr1 1 MKRAS---AGGSRL~~LA~~WV~~WL~~QAWQVAAPCPGACVCYNEPKVTTSC**POOGLQAVPVGIP**
 Ngr2 1 MLPGLRRLLQAPASACLL~~LL~~MLLALPLAAPS**CPMLCTCYSSP-PTVSCQANNFSSVPLSLP**
 Ngr3 1 MLR-----KGCCVELLLLVAELPLGGG**CP**RDCVCYPAP-MTVSC**QAHNFAAIPEGIP**

Ngr1 57 AASQRIFL**EG**NRISHVPAASFRACRN**LT**LWLHSNV**LAR**IDAAAF**TGLALLEQLDLSDNA**
 Ngr2 60 PSTQRLFL**QNN**LIRTLRP**GT**FG--SN**LT**LWLFSNN**LT**IYPGTFR**HLQALEELDLGDNR**
 Ngr3 54 VDSERVFL**QNN**RIGLL**QP**GHFS--PAMVTLWIYSNNITYI**HPSTFEGFVHLEELDLGDNR**

Ngr1 117 QLRSD**PA**TF**EG**LGR**LH**TL**HL**DR**CG**L**QE**LGPGLFRGLAALQYLYLQDNAL**QALPDDTFRD**
 Ngr2 118 HLRSL**EP**DT**FQ**GLERL**QS**LHL**YRC**QL**SS**LPGNI**FR**GLVS**LQ**YLYLQ**ENS**LL**HLQDDLFAD**
 Ngr3 112 QLR**TLA**PE**TFQ**GLVKL**HAL**YLYK**CG**L**SAL**PAGV**FG**GL**HS**LQYLYLQDN**HIEYLQDDIFVD**

Ngr1 177 LGNL**TH**LFLHG**NRIS****SVPE**RAFRGL**HS**LD**RLL**LL**HQNR**VAHV**HP**AFRDLGR**LM**TYL**LFAN**
 Ngr2 178 LANL**SH**LFLHG**NR**L**RLL**TE**HV**FRGL**GS**LD**RLL**LL**HGN**R**LQ**GVHRAAFRGL**SRL**TILY**LFNN**
 Ngr3 172 LVNL**SH**LFLHG**NK**LS**LGP**GTFRGLVN**LD**RLL**LLH**EN**QLQ**WV**HKA**FDL**RRL**TT**LF**LFNN

▼

Ngr1 237 NLSALPTEALAPLRAL**QY**LRLNDNPW**V**CD**CR**ARPLWAWLQ**KFRG****SSSE**VPC**SLP**QRLAGR
 Ngr2 238 SLASLPGEALAD**PS**LE**FL**RRLNANPW**A**CD**CR**ARPLWAW**FQ**RARV**SSSD**VT**CAT**PPERQGR
 Ngr3 232 **SLSE**L**QGE**CLAPL**GA**LE**FL**RRLNGNPWD**CG**CRAR**SLWE**WLQ**RFRG**SSSA**VPC**V**SP**GLRH**GQ**

Ngr1 297 DLKRLAAND**LQ**CAVATG**PY**HP**I**WTGRA**TDEE**PLGLPKCC**Q**PDAA**DKA****SVLE**PGRPAS**SAG**
 Ngr2 298 DL**RAL**READ**FQ**ACP-**PAA**PTR-**PG**SRARG**SS**-----SN**LY**GV**AE**A-----**GAP**PAD**PS**
 Ngr3 292 DLKLLRAED**FR**NCTGPAS**PE**Q-**IK**SET**LT****TTD**RAARKE**HS**PHGP**TRS**----**KGH**PHGPR

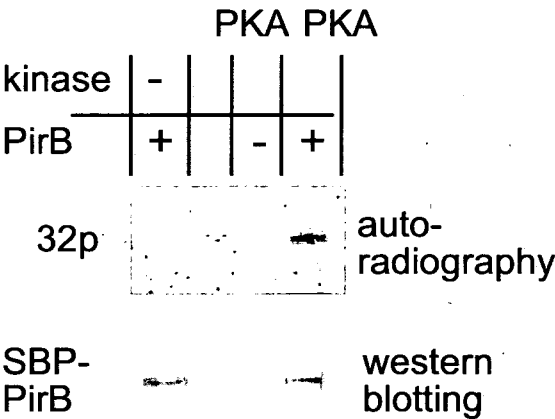
Ngr1 357 NALKGRVPPGDS**PP**GN**GS**CP**RE**INDSP**FG**TL**PG**SAEPPLTAVR**PEG**SEPPGFPT**SG**PRRR
 Ngr2 346 **TL**YR-----DL**PA**EDSRGR**Q**-----G**DA**PT**EDD**YWG**GYG**-----**GED**Q**RG**
 Ngr3 347 **PG**HR----**KPG**K**NCT**N**PR**NR**NQ**ISKAGAG**KQ**AP**EL**PDYAPDY**QH**K**FS**FDI-MPTAR**PKRK**

Ngr1 417 **PG**CS**RK**NRTR**SE**CRL**Q**AG**SG**GGGTGD**SE**GS**AL**PS**LT**CS**LT**PLGLALVLWTVL**GPC**
 Ngr2 382 **EQ**MC**PG**AAC**Q**APPDSRG**P**-----**AL**SAG**LP**SP**LL**CLLL**VP**PH**L**
 Ngr3 402 **GK**CARTPIR**AP**SGV**Q**QA-----**SS**ASS**LG**AS**LL**AWTLGLAVTL**R**

☐ CK2 target site (T/S X X D/E)

Figure 9

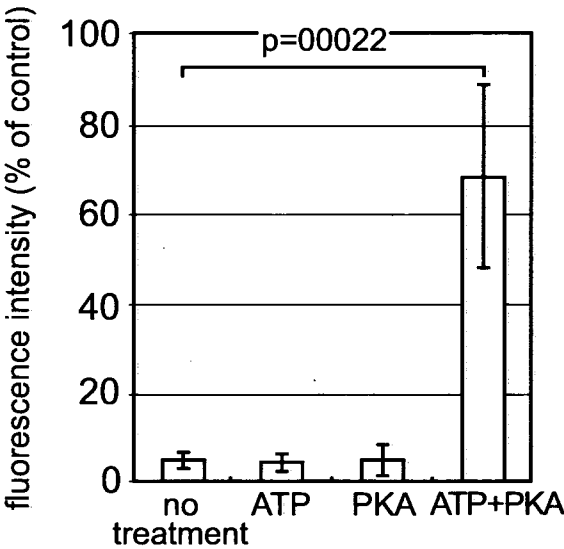
A



B

mPirB 418	SGLSKPSLLTHQGHILDPGMTLTLQCF	NM011095
hLILRB1 220	LGVSKKPSLSVQPGPIVAPEETLTLQCG	NM000669
hLILRB2 219	PGVSKKPSLSVQPGPVVAPGESLTLQCV	NM005874
hLILRB3 219	SGVSRKPSLLTLQGPVLA PGQSLTLQCG	NM001081450
hLILRB5 219	PGVSRKPSLLIPOG SVVARGGSLTLOCR	NM001081442
rPirB 417	SGPSRKPSLLSHQGHILEPGMSLTLOCY	NM001037357

Figure 10B



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Figure 10A

