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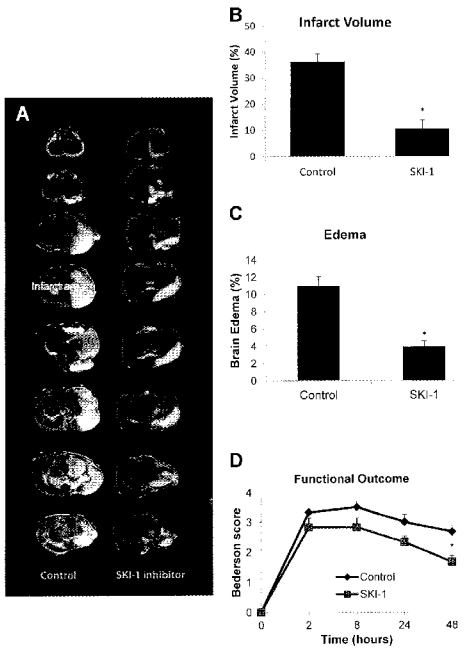
(54) Title: METHODS FOR PROMOTING NEURON SURVIVAL, AXONAL GROWTH AND/OR REGENERATION

Figure 15



(57) Abstract: Methods of stimulating neuron survival, axon growth and/or promoting axon regeneration of a neogenin expressing cell in a subject in need thereof are provided. The methods involve for example, administering to a subject in need thereof a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor. Cleaved RGMa inhibits neogenin mediated axon growth. The SKI-1 inhibitor and/or furin inhibitor inhibit cleavage of RGMa, thereby stimulating neuron survival, axon growth and/or promoting axon regeneration.

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Methods for Promoting Neuron Survival, Axonal Growth and/or Regeneration

Field

[0001] Provided are methods for promoting neuronal survival, axonal growth and/or regeneration and more specifically use of SKI-1 and/or furin inhibitors for promoting axonal growth and/or regeneration.

Introduction

[0002] Axonal outgrowth is precisely orchestrated during development to ensure correct connectivity within the nervous system (Tessier-Lavigne and Goodman, 1996). Surprisingly, however, there are a relatively limited number of known guidance proteins (Thanos and Mey, 2001). Post-translational modification is one strategy to generate multiple activities from a single protein. In theory, complex proteolytic processing with alternate use of multiple specific cleavage sites could dramatically increase diversity (Zisman et al., 2007). Semaphorins, which have several cleavage sites, are one example (Adams et al., 2007), but the extent to which this strategy is utilized and the effects on activity, receptor specificity, and/or short (membrane –bound) versus long-range (soluble) effects are largely unknown.

[0003] The GPI- anchored protein RGMa is key to the development of various projections within the CNS and is thought to act solely as a membrane-bound protein (Monnier et al., 2002). Its activity is similar to that of the ephrins, as it inhibits retinal ganglion cell (RGC) outgrowth (Monnier et al., 2002). RGMa acts through the transmembrane receptor Neogenin, which is expressed in a high-temporal low-nasal gradient in RGC axons (Rajagopalan et al., 2004). In vitro, temporal axons that express Neogenin avoid RGMa-expressing cells, whereas Neogenin-deficient nasal axons are not affected by RGMa (Rajagopalan et al., 2004). In vivo, perturbation of the RGMa gradient in the optic tectum causes pathfinding mistakes for temporal fibers (Matsunaga et al., 2006). Thus, Neogenin and RGMa provide positional information for retinal axons invading the tectum. Besides its contribution to the establishment of the neuronal architecture, RGMa is a major impediment to neuronal regeneration. Strikingly, antibodies that block RGMa activity promote functional recovery after spinal cord injury (Hata et al., 2006). Thus, to optimize therapeutics that promote axonal regeneration it is critical to understand how RGMa contributes to the non-permissive regenerative milieu of the CNS.

SUMMARY

[0004] In an embodiment, the disclosure includes a method of inhibiting RGMa cleavage comprising contacting the RGMa with a proprotein convertase (PPC) inhibitor, optionally a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor and/or a furin inhibitor.

[0005] In an embodiment, disclosure includes a method for inhibiting RGMa cleavage at RTFT↓D (amino acid 175 in human) comprising contacting the RGMa with a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor.

[0006] In an embodiment, inhibition of RGMa cleavage at RTFT↓D (amino acid 175 in human) inhibits formation of RGMa37.

[0007] In another embodiment, the SKI-1 inhibitor is a serine protease inhibitor, optionally a membrane permeable serine protease inhibitor.

[0008] In yet a further embodiment, the RGMa is attached to a cell and the serine protease inhibitor is a membrane permeable serine protease inhibitor.

[0009] In an embodiment, the disclosure includes a method of inhibiting soluble NRGMa or NNRGMa release from a cell comprising contacting the cell with a PPC inhibitor such as a SKI-1 inhibitor and/or a furin inhibitor.

[0010] In an embodiment, the inhibitor is a membrane permeable serine protease inhibitor.

[0011] In yet another embodiment, the PPC inhibitor is selected from a membrane permeable serine protease inhibitor, optionally AEBSF, an ER permeable serine protease inhibitor and RVKR peptide.

[0012] In a further embodiment, the SKI-1 inhibitor is selected from RRLL peptide (SEQ ID NO:4), PF-429242, peptide CIYISRRLLC (SEQ ID NO:5; optionally with terminal "C" residues cyclized) and/or prosegment inhibitor R134E.

[0013] Another aspect includes a method for stimulating neuron survival, axon growth and/or promoting neuron axon regeneration of a neural cell in a subject in need thereof, the method comprising: providing neural tissue with a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor.

[0014] In an embodment, the furin inhibitor is a furin prosegment inhibitor.

[0015] In an embodiment, the method is for inducing long range neuron survival, axon growth and/or regeneration.

[0016] In an embodiment, the neural cell is provided with the SKI-1 inhibitor and/or the furin inhibitor by administering the inhibitor to the subject. In an embodiment, the subject has Multiple sclerosis.

[0017] In another embodiment, the subject has a nerve injury. In an embodiment, the nerve injury is a spinal cord injury.

[0018] In another embodiment, the subject is suffering or has suffered an ischemic neuronal injury.

[0019] In a further embodiment, the subject has glaucoma, Alzheimer's disease, Parkinson's disease and/or a CNS immune response.

[0020] Also provided in another aspect is a method for stimulating neuron survival, axon growth and/or regeneration in a subject in need thereof, the method comprising administering to the subject a PPC inhibitor, optionally a SKI-1 and/or Furin inhibitor, in sufficient amount to promote neuron survival, axon growth and/or regeneration of the neuron.

[0021] A further aspect includes treating nerve damage comprising administering to a subject in need thereof a composition comprising a PPC inhibitor such as a SKI-1 inhibitor and/or a Furin inhibitor.

[0022] In an embodiment, the nerve damage is a neurodegenerative disease. In an embodiment, the neurodegenerative disease is multiple sclerosis or glaucoma. In a further embodiment, the nerve damage is a spinal cord injury, ischemic injury such as a stroke or CNS immune injury.

[0023] In an embodiment, the damaged nerve comprises a sensory neuron or a motor neuron.

[0024] In a further embodiment, the method is for inhibiting an immune response in CNS tissues, e.g. brain and spinal cord in multiple sclerosis patients comprising administering a PPC inhibitor such as a SKI-1 inhibitor and/or a furin inhibitor.

[0025] In an embodiment, the method is for inhibiting demyelination in a subject in need thereof comprising administering a SKI-1 inhibitor and/or a furin inhibitor.

[0026] In an embodiment, the SKI-1 inhibitor and/or the Furin inhibitor is administered to the lesion site directly. In another embodiment the inhibitor is administered systemically.

[0027] In an embodiment, the PPC inhibitor is selected from a membrane permeable serine protease inhibitor, optionally AEBSF, an ER permeable serine protease inhibitor and RVKR peptide.

[0028] In yet another embodiment, the SKI-1 inhibitor is selected from RRLL peptide (SEQ ID NO: 4), PF-429242, peptide CIYISRRLLC (SEQ ID NO: 5; optionally with terminal "C" residues cyclized) and/or prosegment inhibitor R134E.

[0029] In another embodiment, the inhibitor(s) and/or composition is/are administered by intravenous, intraspinal and/or intracranial infusion. In another embodiment he inhibitor(s) and/or composition is/are administered by Intraperitoneal and/or intrathecal application.

[0030] A further aspect is an isolated polypeptide comprising: a RGMa fragment selected from or corresponding to:

a) a RGMa fragment no longer than amino acid 1 to 168 of human RGMa ($RGMa_{1-168}$), optionally no longer than amino acid 47 to 168,

b) a RGMa fragment no longer than amino acid 1 to 127 of human RGMa ($RGMa_{1-127}$), optionally no longer than amino acid 47 to 127,

c) a RGMa fragment no longer than amino acid 1 to 133 of human RGMa ($RGMa_{1-133}$), optionally no longer than amino acid 47 to 133,

d) a RGMa fragment no longer than amino acid 1 to 175 of human RGMa ($RGMa_{1-175}$), optionally no longer than amino acid 47 to 175;

e) a RGMa fragment no longer than amino acid 128 to 175 of human RGMa ($RGMa_{128-175}$)

f) a RGMa fragment no longer than amino acid 169 to 450 of human RGMa ($RGMa_{169-450}$),

g) a RGMa fragment no longer than amino acid 176 to 450 of human RGMa ($RGMa_{176-450}$);

h) a RGMa fragment no longer than amino acid 128 to 450 of human RGMa ($RGMa_{128-450}$)

i) a RGMa fragment no longer than amino acid 201-290 of human RGMa($RGMa_{201-290}$),

j) corresponding species fragments of anyone of a) to i), and/or

k) conservative variants of any one of a) to j).

[0031] In an embodiment, the $RGMa_{1-168}$ consists of: residues 1 to 168 of SEQ ID NO: 1 or residues 47 to 168 of SEQ ID NO: 1; $RGMa_{1-175}$ consists of: residues 1 to 175 of SEQ ID NO: 1 or residues 47 to 175 of SEQ ID NO: 1 or fragments thereof, $RGMa_{1-133}$ consists of residues 1 to 133 of SEQ ID NO: 1 or residues 47 to 133 of SEQ ID NO: 1; $RGMa_{169-450}$ consists of residues 169 to 450 of SEQ ID NO: 1; $RGMa_{176-450}$ consists of residues 176 to 450 of SEQ ID NO: 1; $RGMa_{128-450}$ consists of residues 128to 450 of SEQ ID NO: 1; or $RGMa_{201-290}$ consists of residues 201 to 290 of SEQ ID NO: 1, and/or a conservative variant thereof.

[0032] In an embodiment, the fragment is $RGMa_{1-168}$, $RGMa_{1-133}$ and/or $RGMa_{201-290}$ and/or a corresponding species fragment and/or a conservative variant thereof, wherein the conservative variant retains the ability to bind neogenin.

[0033] In yet a further embodiment, the isolated polypeptide comprises RGMa128-175 in combination with a second fragment of RGMa, optionally no longer than amino acids 169 to 450 of human RGMa (RGMa169-450) wherein the isolated polypeptide is combined with the second fragment via a disulphide bridge forming a species that is approximately 37kDa when separated electrophoretically on a non-reducing agarose gel.

[0034] In an embodiment, the isolated polypeptide is glycosylated and/or further comprises a GPI anchor.

[0035] In an embodiment, the isolated polypeptide comprises one or more of a linker peptide and a tag such as a FLAG®-tag.

[0036] Also provided in another aspect is an antibody that specifically binds an epitope comprising amino acid 175 of SEQ ID NO: 1 and/or blocks cleavage of RGMa at amino acid 175 by SKI-1 or minds a D168 or H170 mutant. In an embodiment, the isolated peptide or the antibody is attached to a substrate.

[0037] A further aspect includes a composition or kit comprising an isolated polypeptide, inhibitor or the antibody described herein, optionally for use in a method described herein.

[0038] Other features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the disclosure are given by

way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

Brief description of the drawings

[0039] An embodiment of the disclosure will now be described in relation to the drawings in which:

[0040] Figure 1. Analysis of RGMa processing. (A) Schematic representation of the 7 RGMa peptides identified in the analysis. In membranes under reducing (+DTT) or non-reducing (-DTT) conditions, an anti C-RGMa revealed 4 RGMa fragments. In cell supernatant (-DTT), an anti-N-RGMa revealed that 3 RGMa fragments are released. Molecular weights are indicated on the right; UG unglycosylated. Black arrow head indicates autocatalytic cleavage site, black arrow represents known shedding cleavage site. Gray arrows indicate new calculated cleavage sites. (B) Western blots with an anti-C-RGMa on membranes from RGMa transfected COS-7 cells, the developing chick brain, and RGMa transfected HEK-293 cells. Analysis was performed under non-reducing conditions (-DTT) to preserve the disulfide bridge or under reducing conditions (+DTT). Under non-reducing conditions, one band is visible in COS-7 cells (60 kDa), whereas 3 bands (33 kDa, 37 kDa and 60 kDa) are visible in chick brain and HEK-293. Under reducing conditions 2 bands (33 kDa and 60 kDa) are visible in membranes. This revealed 4 membrane bound RGMa proteins in chick brain (represented in A; see also Figure 2). (C) Western blots (-DTT) performed with an anti-N-RGMa on brain matrix extracts (urea 3M) revealed a 30 kDa band. In supernatants from RGMa transfected HEK cells, 2 bands at 60 kDa and 30 kDa are apparent. PNGaseF (NGase) treatment to remove N-glycosylations (UG) reduced the 60 kDa to a 45 kDa band and the 30 kDa to 22- and 18 KDa bands(arrow heads) (represented in A). (D, E) Temporal retinal explants cultured on supernatants from cells transfected with control (Mock, D) or RGMa (E). Bar, 200µm. (F) Average axonal length was significantly shorter on supernatants from RGMa transfected cells vs control (Mock; $^{**}p<0.0005$). Data are average ± SEM from 3 independent experiments.

[0041] Figure 2. RGMa is processed differentially in SH-SY5Y and DF1 cells. Western Blots with an anti-C-RGMa on membranes from, RGMa transfected SH-5YSY-, and DF1-cells. Analysis was performed under non-reducing conditions (-DTT) to preserve the disulfide bridge or under reducing conditions (+DTT). RGMa transfected SH-SY5Y cell membranes displayed the same pattern as brain membrane with 3 fragments at 60kDa, 37kDa and 33 kDa under non reducing conditions and 2 bands at 33kDa and 60kDa under reducing conditions. In Western blots performed with membranes from RGMa transfected DF1 cells, one band was apparent at 60kDa under non

reducing conditions, whereas under reducing conditions 2 bands at 33kDa and 60kDa were apparent. Thus, in DF1 cells RGMa processing is similar to the one observed in COS-7 cells. Molecular weights are indicated at the left of each gel. In cell supernatants, anti-C-RGMa recognized only a 60kDa band. HEK cell supernatants were transfected with RGMa and supernatants were blotted with an anti-C-RGMa. Only the full length 60kDa band is apparent.

[0042] Figure 3. Furin and SKI-1 process RGMa and are involved in pathfinding. (A) RGMa expressing cells were treated with the protease inhibitors AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) or RVKR (SEQ ID NO: 3), or DMSO (control) and membranes blotted. AEBSF and RVKR both strongly reduced RGMa cleavages, shown by the reduction of the 33 and 37 kDa bands (Asterisks) (see also Figures 4A,B). (B) RGMa expressing cells were treated with RVKR or RRLL (SEQ ID NO:4) protease inhibitors or DMSO (control). Blots on membranes under reducing (+DTT) and non-reducing (-DTT) conditions show that RVKR inhibits the two cleavage events (Asterisks) whereas the SKI-1 inhibitor RRLL inhibited generation of the 33kDa protein only (Asterisk). In the presence of DTT a 33kDa band appears indicating that auto-catalytic cleavage is not sensitive to treatments. (C) RGMa was co-transfected with R134E or empty plasmid (control). In membranes, R134E strongly reduced formation of the 33 kDa protein (Asterisk), confirming that it is the result of SKI-1 activity. (D) RGMa was co-transfected with ppFurin or empty plasmid (Ctrl). ppFurin strongly reduced formation of the 37 kDa fragment, confirming that it results from Furin activity (see also Figure 4). (E) RGMa expressing cells were treated with DMSO (control) or RVKR and supernatants were blotted with anti-N-RGMa. RVKR suppressed release of N-terminal proteins (Asterisk). F-H) *In situ* analysis demonstrated that Furin and SKI-1 are expressed in the tectum in RGMa expressing radial glial cells (arrows). Controls (sense) did not show any staining (H). (I) Injection of empty virus did not induce axonal phenotypes and all axons established terminal arbors within the Terminal Zone (TZ). (J) Ectopic expression of a SKI-1 pro-form (R134E) induced axonal phenotypes with numerous fibers establishing arbors outside the TZ. (K) Ectopic expression of ppFurin to neutralize Furin induced axonal phenotypes with numerous fibers establishing arbors outside the TZ (arrow heads).

[0043] Figure 4.RGMa is processed by Proprotein Convertase Furin between the ER and the Golgi. A) RGMa was expressed overnight in HEK cells in the presence of protease inhibitors. Membrane preparations were submitted to Western Blot analyses in the absence of DTT to preserve the disulfide bridge. This showed that, none of the inhibitor tested led to a reduction in RGMa cleavage and the intensity of the 33- and 37 kDa bands remained the same as in the control. The name of the inhibitor is indicated for each line. B) Soluble RGMa was treated for 3 hours with purified Furin (10 U for 3 hours) and blotted using an anti-C-RGMa antibody. As expected this

treatment led to the formation of a 37 kDa band, indicating that Furin cleaves RGMa within its N-terminal portion. C) RGMa was expressed in HEK cells in the presence of Brefeldin A and Golgicide A. In the presence of these inhibitors, RGMa was still processed by HEK cells (presence of the 33 and 37 kDa bands) indicating that RGMa processing occurs before it enters the Golgi.

[0044] Figure 5. RGMa Cleavage Is Required for RGMa Inhibition through Neogenin(A) RGMa proteins tested.(B) Expression of Neogenin-AP (Neo-AP) and RGMa constructs in COS-7 and HEK293 cells. The introduction of a single mutation (H151A, D149A) next to autocatalytic cleavage site (aa 150) abolished cleavage in COS-7 cells. The same mutations abolished all cleavage events in HEK293 cells.(C) Microtiter plates were coated with transfected COS-7-membranes and Neogenin-AP binding studied. Binding is abolished in noncleavable mutants (D149A, H151A) versus wtRGMa (wt; see also Figures S3A–S3C).(D) Temporal explants grown on Mock, wtRGMa (wt), and noncleavable mutants (D149A; H151A) membranes. Axonal growth is reduced on wtRGMa versus Mock and mutants. Bar, 150 μm.(E) In four independent experiments, mutation of the cleavage site abolished RGMa inhibition on temporal axons ($^{**}p < 0.05$). Nasal axons that express less Neogenin were less inhibited.(F) NIE-115 cells were grown on Mock, wtRGMa (wt), D149A, and H151A membranes, after transfection with GFP plasmid plus a control shRNA (Ctrl-shRNA) or a Neogenin shRNA (Neo-shRNA). NIE-115 cells transfected with Ctrl-shRNA extend shorter neurites on wtRGMa versus Mock, D149A, and H151A. Bar, 30 μm.(G) D149A and H151A significantly increased length ($^{**}p < 0.001$) compared to wtRGMa. Neogenin silencing with Neo-shRNA suppressed wtRGMa inhibition. All data are average ± SEM from three independent experiments (see also Figure 8). (H) Ectopic expression of RGMa induced axonal phenotypes with numerous fibers establishing arbors outside the Terminal Zone (TZ; arrowheads).(I) Ectopic expression of H151A did not induce axonal phenotypes and all axons established terminal arbors within the TZ.

[0045] Figure 6.**A-C**) Cell surface localization of RGMa constructs in COS-7 cells. COS-7 cells were transfected with RGMa mutant proteins and RGMa localization was studied with an anti-C-RGMa antibody. Staining was done after paraformaldehyde fixation and without treatment with detergent to stain only cell surface proteins. (**A,B**) As indicated by the strong cell surface staining, the two cleavage mutant proteins H151A and D149A localize at the cell surface. (**C**) Control experiments in which Mock transfected cells were stained only show background staining. **D**) Western Blot analysis of Noegenin silencing in NIE-115 cells that were transfected with a control-(Ctrl.shRNA) or a Neogenin- shRNA (Neo.shRNA) was performed. Analysis with an anti-Neogenin antibody revealed a lower Neogenin amount in Neo.shRNA treated cells.

[0046] Figure 7. C-RGMa induces Neogenin mediated inhibition. (A) Representation of RGMa with a TEV site. (B) Under non-reducing conditions, RGMa appears as a 60 kDa band, whereas RGMa-TEV is cleaved by TEV and only C-RGMa (33kDa) is apparent. (C,D) Neogenin-AP binds saturably to wtRGMa (C) and C-RGMa (D). Binding of Neogenin–AP or RGMa-AP to microtiter wells coated with C-RGMa and wtRGMa membranes. Neogenin–AP binding to BSA was less than 5% of these levels. Calculated *Kd* of Neogenin-AP is indicated. Data from 6 determinations. (E) Scatchard of Neogenin-AP binding (see also Figure 8). (F) Temporal-axon growth was reduced on wtRGMa and C-RGMa membranes vs Mock. Bar, 150µm. (G) C-RGMa and wtRGMa significantly reduced length (**p<0.001) of temporal axons. Nasal axons were inhibited to a lesser extent. Data are average ± SEM from 4 independent experiments. (H) NIE-115 cells transfected with Ctrl-shRNA+ GFP extend shorter neurites on C-RGMa vs Mock. Transfection of Neo-shRNA+ GFP restored growth. Bar, 30µm. (I) C-RGMa and wtRGMa reduced NIE-115-neurite length (**p<0.0001) in Ctrl-shRNA cells. Neo-shRNA suppressed this inhibition. Data are average ± SEM from 3 independent experiments. (J) Binding study of RGMas to Neogenin. RGMas were expressed as AP fusion proteins and tested in ELISA plates coated with the extracellular domain of Neogenin (binding; -, base line; ++, > 5 x base line; +++, > 10 x base line). (K) Supernatants (Sup.) from cells transfected with C-RGMa$^{182-271}$ were pulled down (IP) by Neogenin- but not BSA- coated beads. (L) C-RGMa$^{182-271}$ reduced axonal length (**p<0.005). Nasal axons were less inhibited. Data are average ± SEM from 3 independent experiments. (M-O) Ectopic expression of RGMa$^{182-271}$ induced pathfinding errors in the visual pathway. In control experiments (M), all the fibers targeted the terminal zone (TZ), whereas in RGMa$^{182-248}$ experiments fibers displayed aberrant paths (arrows) and connections (arrow heads) outside the TZ (N,O).

[0047] Figure 8: The C-terminal part of RGMa, expression and function. **A)** Cell surface localization of RGMa-TEV constructs in COS-7 cells. COS-7 cells were transfected with RGMa-TEV and its localization was studied with an anti-C-RGMa antibody. Staining was done after paraformaldehyde fixation and without treatment with detergent to stain only cell surface proteins. As indicated by the strong cell surface staining, RGMa-TEV localized at the cell surface. **B)** The C-RGMa$^{182-271}$ protein inhibits axonal growth. Temporal axons were cultured on laminin or laminin + C-RGMa$^{182-271}$. The presence of C-RGMa$^{182-271}$ strongly reduced axonal growth. **C-F**) *In vivo* expression of C-RGMa$^{182-271}$ fragments was cloned as His-Tag proteins in RCAS viral vector to infect E1.5 chick developing tecta. At E10, tecta were removed, fixed and sections stained with an anti-His antibody. (**B,D**) DAPI staining of the immunostaining presented in the right panels. (**C**) Sections from Mock (empty RCAS) infected embryos displayed background staining. (**E**) Sections

from animals infected with RCAS constructs that expressed C-RGMa$^{182-271}$ showed a strong staining, which indicated that this fragment was expressed in the chick tecta.

[0048] Figure 9. Soluble RGMa proteins display Neogenin dependent inhibition. (A) RGMa proteins tested. (B) Temporal explants on laminin, laminin+5µg/ml N-RGMa, 2.5µg/ml NN-RGMa, and 10µg/ml RGMaΔ. The 3 RGMas inhibited growth. Bar, 200µm. (C) RGMa proteins significantly decreased growth (**p<0.0001). Proteins displayed a concentration dependent effect, NN-RGMa having the strongest effect. Nasal axons were less inhibited. Data are average ± SEM from 4 independent experiments. Bar, 150µm (D) NIE-115 cells on laminin, laminin+5 µg/ml NN-RGMa, with control shRNA (Ctrl-shRNA)+GFP or a Neogenin shRNA (Neo-shRNA) +GFP. Cells transfected with Ctrl-shRNA extended shorter neurites on laminin+NN-RGMa, compared to Laminin. Neo-shRNA restored growth. Bar, 40µm. (E) RGMaΔ, N-RGMa, and NN-RGMa inhibited growth. Neo-shRNA increased the average neurite-length on N-RGMa, NN-RGMa, and RGMaΔ, when compared to Ctrl-shRNA (**p<0.05). Data are average ± SEM from 4 independent experiments. (F) Supernatants (Sup.) from cells transfected with N-RGMa or NN-RGMa were pulled down (IP) by Neogenin- but not BSA- coated beads. (G,H,I) Neogenin-AP (2 µg/well) bound saturably to purified RGMaΔ (G), N-RGMa (H), and NN-RGMa (I). RGMa-AP (2 µg/well) did not bind to RGMa proteins. Neogenin–AP binding to BSA was less than 5% of these levels. *Kd* of Neogenin-AP is indicated. (J) Scatchard plot of RGMa proteins binding. Data are average ± SEM from 4–8 determinations. (see also Figure 10).

[0049] Figure 10. **A**) RGMa and Neogenin soluble proteins used in our studies. RGMa and Neogenin constructs were cloned with an His tag, expressed in COS-7 cells, purified on Nickel agarose, and separated in SDS-PAGE electrophoresis before staining with coomassie. All proteins used in our studies had an apparent purity of >90% after coomassie staining. RGMaΔ (full length soluble RGMa) was loaded under non-reducing and reducing conditions to demonstrate that the two N- and C-fragments are linked by a disulfide bridge. All other proteins were loaded under reducing conditions. Molecular weights are indicated in the left. **B-C**) N-RGMa-AP (**B**) NN-RGMa-AP (**C**) binding to Neogenin. Microtiter wells were coated with purified extracellular domain of Neogenin at concentration from 0nM to 80nM. Binding of N-RGMa–AP (C; 2 µg/well) or NN-RGMa-AP (D; 2 µg/well) to the coated wells were assessed after incubation at 20 °C for 3 h. Binding of both N-RGMa–AP and NN-RGMa-AP to BSA-coated wells was less than 5% of these levels. Calculated affinity of N-RGMa-AP and NN-RGMa-AP for the ectodomain of Neogenin is indicated on the graph. NN-RGMa displayed a much higher affinity to Neogenin when compared to N-RGMa. Data are the mean ± SEM from 6 determinations.

[0050] Figure 11. Over-expression of soluble RGMa proteins perturbs axonal pathfinding in the embryonic chicken visual system. At embryonic day 1.5 (E1.5), RCAS-virus (control) and RCAS-RGMa (positive control), RCAS-N-RGMa, RCAS-NN-RGMa were injected into the developing optic tectum. At E15, a DiI crystal was placed in the temporal retina to label fibers. (A) In control experiments, all axons converge towards a well-defined terminal zone (TZ). (B-D) Infections with RGMa, N-RGMa and NN-RGMa expressing RCAS-virus, induced i) the absence of terminal zone, ii) the presence of ectopic anterior terminations, iii) aberrant turns and ectopic posterior terminations. All errors are indicated by arrow heads. The insets represent a drawing of the flat-mounted retina to indicate the location of the DiI crystal in a dorso-temporal position of the retina and the path of axons towards the optic fissure. t, temporal; d, dorsal; v, ventral; n, nasal. The hypothetical TZ is represented in each panel (see also Figure 12). (E) Quantification of axonal phenotypes. -TZ= no terminal zone; AT= ectopic anterior terminations; PT= ectopic posterior terminations; Ab= aberrant turns.

[0051] Figure 12. *In vivo* expression of N-RGMa and NN-RGMa: N-RGMa and NN-RGMa fragments were cloned as His-Tag proteins in RCAS viral vector to infect E1.5 chick developing tecta. At E10, tecta were removed, fixed and sections stained with an anti-His antibody. **A,C,E**) DAPI staining of the immunostaining presented in the right panels. (**B**) Sections from Mock (empty RCAS) infected embryos displayed background staining. (**D,F**) Sections from animals infected with RCAS constructs that express N-RGMa (**D**) and NN-RGMa (**F**) showed a strong staining, which indicated that N-RGMa fragments were expressed in the chick tecta. All proteins expressed in vivo were cloned as His-Tag proteins and expression was demonstrated using an anti-His antibody.

[0052] Figure 13. N- and C-RGMa inhibit axonal outgrowth by binding to the same Neogenin domain. (**A**) The extracellular portion of Neogenin consists of 4 Immunoglobulin like (4Ig) and 6 fibronectin type III (6FNIII) domains. ELISA plates were coated with RGMa proteins and binding of Neogenin-AP was studied. (Binding; -, base line; +> 2 x base line; ++ > 3 x base line; +++> 5x base line). The 3-4 fibronectin (FNIII(3-4)) domains sufficed for binding to RGMa proteins. (**B**) ELISA plates were coated with N-RGMa or C-RGMa and binding of 6FNIII-AP was assessed after incubation with C-RGMa$^{182-271}$, N-RGMa or NN-RGMa. Pre-incubation with either N- or C-RGMa proteins altered 6FNIII-AP binding to both C- and N-RGMa proteins. (**C**) Temporal axons grown on combinations of N- and C-RGMa. (**D**) Quantification of axonal growth on 5 µg/ml of N-RGMa, 5 µg/ml C-RGMa$^{182-271}$ and 2.5 µg/ml N-RGMa + 2.5 µg/ml C-RGMa$^{182-271}$. (**$p<0.005$). Data are average ± SEM from 3 independent experiments. (**E**) Temporal axons grown on N- and C-RGMa in the presence or absence of FNIII(3-4). (**F**) Quantification of axonal growth on laminin, laminin + 2.5 µg/ml of N-RGMa, and laminin + 2.5 µg/ml C-RGMa$^{182-271}$ in the presence or absence of

FNIII(3-4). FNIII(3-4) significantly restored axonal growth on N-RGMa, and C-RGMa$^{182-271}$ (**p<0.01). Data are average ± SEM from 3 independent experiments. (**G**) Model for RGMa action on Neogenin. SKI-1 and Furin digest RGMa into C- and N-terminal proteins all interact with the fibronectin domain (3-4) of Neogenin (middle dark grey rectangles) to inhibit axonal growth.

[0053] Figure 14. Western blot showing RGMa release after treatment with RRLL or RVKR.

[0054] Figure 15. SKI-1 inhibitor reduces cerebral infarct volume and edema and improves functional outcome following stroke. (A) Representative brain slices showing infarct area in Control and SKI-1 inhibitor groups after focal cerebral ischemia in rat. Quantification of (B) brain infarct volume and (C) edema in Control and SKI-1 inhibitor-treated animals. (D) Quantification of neurological outcome using Bederson's test at different time points in Control and SKI-1 inhibitor treated groups after focal cerebral ischemia in rat. Data are means + SEM; *p<0.05 vs. Control.

[0055] Figure 16. Inhibition of SKI-1 promotes cell survival and restores axonal outgrowth. Temporal retinal explants cultured on supernatant from cells transfected with Mock (Control; called Laminin in figure) or RGMa and treated with either DMSO or 10 μM PF429242 (SKI-1 inhibitor). After overnight incubation, membranes from Control, RGMa+DMSO and RGMa+PF429242 cells were prepared and used as a substrate for growing retinal fibers. Retinal explants were put on membranes with laminin and cultured overnight before fixation, then stained with Alexa-488 phalloidin and number of fibers/explant was measured and neurite length was quantified. Quantification of (A) number of fibers and (B) neurite length. Data are average ± SEM. *p<0.05 vs.

Detailed description

I. Definitions

[0056] The term "a cell" as used herein includes a single cell as well as a plurality or population of cells. Similarly, "a neuron" as used herein includes a single cell as well as a plurality or population of neurons.

[0057] The term "administered" as used herein means administration of a therapeutically effective dose of a compound or composition of the disclosure to a cell either in cell culture or in a subject e.g. patient.

[0058] The term "antibody" as used herein is intended to include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies. The antibody may be from recombinant sources and/or produced in transgenic animals. Antibodies can be fragmented using conventional techniques. For example, F(ab')2 fragments can be generated by treating the antibody with pepsin.

The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques. Antibody fragments mean binding fragments.

[0059] Antibodies capable of binding (e.g. having specificity for) a specific RGMa fragment or mutatnt such as the H151A or D149A mutant RGMa, (chick mutants, corresponding human positions being D168A and H170A, human cleavage site is at 169 of SEQ ID NO: 1) may be prepared by conventional methods. A mammal, (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of a RGMa fragment disclosed herein or a specific immunogenic peptide within the RGMa fragment which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

[0060] To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g. the hybridoma technique originally developed by Kohler and Milstein (Nature 256:495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol.Today 4:72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., Methods Enzymol, 121:140-67 (1986)), and screening of combinatorial antibody libraries (Huse et al., Science 246:1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

[0061] The term "conservative variant" refers to a variant of a polypeptide such as a variant of a RGMa fragment, which comprises one or more amino acid substitutions or deletions (for example, 1, 2, 3, 4, 5, 6, 7, 8, 9 , 10 or more, optionally 11-20, 21-30 or more, for example upto 10% of a polypeptide or nucleic acid) that do not substantially affect the character of the variant polypeptide relative to the starting polypeptide. For example, polypeptide character is not substantially affected if the substitutions or deletions do not preclude specific binding of the variant

peptide to a specific binding partner of the starting polypeptide. For example, the following are non-limiting examples of conservative amino acid substitutions:

1. Alanine (A), Serine (S), Threonine (T);
2. Aspartic acid (D), Glutamic acid (E);
3. Asparagine (N), Glutamine (Q);
4. Arginine (R), Lysine (K);
5. Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6. Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (see, e.g., Creighton, Proteins (1984)).

[0062] Conservative mutations may be made for example in non-Neogenin binding regions and in non cleavage domains, and include for example conservative changes that do not impact RGMa axon inhibition activity or RGMa survival inducing activity determinable for example using an assay described herein.

[0063] The term "active fragment" as used herein means a portion of a RGMA fragment such as $RGMa_{169-450}$ that is immunogenic, binds neogenin, promotes neuron cell survival and/or inhibits axon outgrowth

[0064] The term "furin" as used herein means a member of the subtilisin-like proprotein convertase family, having typically RX(K/R)R consensus motif and includes without limitation all known furin molecules including naturally occurring variants and for example those deposited in Genbank with the accession numbers CAA27860 CAA27860.1, CAA37988.1, NP_062204.1, NP_003782.1, NP_001161382.1, which are specifically incorporated by reference. Furin is also known as Pace and PC1, PCSK3 SPC1.

[0065] The term "furin inhibitor" as used herein refers to any peptide, small molecule or other inhibitor that reduces furin enzymatic activity by at least 40%, 50%, 60%, 70%, 80%, 90% or more. Non-limiting examples include furin inhibitors disclosed in U.S. Patent Application Publication No. **20090131328** titled "FURIN INHIBITORS and/or U.S. Patent Application Publication No. **20110059896** titled "USE OF FURIN CONVERTASE INHIBITORS IN THE TREATMENT OF FIBROSIS AND SCARRING" each of which are specifically incorporated by reference. Non-limiting examples of furin inhibitors include peptidyl chloroalkylketones with peptide moieties that mimic the convertase enzyme cleavage site, such as decanoyl-RVKR-cmk (also referred to as RVKR (SEQ ID

NO:3) and derivatives thereof. The inhibitors include modifications that improve membrane solubility such as attaching a moiety such as for example a decanoyl moiety or an HIV TAT peptide, as is known in the art. Serine proteases and/or other PPC inhibitors that reduce furin enzymatic activity by at least 40%, 50%, 60%, 70%, 80%, 90% or more are also contemplated. The furin inhibitor can also for example be a furin-prosegment inhibitor.

[0066] The term "furin- prosegment inhibitor" as used herein refers to an inactive form or fragment of furin that comprises an N-terminal prosegment that acts as a dominant negative form of Furin. Proprotein convertases (PCs) of the subtilisin/kexin family contain an N-terminal prosegment that has been suggested to act both as an intramolecular chaperone and an inhibitor of its parent enzyme. As demonstrated in Zhong et al. 1999 Furin prosegment is a potent inhibitor of furin as are small, synthetic decapeptides derived from the C termini of the prosegments are also potent inhibitors. The fragment can be for example at least 10-20 amino acids, for example 10 amino acids and/or including any one of the furin prosegment derived peptides described in Table 2 of Zhong et al 1999 and/or US Patent **7,211,424 (Seidah et al)**, each herein specifically incorporated by reference.

[0067] The term "isolated polypeptide" as used herein refers to a proteinaceous agent, such as a peptide, polypeptide or protein, which is substantially free of cellular material or culture medium when produced recombinantly, or chemical precursors, or other chemicals, when chemically synthesized.

[0068] The term "polypeptide" as used herein refers to a sequence of amino acids consisiting of naturally occurring residues, and non-naturally occurring residues.

[0069] The term "isolated nucleic acid" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated nucleic acid" is also substantially free of sequences which naturally flank the nucleic acid (i.e. sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived.

[0070] The term "nucleic acid" as used herein includes a sequence of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages, and is intended to include DNA and RNA and can be either double stranded or single stranded represent the sense or antisense strand.

[0071] The term "proprotein convertase inhibitor" and or "PPC" as used herein refers to any peptide, polypeptide, small molecule or other inhibitor that reduces furin and/or SKI-1 enzymatic

activity by at least 40%, 50%, 60%, 70%, 80%, 90% or more, and includes for example serine protease inhibitors, optionally a membrane permeable serine protease inhibitor, such as AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), RVKR peptide (SEQ ID NO:3), decanoyl-RVKR-cmk and derivatives, specific SKI-1 inhibitors and/or furin inhibitors as well as inhibitors that inhibit both SKI-1 and furin and optionally other PPCs. The inhibitors include modifications that improve membrane solubility such as attaching a moiety such as a decanoyl moiety or a HIV TAT peptide. Polypeptide PPCs include for example prosegment inhibitor R134E which inhibits SKI-1, as well as furin- prosegment inhibitor which inhibits furin.

[0072] The term "prosegment inhibitor R134E" as used herein refers to an inactive form of SKI-1 that acts as a dominant negative inhibitor. R134E is in a conserved motif across species including human and murine.

[0073] The term "providing neural tissue comprising neurons and RGMa expressing cells with a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor" means providing the PPC inhibitor or optionally a SKI-1 inhibitor and/or a furin inhibitor, to a site where RMGa expressing cells are present and in contact with neurons (e.g. signaling contact and/or physical contact) and where inhibition of RGMa cleavage in the RGMa expressing cell interrupts RGMa axonal outgrowth inhibition, thereby allowing neuronal axonal growth and/or regeneration. As demonstrated herein, the presence of cleaved RGMa on cells and/or soluble RGMa in the vicinity of neuron cells inhibits axon outgrowth. Inhibiting RGMa cleavage, interrupts the inhibitory signal providing an environment permissive for axon regrowth (see for example Figure 16). RGMa is expressed by astroglia cells and oligodendrocytes, which are the cells that secrete inhibitors that hamper axonal regeneration (Schwab et al., 2005). The PPC inhibitor can be provided to the neural tissue by a number of methods depending on the location of the neural tissue. For example, the PPC inhibitor, optionally the SKI-1 inhibitor can be provided by injection, for example to a site of injury, such as a spinal cord lesion or a stroke infarct lesion.The inhibitor could be administered for example, intrathecally (e.g. using osmotic pumps) or by systemic application. In an embodiment, the cells are contacted with the PPC inhibitor in vitro. In an embodiment, the neural tissue is neogenin expressing neural tissue.

[0074] The term "RGMa" as used herein refers to Repulsive Guidance Molecule A and includes without limitation all known RGMa molecules including naturally occurring variants and for example those deposited in Genbank with accession numbers AAH15886.2, CAD89718.1, NP-001100994.1, and/or NP-989868.1, which are herein specifically incorporated by reference as well as conservative amino acid containing forms and functional mutants (e.g. mutations that have a

functional effect), for example those described herein. A human amino acid sequence of RGMa is provided in SEQ ID NO:1. The sequence provided by SEQ ID NO:1 includes a signal peptide comprising amino acids (aa) 1 to 46. RGMa is cleaved for example providing a N-terminal RMGMa which is aa 47-168, C-terminal is aa. 169-450 including the GPI anchor sequence as well as other fragments described herein. Mutant forms include for example, RGMa mutated at residues within the SKI-1 and furin cleavage sites (e.g. residue 175 of SEQ ID NO:1) (e.g. uncleavable mutants) and forms comprising mutated residues flanking the autocatalytic site (e.g. H151A or D149A in chick, corresponding human positions being D168A and H170A).

[0075] The term "RGMa fragment as used herein refers to a RGMa fragment selected from or corresponding to:

l) a RGMa fragment no longer than amino acid 1 to 168 of human RGMa ($RGMa_{1-168}$), optionally no longer than amino acid 47 to 168,

m) a RGMa fragment no longer than amino acid 1 to 127 of human RGMa ($RGMa_{1-127}$), optionally no longer than amino acid 47 to 127,

n) a RGMa fragment no longer than amino acid 1 to 133 of human RGMa ($RGMa_{1-133}$), optionally no longer than amino acid 47 to 133,

o) a RGMa fragment no longer than amino acid 1 to 175 of human RGMa ($RGMa_{1-175}$), optionally no longer than amino acid 47 to 175;

p) a RGMa fragment no longer than amino acid 128 to 175 of human RGMa ($RGMa_{128-175}$)

q) a RGMa fragment no longer than amino acid 169 to 450 of human RGMa ($RGMa_{169-450}$),

r) a RGMa fragment no longer than amino acid 175 to 450 of human RGMa ($RGMa_{176-450}$);

s) a RGMa fragment no longer than amino acid 128 to 450 of human RGMa ($RGMa_{128-450}$)

t) a RGMa fragment no longer than amino acid 201-290 of human RGMa($RGMa_{201-290}$),

u) corresponding species fragments of anyone of a) to i), and/or

v) conservative variants of any one of a) to j).

[0076] Various fragments have been identified. It is demonstrated herein that SKI-1 cleaves human RGMa after residue 175 of SEQ ID NO:1 and furin cleaves human RGMa after residue 127 of SEQ ID NO:1 (e.g. RLR sequence). The RGMa fragment 1-133 was identified and used to identify the fragment produced upon furin cleavage.

[0077] The amino acid numbers can be e.g. 1 to 168 for $RGMa_{1-168}$ in reference to the human amino acid sequence (e.g. SEQ ID NO:1). A person skilled in the art would readily be able through sequence alignment, be able to determine the corresponding species fragments e.g. determine the amino acid positions of corresponding fragments in other species. For example, the corresponding amino acids for the RGMa fragment no longer than amino acids 1 to 168 of human RGMa corresponds to amino acids 1 to 150 in chick RGMa; and for the RGMa fragment no longer than amino acids 201-290 of human RGMa corresponds to amino acids 182-271 in chick RGMa. The fragment can be any length within the stipulated range or the full length of the stipulated range. For example $RGMa_{1-168}$ fragment can be about 80, 90, 100, 110, 120, 122, 130, 140, 150 160, or 168 or any number of amino acids in between 10 and 168 wherein the fragment retains neogenin binding and/or signaling activity (for example including or excluding the signal sequence). Full length $RGMa_{1-168}$ corresponds for example to the 22 kDa fragment illustrated in Figure 1. $RGMa_{1-127}$ fragment can be about 60, 70, 80, 87, 90, 100, 110, 120, or 127 amino acids or any number of amino acids in between 10 and 127 wherein the fragment is antigenic, retains neogenin binding and/or signaling activity (and can for example including or excluding the signal sequence). Full length $RGMa_{1-127}$ corresponds to the 18 kDa fragment illustrated in Figure 1 (1-46 amino acides being the signal sequence). $RGMa_{1-133}$ fragment which was initially identified and led to determination of the furin cleavage site, can be about 60, 70, 80, 87, 90, 100, 110, 120, 130 or 133 amino acids or any number of amino acids in between 10 and 133 wherein the fragment retains neogenin binding and/or signaling activity for example including or excluding the signal sequence). $RGMa_{1-175}$ fragment can be about 80, 90, 100, 110, 120, 129, 130, 140, 150 160, 170 or 175 or any number of amino acids in between 10 and 175, or any number of amino acids in between 10 and 175 wherein the fragment is antigenic, retains neogenin binding and/or signaling activity. $RGMa_{128-175}$ fragments can be about 10, 20, 30, 40 or 46 amino acids or any number of amino acids in between 10 and 45 wherein the fragment is antigenic. $RGMa_{176-450}$ fragments can be about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270 or 274 amino acids or any number of amino acids in between 10 and 274 wherein the fragment retains neogenin binding and/or signaling activity. $RGMa_{169-450}$ fragments can be about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270 or 281 amino acids or any number of amino acids in between 10 and 281

wherein the fragment retains neogenin binding and/or signaling activity. Full length $RGMa_{169-450}$ corresponds to the C-terminal molecule of RGMa (e.g. the Cterm molecule of RGMa fragment illustrated in Figure 1) and fragments can be for example about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270 or 281 amino acids. $RGMa_{128-450}$ fragments can be about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 322 amino acids or any number of amino acids in between 10 and 322 wherein the fragment retains neogenin binding and/or signaling activity. Similarly, $RGMa_{176-290}$ fragments can be about 40, 50, 60, 70, 80, 90, 100, 110 or 114 amino acids or any number of amino acids in between 10 and 114 wherein the fragment retains neogenin binding and/or signaling activity $RGMa_{201-290}$ fragments can be about 40, 50, 60, 70, 80 or 89 amino acids or any number of amino acids in between 10 and 89 wherein the fragment retains neogenin binding and/or signaling activity.

[0078] The term "RGMaΔ" or "RGMa delta" as used herein refers to a 60 kDa species that results from RGMa shedding from the membrane (RGMaΔ) by a phospholipase. RGMa is a membrane protein, however, soluble full length protein is found in RGMa transfected cell supernatant, indicating that it can be released.

[0079] The term "RGMa37" as used herein refers to a RGMa polypeptide, optionally membrane bound, which is schematically illustrated in Figure 1. It is a 37 kDa species ("RGMa37") which is observed on non-reduced membrane preparations from chick brain as described in Example 1, and can include for example a Cterm RGMa 33kDa fragment in combination with a 4kDa RGMa fragment, which are associated via a disulphide bridge. RGMa37 corresponds to amino acids about 128-450 or optionally about134-450 of human RGMa (for example plus or minus 8 amino acids and starting optionally at 126).

[0080] The term "$RGMa_{1-168}$" (e.g.22 kDa fragment) also referred to as "N-RGMa", as used herein means a RGMa fragment no longer than amino acid 1 to 168 of human RGMa, for example about 80, 90, 100, 110, 120, 122,130, 140, 150 160, or 168 or any number in between 10 and 168 that retains neogenin binding and/or signaling activity. Full length $RGMa_{1-168}$ (and/or RGMa deleted of the signal sequence e.g. corresponding to aa 47-168) corresponds to the 22 kDa fragment illustrated in Figure 1.

[0081] The term "$RGMa_{1-133}$" (e.g.18kDa fragment) also referred to as "NN-RGMa" as used herein means a RGMa fragment no longer than amino acids 1 to 133 of human RGMa, for example about 60, 70, 80, 87, 90, 100, 110, 120, 130 or 133 amino acids or any number of amino acids in between 10 and 133 wherein the fragment retains neogenin binding and/or signaling activity. Full length $RGMa_{1-133}$ (and/or RGMa deleted of the signal sequence e.g. corresponding to aa 47-133)

corresponds to the 18 kDa fragment illustrated in Figure 1. "$RGMa_{1-133}$" was used to identify the furin cleavage site that results in "$RGMa_{1-127}$".

[0082] The term "$RGMa_{1-127}$" also referred to as "NN-RGMa" as used herein means a RGMa fragment no longer than amino acids 1 to 127 of human RGMa, for example about 60, 70, 80, 86, 90, 100, 110, 120, or 127 amino acids or any number of amino acids in between 10 and 127 wherein the fragment is antigenic and/or retains neogenin binding and/or signaling activity. Full length $RGMa_{1-127}$ is amino acids 1 -127 of SEQ I D NO:1 and/or deleted of the signal sequence e.g. corresponding to aa 47-127. Full length $RGMa_{1-128}$ (and/or RGMa deleted of the signal sequence e.g. corresponding to aa 47-127) corresponds to the 18 kDa fragment illustrated in Figure 1.

[0083] The term "$RGMa_{1-175}$", as used herein means a RGMa fragment no longer than amino acid 1 to 175 of human RGMa, for example about 80, 90, 100, 110, 120, 129, 130, 140, 150 160, 170 or 175 or any number in between 10 and 175 that retains neogenin binding and/or signaling activity. $RGMa_{1-175}$ and/or RGMa deleted of the signal sequence e.g. corresponding to aa 47-175 is considered full length $RGMa_{1-175}$.

[0084] The term "$RGMa_{128-175}$", as used herein means a RGMa fragment no longer than amino acid 128 to 175 of human RGMa, for example about 10, 20, 30, or 47 or any number in between 10 and 47 that is antigenic. This fragment corresponds to for example the 4kDa RGMa fragment in Figure 1.

[0085] The term "$RGMa_{169-450}$" as used herein means a RGMa fragment no longer than amino acids 169 to 450 of human RGMa, for example about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270 or 281 amino acids or any number of amino acids in between 10 and 281 wherein the fragment retains neogenin binding and/or signaling activity. Full length $RGMa_{169-450}$ corresponds to the C-terminal molecule of RGMa illustrated in Figure 1.

[0086] The term "$RGMa_{176-450}$" as used herein means a RGMa fragment no longer than amino acids 176 to 450 of human RGMa, for example about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270 or 274 amino acids or any number of amino acids in between 10 and 274 wherein the fragment retains neogenin binding and/or signaling activity.

[0087] The term "$RGMa_{128-450}$" as used herein means a RGMa fragment no longer than amino acids 128 to 450 of human RGMa, for example about 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320 or 322 amino acids or any number of amino acids in between 10 and 322 wherein the fragment retains neogenin binding and/or signaling

activity. Full length $RGMa_{128-450}$ corresponds to a C-terminal molecule of RGMa illustrated in Figure 1.

[0088] The term "$RGMa_{176-290}$" as used herein is in reference to human RGMa and means a RGMa fragment a RGMa fragment no longer than 176 to 290 of human RGMa, for example the human RGMa sequence of SEQ ID NO:1. $RGMa_{176-290}$ can be about 40, 50, 60, 70, 80, 90, 100, 110 or 114 amino acids or any number of amino acids in between 10 and 114 wherein the fragment retains neogenin binding and/or signaling activity.

[0089] The term "$RGMa_{201-290}$" as used herein is in reference to human RGMa and means a RGMa fragment corresponding to and no longer than amino acid 182 to 271 of chick RGMa or a RGMa fragment no longer than 201 to 290 of human RGMa, for example the human RGMa sequence of SEQ ID NO:1. $RGMa_{201-290}$ can be about 40, 50, 60, 70, 80 or 89 amino acids or any number of amino acids in between 10 and 89 wherein the fragment retains neogenin binding and/or signaling activity.

[0090] The term "sequence identity" as used herein refers to the percentage of sequence identity between two polypeptide sequences or two nucleic acid sequences. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical overlapping positions/total number of positions.times.100%). In one embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g., for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present application. BLAST protein searches can be performed with the XBLAST program

parameters set, e.g., to score-50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) can be used (see, e.g., the NCBI website). The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

[0091] The term "SKI-1" as used herein means Subtilisin Kexin Isozyme-1, a member of the subtilisin-like proprotein convertase family, and includes without limitation all known SKI-1 molecules including naturally occurring variants and for example those deposited in Genbank with accession number AAD27010.1, AAD27011.1, AAI14556.1, ABT02354.1, XP_003641945, which are herein specifically incorporated by reference. SKI-1 is also known for example as site 1 Protease, and S1P. It is not possible to predict the presence of a SKI-1 cleavage sites in RGMa sequence, for example using prediction programs, with any certainty. The concensus sequence recognized by SKI-1 is reported as (R/K)X(hydrophobic)Z↓ where Z is variable (Seidah and Chretien, 1999). SKI-1 cleaves RGMa at amino acid 175 of SEQ ID NO:1.

[0092] The term "SKI-1 inhibitor" as used herein refers to any peptide, polypeptide, small molecule or other inhibitor that reduces SKI-1 enzymatic activity by at least 40%, 50%, 60%, 70%, 80%, 90% or more. Non-limiting examples include, RRLL peptide (SEQ ID NO:4), PF-429242 (Pasquato et al, 2012) available for example from TOCRIS Science, peptide CIYISRRLLC (SEQ ID NO:5; optionally with terminal "C" residues cyclized) (Pasquato et al, 2011) and/or prosegment inhibitor R134E. The SKI-1 inhibitors include modifications that improve membrane solubility such as attaching a moiety such as a decanoyl moiety or a HIV TAT peptide. Serine proteases and/or other PPC inhibitors that reduce SKI-1 enzymatic activity by at least 40%, 50%, 60%, 70%, 80%, 90% or more are also contemplated.

[0093] The term "soluble RGMa" as used herein means non-membrane bound forms of RGMa including for example N-RGMa, NN-RGMa and RGMaΔ, as well as the 4kDa RGMa fragment and/or fragments of any thereof.

[0094] The term "subject" includes all members of the animal kingdom, including human. In one embodiment, the subject is an animal. In another embodiment, the subject is a human.

[0095] As used herein, and as well understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

[0096] The term a "therapeutically effective amount", "effective amount" or a "sufficient amount" of an inhibitor(s) (e.g. SKI-1 and/or Furin inhibitor) or a composition comprising the inhibitor, is a quantity sufficient to, when administered to a cell or a subject, including a mammal, for example a human, effect beneficial or desired results, including clinical results, and, as such, an "effective amount" or synonym thereto depends upon the context in which it is being applied.

[0097] In understanding the scope of the present disclosure, the term "comprising" and its derivatives, as used herein, are intended to be open ended terms that specify the presence of the stated features, elements, components, groups, integers, and/or steps, but do not exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The foregoing also applies to words having similar meanings such as the terms, "including", "having" and their derivatives. Finally, terms of degree such as "substantially", "about" and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a deviation of at least ±5% of the modified term if this deviation would not negate the meaning of the word it modifies.

[0098] In understanding the scope of the present disclosure, the term "consisting" and its derivatives, as used herein, are intended to be close ended terms that specify the presence of stated features, elements, components, groups, integers, and/or steps, and also exclude the presence of other unstated features, elements, components, groups, integers and/or steps.

[0099] The recitation of numerical ranges by endpoints herein includes all numbers and fractions subsumed within that range (e.g. 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.90, 4, and 5). It is also to be understood that all numbers and fractions thereof are presumed to be modified by the term "about." Further, it is to be understood that "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. The term "about" means plus or minus 0.1 to 50%, 5-50%, or 10-

40%, preferably 10-20%, more preferably 10% or 15%, of the number to which reference is being made.

[00100] Further, the definitions and embodiments described are intended to be applicable to other embodiments herein described for which they are suitable as would be understood by a person skilled in the art. For example, in the above passages, different aspects of the invention are defined in more detail. Each aspect so defined can be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous can be combined with any other feature or features indicated as being preferred or advantageous.

II. Methods and Compositions

[00101] Current understanding of RGMa processing derives from studies in transfected COS-7 cells, but the in vivo cleavage pattern remains uncharacterized (Tassew et al., 2009). It is demonstrated herein that RGMa processing is far more complex than originally thought. Thus, alternative cleavage by the Proprotein Convertases Subtilisin Kexin Isozyme-1 (SKI-1) and Furin generates multiple membrane-bound and soluble forms, and the resulting peptides have dramatically different potencies, including novel released forms. In stark contrast to non-membrane-bound ephrins that do not influence axonal outgrowth, it is demonstrated herein that soluble forms of RGMa are inhibitory. Unexpectedly, distinct RGMa products with no sequence homology operate through the same Fibronectin III (3-4) domains in Neogenin. As RGMa is implicated in human disorders (Nohra et al., 2010; Hata et al., 2006), the discovery of multiple derivatives is expected to have important implications for therapeutic targeting.

[00102] Various fragments were identified as described below. Drugs were screened to see if the two cleavage sites identified could be inhibited. AEBSF, which is an inhibitor of pro protein convertase was effective. RVKR which inhibits the same enzymes was tested and also inhibited the cleavage. A Furin cleavage site was identified that could provide the observed fragment size.

[00103] Accordingly, an aspect of the disclosure includes an isolated polypeptide comprising: a RGMa fragment selected from or corresponding to:

a) a RGMa fragment no longer than amino acid 1 to 168 of human RGMa ($RGMa_{1-168}$), optionally no longer than amino acid 47 to 168,

b) a RGMa fragment no longer than amino acid 1 to 127 of human RGMa ($RGMa_{1-127}$), optionally no longer than amino acid 47 to 127,

c) a RGMa fragment no longer than amino acid 1 to 133 of human RGMa ($RGMa_{1-133}$), optionally no longer than amino acid 47 to 133,

d) a RGMa fragment no longer than amino acid 1 to 175 of human RGMa ($RGMa_{1-175}$), optionally no longer than amino acid 47 to 175;

e) a RGMa fragment no longer than amino acid 128 to 175 of human RGMa ($RGMa_{128-175}$)

f) a RGMa fragment no longer than amino acid 169 to 450 of human RGMa ($RGMa_{169-450}$),

g) a RGMa fragment no longer than amino acid 176 to 450 of human RGMa ($RGMa_{176-450}$);

h) a RGMa fragment no longer than amino acid 128 to 450 of human RGMa ($RGMa_{128-450}$)

i) a RGMa fragment no longer than amino acid 201-290 of human RGMa($RGMa_{201-290}$),

j) corresponding species fragments of anyone of a) to i), and/or

k) conservative variants of any one of a) to j).

[00104] In an embodiment, the $RGMa_{1-168}$ consists of residues 1 to 168 of SEQ ID NO:1 or a fragment thereof, for example deleted of the signal sequence; $RGMa_{1-133}$ consists of residues 1 to 133 of SEQ ID NO:1 or a fragment thereof for example deleted of the signal sequence, $RGMa_{1-127}$ consists of residues 1 to 127 of SEQ ID NO:1 or a fragment thereof for example deleted of the signal sequence, $RGMa_{1-175}$ consists of residues 1 to 175 of SEQ ID NO:1 or a fragment thereof for example deleted of the signal sequence; $RGMa_{128-175}$ consists of residues 128 to 175 of SEQ ID NO:1 or a fragment thereof, $RGMa_{169-450}$ consists of residues 169 to 450 of SEQ ID NO:1 or a fragment thereof, $RGMa_{128-450}$ consists of residues 128 to 450 of SEQ ID NO:1 or a fragment thereof, $RGMa_{176-450}$ consists of residues 176 to 450 of SEQ ID NO:1 or a fragment thereof; $RGMa_{176-290}$ comprises residues 176 to 290 of SEQ ID NO:1 or a fragment and/or $RGMa_{201-290}$ comprises residues 201 to 290 of SEQ ID NO:1 or a fragment thereof; and/or a conservative variant of any thereof. In another embodiment, the RGMa fragment is another species corresponding fragment, e.g. a mouse, rat or chick RGMa fragment corresponding to $RGMa_{1-168}$, $RGMa_{1-133}$, $RGMa_{169-450}$ and/or $RGMa_{201-290}$.

[00105] Fragments that bind Neogenin include for example, N-RGMa (1-168 for human and 1-150 for chick), NN-RGMa (1-133 or 1-128 in human and 1-114 in chick), (as well as fragments that are deleted of the signal peptide) and C terminal -RGMa fragments comprising of 201 to 290 in humans is 182-271 in chick. RGMa fragments 1-175 (and deleted of the signal peptide) and 176-450 are also expected to bind Neogenin.

[00106] In an embodiment, the fragment is full length $RGMa_{1-168}$, $RGMa_{1-175}$, $RGMa_{1-133}$, and/or $RGMa_{1-128}$, and/or a conservative variant thereof, and/or the fragment deleted of its signal peptide (e.g. residues 1-46 are deleted from $RGMa_{1-168}$, $RGMa_{1-128}$, $RGMa_{1-175}$ or $RGMa_{1-133}$) wherein the conservative variant retains for example the ability to bind neogenin.

[00107] In an embodiment, the fragment is full length $RGMa_{201-290}$.

[00108] In another embodiment, the fragment is $RGMa_{128-175}$.

[00109] In another embodiment, the isolated polypeptide comprises a RGMa fragment such as the 4 kDa RGMa fragment in combination with a second fragment of RGMa, optionally the 33 kDa fragment having the combined sequence of RGMa37; wherein the isolated polypeptide is optionally combined with the second fragment via a disulphide bridge forming a species that is approximately 37kDa when separated electrophoretically on a non-reducing agarose gel. In another embodiment, the combination comprises $RGMa_{169-450}$ for example of Figure 1.

[00110] In yet another embodiment, the isolated polypeptide comprises $RGMa_{169-450}$ (RGMa Cterm fragment 169-450 in humans) in combination with a second fragment of RGMa, optionally no longer than amino acids 1 to 168 of RGMa (RGMa1-168 in humans, optionally minus the signal sequence), wherein the isolated polypeptide is optionally combined with the second fragment via a disulphide bridge forming a species that is approximately 60kDa when separated electrophoretically on a non-reducing agarose gel.

[00111] In yet another embodiment, the isolated polypeptide comprises $RGMa_{176-450}$ (RGMa Cterm fragment 176-450 in humans) in combination with a second fragment of RGMa, optionally no longer than amino acids 1 to 175 of RGMa (RGMa1-175 in humans, optionally minus the signal sequence), wherein the isolated polypeptide is optionally combined with the second fragment, for example a fragment described herein.

[00112] The RGMa fragments are demonstrated to be glycosylated and as shown in Figure 1 to comprise GPI anchors (e.g. Cterminal fragments). Accordingly, in an embodiment, the isolated polypeptide is glycosylated. In another embodiment, the isolated polypeptide comprises a GPI anchor or a part thereof.

[00113] The RGMa fragment can for example comprise a tag such as a His tag, a FLAG®-tag or a fluorescent tag (e.g. GFP) or an enzyme tag such as alkaline phosphatase (AP). The tag can for example be linked to the RGMa fragment via a linker peptide, for example, a linker peptide comprising 6, 7, or 8 amino acids. Linker peptides are commonly employed so when attaching a tag so that the native polypeptide folding is maintained.

[00114] Also provided are isolated polypeptides comprising cleavage site RGMa mutants selected from D168A, H170A, (SEQ ID NO: 8) and mutation at 175.

A cleavage site can also be replaced. For example, RGMA is provided wherein RGMa CGLFGDP residues (residues 8-14 of SEQ ID NO: 7 are replaced with ENLYFQS (residues 8-14 of SEQ ID NO:6) (e.g. using AA-PNYTH*CGLFGDP*HLRTFTD (SEQ ID NO:7) and AA-PNYTHENLYFQSHLRTFTD (SEQ ID NO:6). The replaced sequence is for example provided in SEQ ID NO: 9.

[00115] Accordingly, in an embodiment, the isolated polypeptide comprises one or more of a linker peptide and a tag such as a FLAG®-tag, GST tag, His Tag, GFP tag.

[00116] The isolated polypeptides can be provided as recombinant polypeptides and used for example as standards in a kit and/or as ligands for studying neogenin signal transduction. Isolated polypeptides can be made by transfecting a cell such as a bacterial cell or an insect cell with a nucleic acid molecule encoding the polypeptide.

[00117] A skilled person would appreciate that there are numerous methods of introducing one or more exogenous nucleic acid molecules to a cell or cell population, including, for example, calcium phosphate transfection, DEAE-dextran transfection, infection, electroporation, lipofection, heat shock, magnetofection, nucleofection, integrating episome, use of a gene gun or microinjection. Introduction of nucleic acid molecules to a cell or cell population refers to both stable and transient uptake of the genetic material.

[00118] A further aspect includes an isolated nucleic acid molecule encoding an isolated polypeptide described herein.

[00119] Also provided is an antibody that is capable of binding any of the RGMa fragments described herein.

[00120] As described in the Examples, an antibody was generated using a rat RGMa peptide corresponding to residues 309-322 (which correspond to human RGMa residues 308-321).

Accordingly in an embodiment, the antibody is an antibody that is capable of binding amino acids 309- 322 of rat RGMa and/or amino acids 308-321 of human RGMa.

[00121] As disclosed herein, SKI-1 is responsible for cleaving RGMa at amino acid residue 175 of SEQ ID NO: 1, and/or amino acids corresponding thereto.

[00122] A further aspect provides an antibody that specifically recognizes an epitope that comprises amino acid residue 175 of SEQ ID NO: 1 or a corresponding amino acid. In an embodiment, the isolated antibody is a monoclonal antibody and/or a humanized antibody. The epitope can comprise for example 4 or more amino acids surrounding residue 175, and the antibody can be generated using a peptide comprising 4 or more amino acids centered (or comprising) residue 175 of SEQ ID NO:1. Larger epitopes can be used, for example comprising any where from 5 to 25 residues or more, where antibodies that are capable of biding an epitope comprising residue 175 of SEQ ID NO:1 are selected from example using affinity chromatography.

[00123] In an embodiment, the antibody that is capable of binding the epitope comprising amino acid 175 of SEQ ID NO: 1 and/or blocks cleavage of RGMa at amino acid 175 by SKI-1.

[00124] Also provided is a method of producing an antibody, comprising administering a RGMa antigenic peptide comprising for example at least 6 contiguous amino acids of a RGMa fragment described herein, optionally comprising for example a mutation site described herein, to a non-human mammal and isolating antibodies capable of binding the administered antigenic peptide. The isolated polypeptide and/or antibody can for example be attached to a substrate, such as a plate well or bead.

[00125] A further aspect includes a composition comprising the isolated antibody and/or isolated polypeptide and/or the isolated nucleic acid. The composition can comprise for example a suitable vehicle or diluent. In an embodiment, the suitable vehicle or diluent is a pharmaceutically suitable vehicle or diluent.

[00126] As demonstrated in the Examples below, proprotein convertases, Furin and SKI-1 combine with autocatalytic-cleavage and a disulphide-bridge to generate four membrane-bound and three soluble forms of the Repulsive Guidance Molecule (RGMa).

[00127] Accordingly, a further aspect includes a method of inhibiting RGMa cleavage comprising contacting the RGMa with a proprotein convertase (PPC) inhibitor.

[00128] In an embodiment, the PPC inhibitor comprises a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor and/or a furin inhibitor.

[00129] In an embodiment, the RGMa is GPI anchored to a cell.

[00130] An embodiment includes a method of inhibiting RGMa cleavage at RTFT↓D (SEQ ID NO: 10) (amino acid 175 in human) comprising contacting the RGMa with a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor.

[00131] A further embodiment includes a method of inhibiting RGMa cleavage at RLR, comprising contacting the RGMa with a furin inhibitor.

[00132] There are several RGM protiens including RGMa, RGMb, and RGMc. RGMc, also known as hemojuvelin, (having for example, Accession: NP_998818.1 GI: 47458048 and/or Q6ZVN8.1) is also cleaved by furin. Accordingly methods described herein comprising inhibiting furin can be used for example with RGMc as well. RGMc is involved in hemochromatosis which is a blood iron condition. In an embodiment, the methods and compositions described herein can be used to inhibit RGMc cleavage, for example in a subject afflicted by hemochromatosis.

[00133] Inhibition of SKI-1 and/or furin mediated cleavage of RMGa, for example RMGa tethered to a cell, inhibits formation of RGMa37 and/or release of, $RGMa_{1-168}$ (e.g. 22kDa fragment) and/or $RGMa_{1-133}$ $RGMa_{1-128}$ (e.g. 18kDa fragment).

[00134] Accordingly another aspect includes a method of inhibiting soluble NRGMa ($RGMa_{1-168}$ (e.g. 22kDa fragment)) or NNRGMa (e.g. RGMa1-128 18kDa fragment) release from a cell (e.g. a RGMa expressing cell) comprising contacting the cell with a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor.

[00135] A number of SKI-1 and furin inhibitors are described herein. A person skilled in the art could readily test if a putative PPC inhibitor prevents cleavage of RGMa using assays, polypeptides and cells described herein.

[00136] Where the cell is in vivo in a subject, contacting can be performed for example by administering the PPC inhibitor to the subject, for example intravenously and/or to the site of RGMa expressing cells.

[00137] It is also disclosed herein that both Nterm RGMa fragments and Cterm RGMa fragments bind neogenin fibronectin domain, specifically fibronectin domain 3-4. RGMa fragments, neogenin and/or fibronectin 3-4 domain comprising fragments can be used to screen for compounds that decrease or increase RGMa and neogenin interaction and/or that promote neuron survival, axon growth and/or regeneration.

[00138] Accordingly a further aspect of the disclosure includes an assay for identitying compounds that inhibit RGMa:neogenin binding and/or activation comprising:

a. a. contacting a neogenin polypeptide comprising firbronectin domain 3-4 and a RGMa fragment polypeptide that binds neogenin fibronectin domain 3-4 with a test compound;

[00139] b. determining whether the test compound interferes with RGMa: Neogenin binding and/or activation. The assay can for example be performed in cell frees assay using polypeptides described herein and/or using cells expressing the polypeptides described herein. The test comound may be added prior to the addition of the RGMa fragment.

[00140] Determining whether the test compound interferes with RGMa:Neogenin binding can comprise for example determining if the RGMa fragment polypeptide binds to the neogenin comprising fibronectin domain 3-4 polypeptide with the same affinity or the same extent. This can be assessed using a number of techniques known in the art for assessing protein protein interaction such as immunoprecipitation, yeast 2-hybrid systems and GST protein interaction.

[00141] Determining whether the test compound interferes with RGMa Neogenin activity can be performed for example using cells expressing a Neogenin receptor, contacting the cells with soluble RGMa (or tethered RGMa), and the test comound and assessing for example axon growth in the presence and absence of test compound using for example methods as described herein. For example, retinal explants can be tested in the presence of the test compound plus from cells that were either Mock transfected or transfected with full length RGMa.

[00142] Additional controls can be used for example RGMa fragments and/or neogenin fragments and/or mutants that are not involved in RGMa: neogenin binding.

[00143] Inhibiting RGMa cleavage and for example inhibiting release of soluble RGMa can be used to stimulate neuron survival, axon growth and/or regeneration and/or inhibit ischemic injury.

[00144] In vivo evidence described in the Examples, demonstrates that SKI-1 and furin proprotein-convertases are involved in axonal growth and/or survival and that RGMa-cleavage is required for Neogenin-mediated outgrowth inhibition. Figure 15 demonstrates for example that SKI-1 inhibition can reduce infarct size, edema and improves neurological outcome in a stroke animal model. As described in the examples, embolization of a preformed clot resulted in an infarction in the ipsilateral hemisphere, mainly located in the MCA-irrigated region. As shown in Figure 15, SKI-1 inhibitor significantly reduced the infarct volume and edema compared to Control. Additionally, neurological outcome was markedly improved in animals treated with SKI-1 inhibitor at 48h post-

stroke induction. Because the SKI-1 inhibitor reduced brain infarct volume and edema and improved functional outcome, it is possible that SKI-1 inhibitor acts on the brain's perfusion deficits for improvement in functional outcome following stroke. Further in vivo evidence for the effect of SKI-1 inhibitors is provided in Figure 16. Figure 16 demonstrates SKI-1 inhibition restores the deleterious effect of RGMa on fibre number and axonal outgrowth. These data provide in vivo evidence that inactivation of SKI-1, which prohibits the cleavage of full-length RGMa into its active fragments and thus binding to Neogenin, promotes cell survival and axonal outgrowth.

[00145] Accordingly, a further aspect includes a method of stimulating neuron survival, axon growth and/or promoting neuron axon regeneration and/or inhibiting ischemic injury (e.g. of neural tissues) in a subject in need thereof, the method comprising: providing neural tissue with a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor. In an embodiment, the neuron is a neogenin expressing neuron. The neural tissue comprises for example neurons and RGMa expressing cells. Providing the neural tissue with the PPC inhibitor, for example by administration to the subject, optionally by injection (for example intravenously or by injection into the neural tissue e.g. intraspinal injection), to promote axon survival, growth and/or axon regeneration, for example post-natal axon survival, growth and/or regeneration, by inhibiting RGMa anti-outgrowth signals.

[00146] Also provided is use of a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor for stimulating neuron survival, axon growth and/or promoting neuron axon regeneration and/or inhibiting ischemic injury.

[00147] A further embodiment comprises a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor for use in stimulating neuron survival, axon growth and/or promoting neuron axon regeneration and/or inhibiting ischemic injury.

[00148] The PPC inhibitor can for example be comprises in a composition, for example comprising a pharmaceutically acceptable diluent or carrier.

[00149] Neogenin expressing neurons include for example cortico-spinal neurons, dorsal root ganglion (DRG) neurons, Retinal Ganglion Cells, and hippocampal neurons.

[00150] RGMa expressing cells include for example retinal ganglion cells, DRGs, hippocampal, cortico-spinal neurons. Tissues comprising said cells, and/or diseases and/or injuries involving said cells can be treated with a a PPC inhibitor such as a SKI-1 and/or inhibitor to promote neuron survival, axon growth and/or regeneration and/or treat the disease involving said cells.

[00151] Another aspect includes a method of promoting neuron survival, axon growth and/or regeneration in a subject in need thereof, the method comprising the step of contacting neural tissue with a PPC inhibitor, optionally a SKI-1 and/or Furin inhibitor in an amount sufficient to promote neuron survival, axon growth and/or regeneration of the neuron.

[00152] As demonstrated herein, soluble $RGMa_{1-133}$ (or $RGMa_{1-128}$; e.g.18kDa fragment) is the most potent inhibitor of axonal growth of the RGMa ligands followed by soluble $RGMa_{1-168}$ (e.g. 22kDa fragment) (see for example Fig 9). Without wishing to be bound by theory, it is contemplated that soluble RGMa fragments can provide long range signals. SKI-1 and/or furin by inhibiting release of soluble RGMa fragments, can promote long range neuron survival and axon growth. Accordingly, in an embodiment the method is for stimulating long range neuron survival, axon growth and/or regeneration.

[00153] In an embodiment, the neural tissue is provided with the SKI-1 inhibitor and/or the furin inhibitor by administering the inhibitor or a composition comprising the inhibitor to the subject by injection into the neural tissue. In an embodiment, the neural tissue is provided with SKI-1 inhibitor and/or the furin inhibitor by intravenous injection.

[00154] Another aspect includes a method of promoting neuron survival, axon growth and/or regeneration in a subject in need thereof, the method comprising administering to the subject a SKI-1 and/or Furin inhibitor sufficient to promote growth and/or regeneration of neuron axons.

[00155] Axonal growth and regeneration can be important for improving nerve signal transduction in damaged nerves, damaged for example by trauma and/or disease. The central nervous system has limited capacity to repair damaged nerves.

[00156] Nerves comprise bundles of axons from different neurons. A neuron consists of a cell body, branch-like extensions called dendrites, and at least one longer extension called an axon. The dendrites conduct signals toward the neuron cell body and the axon carries messages away from the cell body toward the terminal end of the axon to communicate with other cells. Nerves connect the brain to effector cells. The signal between the cell body and effector cells is interrupted if neurons are severely damaged. Nerve damage can be a result of nerve injuries resulting from nerve trauma such as a spinal cord injury.

[00157] Nerve trauma can for example be incurred through high impact accidents such as motor vehicle accidents, severe falls and lacerations that lead to nerve compression and/or nerve severance. For example, damage to the lower spinal cord if severe can lead to paraplegia,

paralysis of the lower extremities. Severe damage to the upper spinal column may lead to quadriplegia, paralysis of all four extremities.

[00158] Accordingly, in an embodiment, the subject has a nerve injury and/or nerve damage.

[00159] A further aspect includes a method of treating nerve damage in a subject in need thereof, the method comprising the step of administering to the subject a SKI-1 and/or Furin inhibitor sufficient to promote regeneration of neuron axons. Also provided is use of a SKI-1 and/or Furin inhibitor for promoting survival and/or regeneration of neuron axons and/or treat nerve damage in a subject in need thereof. In another embodiment, the disclosure includes a SKI-1 and/or Furin inhibitor for use to promote survival and/or regeneration of neuron axons and/or treat nerve damage in a subject in need thereof.

[00160] Functional recovery within the CNS, for example due to an injury or disease, relies on cell survival and axonal regeneration.

[00161] In an embodiment, the nerve injury and/or damage is a spinal cord injury. In another embodiment, the nerve damage is damage of the optic nerve for example as seen in multiple sclerosis and/or nerve damage in the spine for example as seen in multiple sclerosis. In another embodiment, the nerve damage is damage of CNS connections following stroke. In a further embodiment, the nerve damage is damage of neuronal networks seen for example in Parkinson disease and Alzheimer's disease.

[00162] Nerve damage can be caused by stroke. Zhang et al reported that the levels of RGMa are significantly elevated after ischemia/reperfusion injury in a rodent model. Olfactory stimulation treatment downregulated the expression of RGMa, reduced infarct volume and improved neurological function. Administration of a SKI-1 inhibitor is demonstrated herein to reduce infarct volume and edema and improve neurological outcome.

[00163] Accordingly in an embodiment, the subject has suffered and/or is suffering a stroke (e.g. ischemic neuronal damage).

[00164] An embodiment, includes a method of reducing ischemic injury in a subject in need thereof comprising administering a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor to the subject.

[00165] Also provided is use of a PPC inhibitor, for example a SKI-1 and/or Furin inhibitor to reduce ischemic injury in a subject in need thereof. In another embodiment, the disclosure includes

a PPC inhibitor, for example a SKI-1 and/or Furin inhibitor for use to reduce ischemic injury in a subject in need thereof.

[00166] Another aspect includes a method of treating an ischemic neuronal injury comprising administering to a subject in need thereof a composition comprising a SKI-1 inhibitor and/or a Furin inhibitor.

[00167] Also provided is use a SKI-1 and/or Furin inhibitor to treat ischemic injury in a subject in need thereof. In another embodiment, the disclosure includes a SKI-1 and/or Furin inhibitor for use to treat ischemic injury in a subject in need thereof.

[00168] Nerve damage can be caused by neurodegenerative diseases including but not limited to multiple sclerosis, Alzheimer's disease, dementia, diabetes, Parkinson's disease and Huntington's disease. The need for neuron survival, axonal growth and/or regeneration is a common theme in neurodegenerative disease.

[00169] Another aspect includes a method of treating a neurodegenerative disease, the method comprising administering to a subject in need thereof a PPC inhibitor, optionally a SKI-1 inhibitor and/or a Furin inhibitor, and/or a composition comprising the PPC inhibitor.

[00170] Also provided is use of a PPC inhibitor, for example a SKI-1 and/or Furin inhibitor treating a neurodegenerative disease in a subject in need thereof. In another embodiment, the disclosure includes a PPC inhibitor, for example a SKI-1 and/or Furin inhibitor for use in treating a neurodegenerative disease in a subject in need thereof.

[00171] As demonstrated in the Examples and Figure 14, SKI-1 inhibitors are sufficient to prevent the release of soluble RGMa, such as N-RGMa fragments and NN-RGMa. As demonstrated in Figure 15, administration of a SKI-1 inhibitor significantly reduced the infarct volume and edema compared to Control. Additionally, neurological outcome was markedly improved in animals treated with SKI-1 inhibitor at 48h post-stroke induction. As demonstrated in Figure 16, inactivation of SKI-1, which prohibits the cleavage of full-length RGMa into its active fragments and thus binding to Neogenin, promotes cell survival and axonal outgrowth.

[00172] The amount administered is for example an effective amount to induce neuron survival, axon growth and/or regeneration.

[00173] Further, RGMa has been implicated in multiple sclerosis. Indeed a genetic link has been suggested between RGMa mutations and the development of the disease in human patients

(Nohra et al., 2010) and RGMa has been shown to activate the immune system in experimental models of MS (Muramatsu et al., 2011).

[00174] Accordingly, in an embodiment, the subject has multiple sclerosis and the method, use or inhibitor is for treating multiple sclerosis.

[00175] Muramatsu et al 2011 also showed neutralizing antibodies to RGMa attenuated clinical symptoms of mouse myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) and reduced invasion of inflammatory cells into the CNS. Silencing of RGMa in MOG-pulsed BMDCs reduced their capacity to induce EAE following adoptive transfer to naive C57BL/6 mice.

[00176] Accordingly a further embodiment includes a method of inhibiting an immune response in CNS tissues, e.g. brain and spinal cord in multiple sclerosis patients comprising administering a SKI-1 inhibitor and/or a furin inhibitor.

[00177] Another embodiment includes a method of inhibiting demyelination in a subject in need thereof comprising administering a SKI-1 inhibitor and/or a furin inhibitor.

[00178] Yet another aspect includes a method of treating a subject with a multiple sclerosis comprising administering to a subject in need thereof a PPC inhibitor, optionally a SKI-1 inhibitor and/or a Furin inhibitor, and/or a composition comprising the PPC inhibitor.

[00179] Accordingly, in an embodiment, the PPC inhibitor is for treating glaucoma, stroke, MS, Parkinson's disease, Alzheimer's disease, nervous system inflammatory conditions, and/or spinal cord and peripheral nerve injuries in a subject in need thereof. As demonstrated in Example 5, administration of a PPC inhibitor significantly reduces tissue damage in a stroke rodent model and improves neurological outcome.

[00180] Treatment efficacy can be monitored for example by conducting neurological tests known in the art, relevant for the particular nerve damage.

[00181] Also included are uses for promoting neuron survival, axon growth and/or regeneration and for treating nerve damage, including spinal cord injuries and damage related to neurodegenerative disease and/or stroke as well as compositions for use in promoting neuron survival, axon growth and/or regeneration treating nerve damage, including spinal cord injuries and damage related to neurodegenerative disease and/or stroke.

[00182] In an embodiment the PPC inhibitor is selected from a serine protease inhibitor, optionally a membrane permeable serine protease inhibitor if the inhibitor is used for applications

involving cells. In an embodiment, the membrane permeable serine protease inhibitor is selected from an AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) peptide and an ER permeable serine protease inhibitor. In an embodiment, the PPC inhibitor is RVKR peptide (SEQ ID NO:3) for example decanoyl-RVKR-cmk and derivatives.

[00183] In an embodiment, the PPC inhibitor is a SKI-1 inhibitor, such as RRLL peptide (SEQ ID NO:4), PF-429242 (Pasquato et al, 2012), peptide CIYISRRLLC (SEQ ID NO:5; optionally with terminal "C" residues cyclized) (Pasquato et al, 2011) and/or prosegment inhibitor R134E.

[00184] The peptide inhibitors, for example RVKR (SEQ ID NO:3), RRLL (SEQ ID NO:4) and CIYISRRLLC (SEQ ID NO:5; optionally with terminal "C" residues cyclized) can be synthesized using methods known in the art . The peptides can also be modified for example by addition of N and C term moieties including for example CMK and Decanoyl to promote cell permeability. To promote stability cyclic peptides can be prepared using methods known in the art

[00185] In another embodiment, the PPC inhibitor is a furin inhibitor. In an embodiment, the furin inhibitor is a furin prosegment inhibitor

[00186] The inhibitors can be suitably formulated into pharmaceutical compositions for administration to human subjects in a biologically compatible form suitable for administration *in vivo*.

[00187] Such compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions that can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle.

[00188] Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (2003 - 20th edition). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more than one pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

[00189] Pharmaceutical compositions include, without limitation, lyophilized powders or aqueous or non-aqueous sterile injectable solutions or suspensions, which optionally further contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially compatible with the tissues or the blood of an intended recipient. Other components that are optionally present in such compositions include, for example, water, surfactants (such as Tween^{TM}), alcohols, polyols, glycerin and vegetable oils. Extemporaneous injection solutions and suspensions

may be prepared from sterile powders, granules, tablets, or concentrated solutions or suspensions. The composition can be supplied, for example, but not by way of limitation, as a lyophilized powder which is reconstituted with sterile water or saline prior to administration to the subject.

[00190] Suitable pharmaceutically acceptable carriers include essentially chemically inert and nontoxic compositions that do not interfere with the effectiveness of the biological activity of the pharmaceutical composition. Examples of suitable pharmaceutical carriers include, but are not limited to, water, saline solutions, glycerol solutions, ethanol, N-(1(2,3-dioleyloxy)propyl)N,N,N-trimethylammonium chloride (DOTMA), diolesyl-phosphotidyl-ethanolamine (DOPE), and liposomes. Such compositions should contain a therapeutically effective amount of the inhibitor(s) together with a suitable amount of carrier so as to provide the form for direct administration to the subject.

[00191] In an embodiment, the inhibitor(s) and/or composition comprising the inhibitor(s) is/are administered, for example, by parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol or oral administration.

[00192] In another embodiment, the disclosure describes a pharmaceutical composition wherein the dosage form is an injectable dosage form. An injectable dosage form is to be understood to refer to liquid dosage forms suitable for, but not limited to, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, or intranasal administration. Solutions of compounds described herein can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Or for example, can be prepared in a sodium chloride solution, for example a 0.9% sodium chloride solution or a dextrose solution for example a 5% dextrose solution.

[00193] Dispersions can also be prepared in glycerol, liquid polyethylene glycols, DMSO and mixtures thereof with or without alcohol, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. A person skilled in the art would know how to prepare suitable formulations. Conventional procedures and ingredients for the selection and preparation of suitable formulations are described, for example, in Remington's Pharmaceutical Sciences (2003 - 20th edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999.

[00194] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersion and sterile powders for the extemporaneous preparation of sterile injectable

solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists.

[00195] In an embodiment, the inhibitor(s) and/or composition is/are administered by intravenous, intraspinal and/or intracranial infusion. In another embodiment he inhibitor(s) and/or composition is/are administered by Intraperitoneal and/or intrathecal application.

[00196] In an embodiment, the PPC inhibitor, optionally SKI-1 inhibitor and/or furin inhibitor is administered in conjunction with another therapy. For example, Wang et al reported that olfactory stimulation reduced levels of RGMa in an ischemia/reperfusion injury, a model for stroke, and was associated with improved response.

[00197] In some embodiments, the PPC inhibitor optionally a SKI-1 inhibitor and/or furin inhibitor, is administered in conjunction with additional agent. For example, in certain embodiments, the additional agent may be administered separately, as part of a multiple dose regimen, from the PPC inhibitor. In other embodiments, these agents may be part of a single dosage form, mixed together with PPC inhibitors in a single composition. In still another embodiment, these agents can be given as a separate dose that is administered at about the same time.

III. Kits

[00198] Also provided in another aspect is a kit comprising one or more of a PPC inhibitor, such as a SKI-1 inhibitor and/or a furin inhibitor, a RGMa fragment, a composition, isolated polypeptide, isolated nucleic acid, and/or an antibody, described herein, instructions for use, and/or a vial for housing the PPC inhibitor, RGMa fragment or antibody. In an embodiment, the RGMa fragment and/or antibody is attached to a substrate. In another embodiment the antibody comprises a detectable label, preferably capable of producing, either directly or indirectly, a detectable signal. For example, the label may be radio-opaque or a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{123}I, ^{125}I, ^{131}I; a fluorescent (fluorophore) or chemiluminescent (chromophore) compound, such as fluorescein isothiocyanate, rhodamine or luciferin; an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase; an imaging agent; or a metal ion.

[00199] The above disclosure generally describes the present application. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the application. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

[00200] The following non-limiting examples are illustrative of the present application:

Examples

Example 1

Summary

The nervous system is enormously complex, yet the number of cues that control axonal growth is surprisingly meager. Post-translational modifications amplify diversity, but the degree to which they are employed is unclear. Here, we show that Furin and SKI-1 combine with autocatalytic-cleavage and a disulphide-bridge to generate four membrane-bound and three soluble forms of the Repulsive Guidance Molecule (RGMa). We provide in vivo evidence that these proprotein-convertases are involved in axonal growth and that RGMa-cleavage is essential for Neogenin-mediated outgrowth inhibition. Surprisingly, despite no sequence homology, N- and C-RGMa fragments bound the same Fibronectin-like domains in Neogenin and blocked outgrowth. This represents an example in which unrelated fragments from one molecule inhibit outgrowth through a single receptor-domain. RGMa is a tethered membrane-bound molecule, proteolytic processing amplifies RGMa diversity by creating soluble versions with long-range effects as well.

Results

Complex in vivo RGMa processing

[00201] Western blotting was performed on membrane preparations under reducing (+DTT) and non-reducing conditions (-DTT) to allow analysis of the RGMa disulfide bridge. As reported, membranes from COS-7 cells transfected with RGMa contained a single 60 kDa protein, which DTT reduced to a 33 kDa C-terminal RGMa fragment ("C-RGMa") due to the presence of a autocatalytic-cleavage site at residue 150 (Figure 1A; B, lanes 1,2; Tassew et al., 2009). Some of the 60 kDa band remained even after DTT treatment (Figure 1B, lane 2) suggesting that membranes contain both cleaved and uncleaved full length RGMa (Figure 1A, CFL-RGMa and U-RGMa, respectively; Tassew et al., 2009). Whereas the C-terminal antibody only detected full length 60 kDa protein on non-reduced COS-7 membranes (Figure 1B, lane 1), this protein plus the 33 kDa C-RGMa fragment and a novel 37 kDa species ("RGMa37") were observed on non-reduced membrane preparations from chick brain (Figure 1B, lane 3). Thus, in brain C-RGMa is present on membranes both as a linked heterodimer with N-RGMa (33 kDa+22 kDa), and on its own (33 kDa alone) (Figure 1B). The RGMa37 fragment disappeared after treatment with DTT (Figure 1B, lane 4) and must, therefore, arise from a second in vivo cleavage site located within the N-terminal part of RGMa, and consists of the 33 kDa C-RGMa fragment disulphide linked to a calculated 4 kDa N-RGMa fragment (Figure 1A). In summary, Western blots with the C-terminal antibody suggest the presence of four

distinct membrane-anchored RGMa proteins in vivo: CFL-RGMa (60 kDa), U-RGMa (60 kDa), C-RGMa (33 kDa) and RGMa37 (37 kDa).

RGMa is processed into soluble proteins that inhibit axonal growth

[00202] To further test and extend these conclusions we sought to identify cell lines which, unlike COS-7, recapitulate the more complex cleavage pattern seen in chick brain. Strikingly, Western blotting with the C-terminal antibody on membranes from HEK293, and SH-SY5Y cells, but not COS-7 or DF1 cells, transfected with RGMa showed a pattern similar to the one observed in brain (Figure 1B, lanes 5,6; Figure 2). While RGMa processing in HEK293 or SH-SY5Y cells did not seem as efficient as in the brain, these model cell lines could be used to study this process. The presence of two cleavage sites within the N-terminal domain should lead to the release of two small N-terminal fragments (Figure 1A, N-RGMa and NN-RGMa, respectively). Thus, we sought to identify these fragments in chick brain matrix extracts using N-terminal anti-RGMa antibodies. Surprisingly, we could only reveal the presence of one 30 kDa band that was bigger than any of the expected N-terminal fragments (Figure 1C). We extended this study to the medium of RGMa-transfected HEK293 cells and detected two RGMa proteins with apparent molecular masses of 60 kDa and 30 kDa (Figure 1C, lane 1). The 60 kDa has been described before and results from RGMa shedding from the membrane (RGMaΔ) by a phospholipase (Hata et al., 2006) and may not be biologically relevant as it is not seen in brain matrices. The 30 kDa band was broad, so we surmised that it represented a glycosylated N-terminal fragment. To test this hypothesis, we removed N-glycosylations using PNGaseF and assessed its resulting molecular mass by Western blot. Once unglycosylated (UG), the 60 kDa was reduced to one 45 kDa band whereas the 30 kDa resulted in 2 bands (18- and 22 kDa) that correspond to N-RGMa and NN-RGMa (Figure 1C). Thus, RGMa processing in HEK leads to the secretion of 3 soluble fragments: N-RGMa (30-; 22 kDa unglycosylated (UG)), NN-RGMa (30-; 18 kDa (UG)) and RGMaΔ (60–; 45 kDa (UG)).

[00203] To address the role of RGMa soluble proteins on growing axons, we cultured retinal explants in the presence of supernatant from cells that were either Mock transfected or transfected with full length RGMa (Figure 1D-F). Interestingly, cells grown on supernatant from RGMa-transfected HEK293-cells showed markedly reduced axonal growth (41.3±6 µm) relative to those grown on media from Mock transfected cells (131±2.1 µm). Thus, RGMa expressing cells release factors that hamper axonal growth.

SKI-1 and Furin process RGMa

[00204] The identification of 33- and 37-kDa species in membranes could be explained by two cleavage events, one within the N-terminal domain and one next to the autocatalytic site, respectively (Figure 1A). To identify the enzyme(s) that cleave(s) RGMa we treated cells with protease inhibitors. Of the many inhibitors tested, only AEBSF, a membrane permeable inhibitor for serine proteases, showed a significant reduction of RGMa cleavage (Figure 3A, 4A). Interestingly, another non-membrane permeable serine protease inhibitor (Aprotinin) did not show any reduction in cleavage. Because this indicates that RGMa is cleaved on its way towards the cell surface, we tested two inhibitors of transport from the endoplasmic reticulum (ER) to the Golgi (Seidah et al., 1999). Both Golgicide A and Brefeldin A did not have any effect on RGMa processing, indicating that it is cleaved within the ER (Figure 4B). This behavior corresponds to the one observed with Proprotein Convertases (PCs), hence, we tested the cell-permeable RVKR peptide, a broad inhibitor for PCs (Seidah and Chretien, 1999). Membranes were analyzed by Western Blotting under non-reducing conditions, which showed that both the 33- and the 37- kDa bands, were completely abolished by RVKR treatments (Figure 3A). PCs normally require two arginines for cleavage, however, we could not identify any PC consensus sequence next to the autocatalytic site. Thus, we suspected that another PC that does not require arginines to induce cleavage was involved in RGMa processing. Because Subtilisin Kexin Isoenzyme-1 (SKI-1) does not require a basic residue at the cleavage site (Pasquato et al., 2006), we tested whether specific SKI-1 inhibition with the cell-permeable RRLL peptide inhibitor could prevent RGMa processing. Indeed, this peptide completely abolished formation of the 33 kDa band but not the 37 kDa suggesting that SKI-1 mediates generation of the former protein (Figure 3B). In all cases, the presence of DTT resulted in the formation of the 33 kDa band indicating that treatments with these inhibitors did not suppress the auto-catalytic cleavage event (Figure 3B).

[00205] To confirm that SKI-1 is involved in RGMa processing, we expressed a prosegment inhibitor of this enzyme (R134E; Figure 3C; Pullikotil et al., 2004) together with RGMa. Here again, SKI-1 inhibition dramatically reduced formation of the 33 kDa band (Figure 3C). Next, we tested whether Furin processes RGMa by using a Furin-prosegment inhibitor construct that specifically prevents Furin cleavage (ppFurin; Zhong et al., 1999). Here, the formation of the 37 kDa species was blocked suggesting the involvement of Furin (Figure 3D). This was confirmed by the fact that soluble RGMa formed a 37kDa band when treated by purified Furin (Figure 4). Finally, to confirm that Furin and SKI-1 generate soluble N-terminal peptides, we studied cell supernatants after treatment with RVKR. As expected, the 60 kDa band but not the 30 kDa band was released by RGMa expressing cells (Figure 3E).

[00206] In summary, RGMa processing is far more complex than previously appreciated; thus, autocatalytic cleavage and proteolytic cleavage by SKI-1 and Furin generate seven distinct species (Figure 1A). Furthermore, RGMa is N-glycosylated, adding to the complexity of its posttranslational modifications (Figure 1).

SKI-1 and Furin are involved in axonal guidance

[00207] Although Furin activates Semaphorins (Adams et al., 2007), its in vivo function on growing axons has never been studied. SKI-1 has no reported action on any guidance molecule. Thus, we assessed the role of these secretory proteases on visual paths. First, we studied expression of these proteins using in situ hybridization in the optic tectum. Our data revealed that both proteins are expressed in RGMa expressing radial glia cells (Figure 3F-H), which fits the idea that they may be involved in retino-tectal pathfinding. Next, we performed ectopic-expression of SKI-1- (R134E) and Furin-inhibitors (ppFurin) in developing chicks (Figure 3I-K) using the RCAS viral vector and studied retinal tracts from the eye to the optic tectum using DiI tracing. As expected, both SKI-1 and Furin inhibitions induced axonal phenotypes within the tectum. We observed that many fibers failed to target the terminal zone and arborized randomly within the tectum, when compared to controls (Figure 3I-K). ppFurin induced aberrant paths in all embryos (n=8) and 70% (7 of 10) of the R134E embryos but none of the controls (n=6) displayed phenotypes. Furin and SKI-1 may regulate the activity of other guidance molecule. Indeed, Furin activates Semaphorins, which are critical for the growth of many axons (Adams et al., 2007). While these experiments do not directly show that RGMa cleavage by Furin and SKI-1 regulates axonal paths, they are consistent with that model and provide in vivo evidence that these enzymes play a critical role in the regulation of axonal growth.

RGMa cleavage is required for Neogenin binding and outgrowth inhibition

[00208] The above insights raise important questions regarding the biological relevance of each of the multiple cleavage sites and the relative activity of each resulting peptide. First we examined whether RGMa cleavage is required for interaction with Neogenin. For this, we generated mutations next to the auto-catalytic cleavage site (D149A, H151A; chick positions. Corresponding positions in human are D168 and H170) and showed that these mutations altered all cleavage events (Figure 5B). These mutations did not change RGMa processing towards the cell surface (Figure 6). We then performed an ELISA assay in which Neogenin-AP interacts with membranes from COS-7 cells transfected with RGMa constructs (Figure 5A-C; 6A-C). In agreement with previous observations, wt RGMa interacted with Neogenin-AP (Rajagopalan et al., 2004), however, the two RGMa mutants did not interact with Neogenin (Figure 5B,C). Binding of

membrane bound RGMa to Neogenin is believed to be necessary for RGMa to exert its inhibition on axonal growth (Rajagopalan et al., 2004). To address this issue, we tested whether the RGMa mutants (D149A, H151A) inhibit axonal growth of Neogenin-expressing temporal axons. As controls, we cultured nasal axons that express lower amounts of Neogenin. As expected, temporal explants grown on wt RGMa membranes extended shorter axons (197.6±7.5 µm) when compared to the otherwise long axons on Mock membranes (407.9±16.7 µm) (Figure 5D,E). Outgrowth on U-RGMa mutants was 70.8% (335.7±11.7 µm; $P<0.0001$; D149A) and 82.2% longer (360±6.1 µm; $P<0.0001$; H151A) than on wt RGMa (Figure 5D,E). Because they express lower amounts of Neogenin, the reduction in length for nasal axons on wtRGMa vs control was lesser than the one observed with temporal axons (Figure 5E).

[00209] To confirm that catalytic cleavage is essential for Neogenin-mediated inhibition, we studied neurite growth on Neogenin-expressing NIE-115 cells (Endo et al., 2009). RGMa-containing membranes inhibited NIE-115 neurite extension compared to control Mock-transfected membranes (Figure 5F,G). This effect was Neogenin-dependent as it was suppressed when cells were treated with a Neogenin shRNA (Figure 5G; 6), but not with control shRNA (Figure 5F,G). Here again, non-cleavable mutants did not inhibit growth (Figure 5F,G).

[00210] We next examined the effect of RGMa cleavage on axonal pathfinding and performed ectopic expression of wt RGMa and U-RGMa (H151A). As expected, tectal infection with a vector that expressed wt RGMa induced strong pathfinding mistakes (Figure 5H). In 100% of the embryos (13 of 13), we observed that axons established terminal arbors outside the predicted terminal zone (TZ) indicating that RGMa over-expression perturbed pathfinding. In contrary, no obvious axonal phenotype was observed when H151A was over-expressed in the tectum (Figure 5I). Here, 89% (8 of 9) of the embryos displayed normal behavior with all fibers terminating their growth within the predicted TZ.

[00211] Together these data demonstrate that RGMa catalytic processing is required for interaction with Neogenin and the resulting neurite/axon growth inhibition.

C-RGMa on its own is sufficient for Neogenin binding and growth inhibition

[00212] It has been assumed that C-RGMa on its own is sufficient to inhibit axonal growth (Rajagopalan et al., 2004). This notion derives from experiments that were performed before the identification of a disulfide bridge between N- and C-RGMa (Tassew et al., 2009), and remains to be tested. C-RGMa (33 kDa) is poorly targeted to the cell surface when expressed on its own (Tassew et al., 2009). Thus, to target C-RGMa to the cell surface, we developed a construct (C-$RGMa^{TEV}$) in which the original autocatalytic cleavage site between N- and C-RGMa was replaced

by a TEV cleavage site (Phan et al., 2002; Figure 7A, 8). In ELISAs, either $RGMa^{wt}$ or $C\text{-}RGMa^{TEV}$ membrane preparations bound Neogenin-AP, but notably $C\text{-}RGMa^{TEV}$ (*Kd*= 6.8 nM) had a higher affinity than $RGMa^{wt}$ (*Kd*= 11.96 nM; Figure 7C-E). These data suggested that C-RGMa may inhibit axonal growth more effectively that the full length protein. Indeed, membranes containing $C\text{-}RGMa^{TEV}$ inhibited temporal axons more potently than $RGMa^{wt}$ (Figure 7F-G). As expected, outgrowth from nasal axons was significantly higher, which is consistent with a role for Neogenin as the receptor (Figure 7G). To confirm that Neogenin mediated C-RGMa inhibition we performed silencing experiments using NIE-115 cells on $C\text{-}RGMa^{TEV}$, $RGMa^{wt}$ and Mock-transfected membranes. In agreement with retinal axon data, $C\text{-}RGMa^{TEV}$ and $RGMa^{wt}$ significantly hampered neurite growth, an effect that vanished in the presence of Neogenin shRNA but not control shRNA (Figure 7H,I).

[00213] To rule out the possibility that the activity in $C\text{-}RGMa^{TEV}$ membranes resulted from unwashed N-RGMa fragments, we sought to identify the C-RGMa domain that interacts with Neogenin (Figure 7J). Using an interaction assay, we identified a domain spanning aa residues 182-271 that is sufficient for interaction with Neogenin (Figure 7J,K). In outgrowth experiments, $C\text{-}RGMa^{182\text{-}271}$ strongly inhibited temporal fibers (Figure 8B). A lesser effect was obtained on nasal fibers suggesting that this effect is mediated by Neogenin (Figure 7L). To assess the function of $C\text{-}RGMa^{182\text{-}271}$ on retino-tectal map formation we performed its ectopic expression in the chick tectum (Figure 8B-E). In 90% (9 of 10) of the embryos, $C\text{-}RGMa^{182\text{-}271}$ expression resulted in aberrant pathfinding (Figure 7M-O). Together, these data demonstrate that C-RGMa on its own is sufficient for Neogenin interaction and inhibition of axonal growth.

Three RGMa soluble proteins inhibit axonal outgrowth via Neogenin

[00214] Because RGMa is GPI anchored and has the same action on growing fibers as ephrins (Drescher et al., 1997), it has been assumed that only membrane bound RGMa inhibits axons (Monnier et al., 2002). The release of three soluble RGMa proteins (RGMaΔ, N-RGMa and NN-RGMa; Figure 1A) has never been reported before, hence their functions remain unknown. To assess the functions of soluble forms of RGMa, we tested purified proteins on growing fibers (Figure 10). Strikingly, RGMaΔ, N-RGMa and NN-RGMa significantly inhibited retinal fibers in a concentration-dependent manner (Figure 9B,C). All proteins restricted outgrowth and displayed a greater activity on temporal vs nasal fibers, indicating that their receptor is present in higher quantities on temporal fibers. Remarkably, the shorter NN-RGMa fragment was the most potent inhibitor of outgrowth as 1 µg/ml of this protein led to 71% inhibition of temporal fibers, similar to that obtained with 10 µg/ml of N-RGMa (74%) or 20 µg/ml of RGMaΔ (72%; Figure 9C).

[00215] Because all fragments displayed stronger effect on temporal vs nasal axons, we tested whether Neogenin mediates their inhibition. NIE-115 cells expressing a control shRNA extended shorter processes on RGMaΔ, N-RGMa and NN-RGMa compared to laminin alone, which was suppressed in cells expressing Neogenin-shRNA (Figure 9D,E). Here also, NN-RGMa (5 µg/ml) was more potent than N-RGMa (10 µg/ml) and RGMaΔ (20 µg/ml; Figure 9E). Together with the temporal/nasal difference described above (Figure 9C) these data show that Neogenin mediates inhibition by N-RGMa, NN-RGMa, and RGMaΔ (Figure 9D,E). Unexpectedly, this revealed that unlike ephrins, soluble RGMa proteins inhibit axonal growth.

RGMa soluble proteins display various affinities to Neogenin

[00216] These data prompted us to examine whether Neogenin interacts directly with soluble N-terminal RGMa proteins. We performed pull down on supernatants from cells expressing N-RGMa or NN-RGMa, and observed co-immunoprecipitation with Neogenin- but not BSA- coated beads (Figure 9F), raising the possibility that they directly interact with Neogenin. Because RGMa proteins displayed varying potency, we determined binding affinities to Neogenin (Figure 9G-J). Purified RGMaΔ bound to Neogenin-AP with a *Kd* of 16.9 nM (Figure 9G). The interaction was specific because RGMa-AP did not bind detectably to RGMa fragments and Neogenin–AP did not bind to BSA-coated wells. In the same assay, N-RGMa exhibited a higher affinity to Neogenin-AP with a calculated *Kd* of 11.8 nM (Figure 9H). Strikingly, the affinity of Neogenin for NN-RGMa was substantially greater with a *Kd* of 1.5 nM, 11-fold greater than the soluble full length RGMaΔ protein (Figure 9I,J). A similar study in which NN-RGMa-AP and N-RGMa-AP interact with Neogenin-coated plates was performed and confirmed NN-RGMa's higher affinity (*Kd*=1.2 nM) for Neogenin compared to N-RGMa (*Kd*=8.2 nM; Figure 10). These affinities correlate with the inhibitory action of RGMa proteins on outgrowth (Figure 9C).

N-RGMa and NN-RGMa perturb pathfinding *in vivo*

[00217] RGMa gain- and loss- of function experiments indicate that it is a key protein for the establishment of visual maps (Matsunaga et al., 2006). However, these experiments did not establish which RGMa fragment(s) is (are) involved in pathfinding. The fact that secreted RGMa proteins may control retinal axon pathfinding was unexpected as RGMa was believed to i) function similar to ephrins, which require polymerization in membranes to guide axons (Egea and Klein, 2007) and ii) use its C-terminal portion to inhibit axonal growth. To assess a role of N-RGMa, and NN-RGMa during retinal-map formation, we performed ectopic expression of these proteins in the developing tectum using viral-mediated expression (Figure 12). In controls (empty virus), retinal fibers directly targeted the predicted terminal zone (TZ; Figure 11A). However, when N-RGMa and

NN-RGMa were over-expressed (Figure 9) pathfinding was greatly perturbed (Figure 11B-D). Abnormal axonal paths included terminations outside the TZ, aberrant turns within the optic tectum, as well as the absence of a clear TZ (Figure 11B-D). *In situ* hybridization has demonstrated that expression patterns of other guidance molecules are not affected by ectopic expression of RGMa (Matsunaga et al., 2006), thus, the axon-targeting phenotype arises from an effect of over-expression of RGMa constructs and not from a modification of other proteins. In summary, ectopic expression of soluble RGMa N-terminal proteins indicates that they are involved in the formation of visual maps.

C- and N- RGMa proteins interact with the same Neogenin FNIII(3-4) domain

[00218] We next sought to identify the Neogenin domain(s) with which C- and N-RGMa proteins interact. The extracellular portion of Neogenin contains four Immunoglobulin like (4Ig) and six Fibronectin type III (6FNIII) domains (Figure 13A). It has been reported that 6FNIII interacts with full length RGMa (Rajagopalan et al., 2004). Using 6FNIII-AP, we showed that C- and N-RGMa proteins interact with 6FNIII (Figure 13A). Next, we sought to identify which sub-region of the 6FNIII domain(s) interact with N- and C-RGMa. To do so, we generated Neogenin constructs that contain only some of the 6 FNIII domains (Figure 13A). In interaction assays, the FNIII (2-5) domains bound to all C- and N-RGMa proteins, which contrasted with domains 1-3 and 4-6 that did not interact with any RGMa protein (Figure 13A). To further refine the location of the binding motif, we generated a construct that only contains the FNIII(3-4) domains, and showed that this segment was sufficient for binding to each one of the RGMa proteins (Figure 13A). Because C- and N-RGMa fragments bind to the same Neogenin subdomain, we tested whether they interfere with each other. In these experiments, we assessed 6FNIII-AP binding to C- or N-RGMa after incubation with either one of these proteins. As expected, pre-incubation of Neogenin with C-RGMa abolished binding to C-RGMa. Interestingly, pre-incubation with N-RGMa also inhibited binding of Neogenin to C-RGMa, indicating that C- and N-RGMa interfere in their binding to Neogenin (Figure 13B). In agreement with these data, binding to N-RGMa was also altered when Neogenin was pre-incubated to either C- or N-terminal fragments (Figure 13B) Next we studied the effect of the combined presence of RGMa proteins on axonal growth. When axons were grown on equimolar concentrations of either N-RGMa (5µg/ml), C-RGMa$^{182-271}$ (5µg/ml, these proteins have the same apparent molecular weight) or N-RGMa (2.5µg/ml) + of C-RGMa$^{182-271}$ (2.5µg/ml), all treatments resulted in inhibitions that did not significantly differ from each other (Figure 13C,D). This represents the third indication that both N- and C-fragments act through FNIII(3-4).

[00219] To further assess the role of FNIII(3-4) in RGMa inhibition, we tested its function-blocking capability. Retinal axons were grown on RGMa proteins in the presence of 1μg/ml of FNIII(3-4). Remarkably, the presence of this fragment restored axonal growth on RGMaΔ, N-RGMa, and C-RGMa, indicating that it blocked the inhibitory activities of these proteins on retinal axons (Figure 13E-F). Together these data represent a unique example in which two unrelated domains of a same guidance molecule inhibit axonal growth through interaction with the same receptor region.

Inhibition of SKI-1 improves functional outcomes of stroke and reverses the deleterious effects of RGMa on cell survival and axonal outgrowth

[00220] As shown in Figure 15, administration of a SKI-1 inhibitor significantly reduced the infarct volume and edema compared to Control. Additionally, neurological outcome was markedly improved in animals treated with SKI-1 inhibitor at 48h post-stroke induction. Additionally, the number of fibres and axonal growth were reduced in cells grown on supernatant from full-length RGMa-transfected HEK293 cells (Figure 16). Importantly, inhibition of SKI-1 by the small molecule PF429242 restored the deleterious effect of RGMa on fibre number and axonal outgrowth. These data provide in vivo evidence that inactivation of SKI-1, which prohibits the cleavage of full-length RGMa into its active fragments and thus binding to Neogenin, promotes cell survival and axonal outgrowth and improves functional outcome of stroke.

Discussion

[00221] RGMa has critical roles in axonal growth, cell differentiation, apoptosis, neuronal regeneration, and bone development (Monnier et al., 2002; Matsunaga et al., 2004, 2006; Hata et al., 2006; Zhou et al., 2010).

[00222] In order to interact with Neogenin, RGMa needs to be cleaved. Because the interaction between RGMa and Neogenin is demonstrated in spinal cord injury and Multiple sclerosis (Hata et al., 2006; Muramatsu et al., 2011) it logic to postulate that preventing RGMa cleavage will promote regeneration in spinal cord injuries and treat MS, respectively.

[00223] The data revealed that proteolytic cleavage together with the formation of a disulfide bridge generate 4 membrane-bound and 3 soluble RGMa species. This level of complexity was not expected as it was believed that only full length RGMa, in which RGMa is either uncleaved or its N- and C-RGMa fragments remain attached by a disulfide bridge, is expressed *in vivo* (Tassew et al., 2009). The activity of individual RGMa proteins was studied and showed that all cleaved protein products inhibit neurite growth via Neogenin, while the full length uncleaved membrane-bound

RGMa is inactive. Hence, proteolysis is required for RGMa activity. Furthermore, we show that two Proprotein Convertases (PCs), namely Furin and SKI-1 are involved in RGMa processing and generate N-terminal soluble and C-terminal membrane bound proteins with different inhibitory activities. Surprisingly, RGMa fragments with no apparent sequence homology all interacted with the same Fibronectin domains in Neogenin to inhibit axonal growth.

[00224] Regulation of the activity of extracellular proteins by proteolytic cleavage is an emerging theme within the CNS (Adams et al., 2007). PCs form a family of nine proteinases with a large array of functions within the CNS (Seidah and Chretien, 1999). Some members of the family have been shown to process extracellular proteins thereby regulating axonal growth. For instance, Furin activates members of the Semaphorin family (Adams et al., 2007). Similarly, PC5/6A cleaves the neural adhesion molecule L1, an event that appears important for L1-dependent neurite growth of cerebellar neurons (Kalus et al., 2003). Thus far, SKI-1 has not been shown to play any role within the CNS. Therefore, this study uncovers a novel aspect of SKI-1 function in the CNS. SKI-1 knock out mice die at embryonic day 2-3 (Mitchell et al., 2001) and that of Furin die of heart defect at embryonic day 11 (Roebroek et al., 1998). Thus, a function for these proteins could not be assessed within the developing CNS. Using local perturbation of SKI-1 and Furin function in the developing tectum, we generated evidence that both proteins regulate axonal growth *in vivo*.

[00225] RGMa and ephrin-A5,-A2 are GPI-anchored proteins that guide retinal fibers (Monnier et al., 2002; Drescher et al., 1997). Thus, they have both been assumed to function as membrane bound cues. As shown here, however, soluble RGMa proteins can also strongly inhibit fiber growth. This is in stark contrast to ephrins, which require oligomerization in membrane clusters to be functionally active (Egea and Klein, 2007). Activity of ephrins is also regulated by proteolytic processing. For instance, ephrin-A2 is cleaved by Kuzbanian after binding to its receptor, a mechanism that leads to axon detachment and termination of signaling (Hattori et al., 2000). Strikingly, release of RGMa from the membrane has the opposite effect since un-cleaved membrane bound protein is inactive, and proteolysis creates active soluble RGMa proteins. Thus this new data invoke a new mechanism of action for RGMa in which the combination of long (soluble) and short (membrane bound) range guidance regulates topographic mapping.

[00226] Surprisingly, unrelated N- and C-terminal fragments inhibit axonal growth via the same Fibronectin domains in Neogenin. At first sight this result confirms a recent study in which we showed that both domains are involved in retino-tectal pathfinding (Tassew et al., 2009). Because in COS-7 cells, N- and C-RGMa are linked to each other by a disulfide bridge, the logical interpretation was that RGMa simultaneously requires N- and C-RGMa for interaction with

Neogenin. It is postulated that pathfinding towards the optic tectum can be controlled by independent and unrelated N- and C-terminal RGMa fragments. It is known for other proteins such as Nogo or NG2 that multiple distinct regions might contribute to their inhibitory activity (Oertle et al., 2003). However, unlike RGMa, each one of these regions uses a distinct receptor to transmit its inhibitory activities (Oertle et al., 2003). Ephrins have two domains that interact with the Eph receptors, however only one of these domains has an inhibitory action on growing fibers (Carvalho et al., 2006). Slit2 is processed into several peptides, however only the D2 inhibitory domain binds to robo-receptor whereas the other inhibitory domain D4 interacts with heparin sulfate (Seiradake et al., 2009). Thus, to our knowledge, RGMa represents the first example in which multiple inhibitory fragments from a single ligand regulate axonal growth through the same receptor domain.

[00227] As well as controlling connections within the developing CNS, RGMa is involved in neurodegenerative diseases. Thus, it is a major impediment to neuronal regeneration and antibodies that neutralize C-RGMa promote regeneration (Hata et al., 2006). Moreover, there is growing evidence that RGMa is a key player in multiple sclerosis (Muramatsu et al., 2011; Nohra et al., 2010). In the light of the new data disclosed herein, multiple RGMa-fragments may contribute to the negative environment that hampers regeneration following CNS injury. Optimal approaches to deactivate RGMa should target all inhibitory C- and N-terminal fragments. The newly generated data indicate that specific SKI-1 inhibition, using for example the RRLL peptide, is sufficient to prevent the formation of C- and N-terminal RGMa peptides (Fig.14).

Experimental procedures

[00228] **Cloning, Expression and Purification.** All RGMa constructs were cloned in pSectag2B vector (Invitrogen) with an N-terminal His-tag. They were then transferred to RCAS BP(B) vector for viral production. SKI-1 and ppFurin were cloned in the bicistronic eGFP-containing pIRES vector (invitrogen) as we published before (Pullikotil et al., 2004).

[00229] C-RGMa-TEV was cloned by inserting a TEV cleavage site between N-RGMa and C-RGMa. Membranes were prepared from transfected cells, washed and resuspended in 1x TEV buffer and cleaved with AcTEV protease (Invitrogen) ON at 4°C. Membranes were washed to remove the cleaved N-terminal part, and re-suspended in PBS.

[00230] Soluble proteins were purified using Ni-NTA agarose (Invitrogen), and dialyzed in PBS. Anti-His (Qiagen), and anti RGMa (8B6; Tassew et al., 2009) were used.

[00231] Binding Assay. A 96 well plate was coated with i) Poly-L-Lysine (100µl, 10µg/ml) and ii) membrane suspensions (100µl) adjusted to an OD of 0.1 (at 220 nm) were added to the wells.

Plates were then centrifuged at 3000rpm (15min at 4oC), blocked with 5% BSA for 1h, and different concentrations of AP-tagged proteins were added for 3h. Wells were washed with PBS, incubated at 65oC for 1h to deactivate endogenous AP and developed with 1mg/ml pNPP.

[00232] For binding assay, wells were coated with purified proteins (3h at RT), and blocked with 5% BSA (1h), followed by incubation with AP-tagged proteins (2µg/well; 3h at RT). To quantify the binding, absorbance at 405nm was measured by using a microplate reader (EL 311SX, Bio-TEK Instruments Inc.). *Kd* and scatchard plots were obtained after fitting the data using a non-linear curve fit by Graphpad Prism 5 software.

[00233] Neogenin silencing and neurite outgrowth. Mouse Neogenin shRNA and control shRNA were gifts from Dr. Yamashita T. NIE-115 cells which endogenously express Neogenin were co-transfected with shRNA and GFP. 24 h later, cells were plated on coverslips coated with membranes from Mock, wtRGMa, D149A and H151A (OD of 0.1 at 220nm). Alternatively, cells were cultured on molar equivalent amounts of soluble proteins, RGMaΔ (20µg/ml), N- RGMa (10µg/mL) and NN-RGMa (5µg/ml). Cells were differentiated in 2% DMSO and neurite length was measured 48h later.

[00234] Retinal explants outgrowth Assay. Glass coverslips were coated with 10µg/ml Poly-L-Lysine, treated with Laminin (10µg/ml), and membrane preparations from transfected cells were added and centrifuged for 15 minutes at 3000xg and 4oC. Alternatively, different concentrations of soluble proteins mixed with Laminin (10µg/ml) were added to the coverslips and incubated for 3h at RT. Explants from the temporal and Nasal retina were then added to either membrane-or protein-coated surfaces in DMEM F-12 media (2% chick serum, 10% FBS) and incubated (37oC, 5% CO2) for 18h. Explants were fixed in 4%PFA, permeablized with 0.1%Triton X100, stained with Alexa488-fluor-phalloidin and viewed under a fluorescence microscope (Zeiss). The number and length of fibers were then quantified using Image Pro 5.0. Only explants which displayed growth were considered.

[00235] Cell treatments: HEK cells transfected with RGMa and grown for 24 h were treated with 50 µM RVKR, 300 µM AEBSF (ENZO life science), 50 µM RRLL (BACHEM) for 12h before membranes or supernatants were processed.

[00236] Pull-down assay. Proteins were coupled to activated-CNBr Sepharose (Pharmacia). Beads were then blocked with 100 mM Tris-HCl, pH 8 and washed. Supernatants from transfected cells were added to coupled beads for 2h at RT. Beads were then washed 6 X with PBS +0.02% Tween 20, and SDS loading buffer was added. Samples were boiled and subjected to Western Blotting.

[00237] Preparation of viral stocks: DF1 cells (DSHB) were transfected with RCAS constructs using Lipofectamine 2000 (Invitrogen). Cultures were expanded and supernatants were collected, pooled, and concentrated by centrifugation (21,000rpm, 2h) in a SW 28 rotor (Beckman). Viral titer was determined by infecting DF1 cells with serial dilutions and staining for the gag protein (AMV-3C2 Ab; DSHB). Titers of $1x10^8$IU/mL were used for infections.

[00238] *In ovo* injection and DiI tracing. Eggs (White Leghorn) were incubated at 38°C in high-humidity. At E1.5 viral solution (viral titers of 1×10^8 IU/ml) was injected in the tectum. At E15, a small DiI crystal (Molecular Probes) was placed in the temporo-dorsal part of the right eye. At E17, tecta were fixed in 4% PFA. DiI tracing was viewed under a fluorescent microscope (Olympus BX61) after cutting the tecta in half. Digital Images were taken and processed using Photoshop (Adobe).

[00239] Cerebral Focal Ischemia Model. Female Sprague–Dawley rats weighing 200–250 g were housed in a 12-h light: 12-h dark cycle and had free access to water and food. Focal cerebral ischemia was induced by injection of a preformed clot into the MCA, the rats was initially anesthetized with 3.0% isoflurane and then maintained with 1.5% isoflurane in a mixture of 30:70 O_2:NO_2 with a face mask during surgery. Body temperature was maintained at 37°C in the normothermic rats with a heating pad for the duration of surgery and immediate postoperative period until the animal recovered fully from anesthesia. A 1.5-cm longitudinal incision was made in the midline of the ventral cervical skin. The right common carotid artery, right internal carotid artery, and right external carotid were exposed. The distal portion of the ECA was ligated and cut. A modified polyethylene-10 catheter, filled with bovine thrombin (Thrombostat, TM Warner-Lambert Co.), was introduced into the lumen of the right ECA via a small puncture. Ten microliters of blood was withdrawn into the catheter and retained for 15 minutes to allow formation of a clot. Once the clot is formed, the catheter was advanced 17 mm into the internal carotid artery until its tip is 1–2 mm away from the origin of the MCA. The preformed clot in the catheter was then injected, and the catheter removed. Surgery was completed within 15 minutes, the wound was closed and the animal was returned to its cage. The animals (n=6) were randomly assigned to receive Saline (Control) or SKI-1 inhibitor (5 mg/kg). (PF-429242). Either saline or drug was administered intravenously through the tail vein immediately or every 12 hours after embolization.

[00240] Quantification of Brain Infarct Volume and Edema. The infarct volume was expressed as a percentage of the total volume from the ipsilateral hemisphere. Brain edema was determined by calculating the volume difference between the 2 hemispheres and dividing by the volume of the left hemisphere. 48 hours after MCA occlusion the anesthetized rats was killed. The brains was

removed from the skull and cooled in ice-cold saline for ~ 5 minutes. For morphometric examination, 2 mm-thick coronal sections were cut using a rat brain matrix. A total of 8 coronal sections was collected, and the sections was stained using a 2% 2, 3, 5-triphenyltetrazolium chloride solution. The infarct appears pale white on a background of red normal brain. The stained brain sections were scanned and the images were analyzed. Determination of infarct volume and edema in 2 groups were blinded. The infarct volume was calculated using the following formula: Infarct volume = [left hemisphere volume − (the right hemisphere volume − measured infarct volume)]/left hemisphere volume. Brain swelling was determined as follows: Swelling (edema) = (right hemisphere volume − left hemisphere volume)/left hemisphere volume. The infarct volume and brain swelling were expressed in percentage.

[00241] Functional Outcome (Neurological Status) Assessment. The neurological status of each rat was evaluated carefully at 0, 2, 8, 24, and 48 hours (four times for each rat) hr after ischemic injury by an observer blinded to the group assignment. Neurological deficits were determined using a modified Bederson's scoring system. In the modified system, the grading scale of 0-4 was used to assess the behavior, which is more clinical relevant with greater capability to assess the behavior compare with the unmodified scoring system (37,38). The animals were assessed and assigned a score based on the following grading system: 0, no observable deficit(normal); 1, forelimb flexion(moderate deficits); 2, forelimb flexion plus decreased resistance to lateral push(moderate deficits); 3, unidirectional circling(severe deficits); and 4, unidirectional circling plus decreased level of consciousness(severe deficits).

[00242] Statistical analysis. Quantifications were done for binding and outgrowth assays from at least 3 independent experiments. Statistical analysis was performed using ANOVA by XLSTAT. Results are expressed as the average ± SEM. Quantification of brain infarction volume and edema was analyzed with independent sample T-test. Neurological scores were analyzed with Wilcoxon Mann-Whitney test. A probability value < 0.05 is considered statistically significant.

Example 2

RGMa release was studied after transfection of HEK-293 cells with full length RGMa. In Untreated cells two RGMa peptides are apparent -soluble full length RGMa (RGMa delta) plus N-RGMa peptides. In the presence of either RRLL Or RVKR, none of the N-RGMa peptide is apparent (Figure 14) indicating that SKI-1 inhibition is sufficient to block release of N-RGMa peptides.

Example 3

[00243] Hata et al 2006 teach that a domain important for functional activity in chick RGM is the COOH-terminal 150–200 amino acids of the active RGM protein. A synthetic peptide (residues 309–322) was selected as immunogen to generate anti–rat RGMa rabbit antisera. The Hata experiments suggest that neutralizing C-RGMa promotes regeneration. The present data show that not only C-RGMa inhibits regeneration but also that N-RGMa is involved and even more potent. To neutralize both activities, inhibitors of SKI-1 that prevent activation into C- and N-terminal fragments are proposed.

Example 4

[00244] Data show that RGMa is upregulated following stroke. RGMa may therefore be involved in the non-regenerative property of the CNS. Schwab et al., 2005 reported following central nervous system injury, RGM, a novel, potent axonal growth inhibitor, is present in axonal growth impediments: the mature myelin, choroid plexus, and components of the developing scar.

[00245] As described above, embolization of a preformed clot resulted in an infarction in the ipsilateral hemisphere, mainly located in the MCA-irrigated region. As shown in Figure 15, SKI-1 inhibitor significantly reduced the infarct volume and edema compared to Control. Additionally, neurological outcome was markedly improved in animals treated with SKI-1 inhibitor at 48h post-stroke induction. Because the SKI-1 inhibitor reduced brain infarct volume and edema and improved functional outcome, it is possible that SKI-1 inhibitor acts on the brain's perfusion deficits for improvement in functional outcome following stroke.

Table of Sequences

Human RGMa

SEQ ID NO:1 - full length
mqpprerlvv tgragwmgmg rgagrsalgf wptlafllcs fpaatspcki lkcnsefwsa
tsgshapasd dtpefcaalr syalctrrta rtcrgdlayh savhgiedlm sqhncskdgp
tsqprlrtlp pagdsqersd speichyeks fhkhsatpny thcglfgdph lrtftdrfqt
ckvqgawpli dnnylnvqvt ntpvlpgsaa tatskltiif knfqecvdqk vyqaemdelp
aafvdgsknq gdkhganslk itekvsgqhv eiqakyigtt ivvrqvgryl tfavrmpeev
vnavedwdsq glylclrgcp lnqqidfqaf htnaegtgar rlaaaspapt apetfpyeta
vakckeklpv edlyyqacvf dllttgdvnf tlaayyaled vkmlhsnkdk lhlyertrdl
pgraaaglpl aprpllgalv pllallpvfc

SEQ ID NO: 2 - RGMa 1 to 175 amino acids
mqpprerlvv tgragwmgmg rgagrsalgf wptlafllcs fpaatspcki lkcnsefwsa
tsgshapasd dtpefcaalr syalctrrta rtcrgdlayh savhgiedlm sqhncskdgp
tsqprlrtlp pagdsqersd speichyeks fhkhsatpny thcglfgdph lrtft

SEQ ID NO: 3 RVKR sequence of an inhibitor; SEQ ID NO: 4 RRLL sequence of an inhibitor

SEQ ID NO: 5 CIYISRRLLC SEQ ID NO: 6 AAPNYTHENLYFQSHLRTFTD (TEV)

SEQ ID NO: 7 AAPNYTH*CGLFGDP*HLRTFTD (wt)

SEQ ID NO: 8
mqpprerlvv tgragwmgmg rgagrsalgf wptlafllcs fpaatspcki lkcnsefwsa 60
tsgshapasd dtpefcaalr syalctrrta rtcrgdlayh savhgiedlm sqhncskdgp 120
tsqprlrtlp pagdsqersd speichyeks fhkhsatpny thcglfgXpX lrtftdrfqt 180
ckvqgawpli dnnylnvqvt ntpvlpgsaa tatskltiif knfqecvdqk vyqaemdelp
aafvdgsknq gdkhganslk itekvsgqhv eiqakyigtt ivvrqvgryl tfavrmpeev
vnavedwdsq glylclrgcp lnqqidfqaf htnaegtgar rlaaaspapt apetfpyeta
vakckeklpv edlyyqacvf dllttgdvnf tlaayyaled vkmlhsnkdk lhlyertrdl
pgraaaglpl aprpllgalv pllallpvfc

amino acids 1-46 can be present or absent; amino acid 168 can be D or A; amino acid 170 can be H or A

SEQ ID NO:9 (includes tev)
mqpprerlvv tgragwmgmg rgagrsalgf wptlafllcs fpaatspcki lkcnsefwsa
tsgshapasd dtpefcaalr syalctrrta rtcrgdlayh savhgiedlm sqhncskdgp
tsqprlrtlp pagdsqersd speichyeks fhkhsatpny thenlyfqsh lrtftdrfqt
ckvqgawpli dnnylnvqvt ntpvlpgsaa tatskltiif knfqecvdqk vyqaemdelp
aafvdgsknq gdkhganslk itekvsgqhv eiqakyigtt ivvrqvgryl tfavrmpeev
vnavedwdsq glylclrgcp lnqqidfqaf htnaegtgar rlaaaspapt apetfpyeta
vakckeklpv edlyyqacvf dllttgdvnf tlaayyaled vkmlhsnkdk lhlyertrdl
pgraaaglpl aprpllgalv pllallpvfc

amino acids 1-46 can be present or absent; amino acid 168 can be D or A; amino acid 170 can be H or A

[00246] While the present application has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the application is not limited to the disclosed examples. To the contrary, the application is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

[00247] All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. Specifically, the sequence associated with each accession number provided herein is incorporated by reference in its entirely

References

1. Adams, R.H., Lohrum, M., Klostermann, A., Betz, H., and Püschel. A. (2007). The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. EMBO J. *16*, 6077-6086.

2. Carvalho, R.F., Beutler,. M, Marler, K.J.M., and Drescher, U. (2006). Silencing of EphA3 through a cis interaction with ephrinA5. Nat. Neurosci. *9*, 322-330.
3. Drescher, U., Bonhoeffer, F., and Muller, B.K. (1997). The Eph family in retinal axon guidance. Curr. Opin. Neurobiol. *7*, 75-80.

4. Egea, J., and Klein, R. (2007). Bidirectional Eph-ephrin signaling during axon guidance. Trends Cell Biol. *17*, 230-238.

5. Endo, M., and Yamashita, T. (2009). Inactivation of Ras by p120GAP via focal adhesion kinase dephosphorylation mediates RGMa-induced growth cone collapse. J. Neurosci. *29*, 6649-6662.

6. Hata, K., Fujitani, M., Yasuda, Y., Doya, H., Yamagishi, S., Mueller, B.K., and Yamashita, T. (2006). RGMa inhibition promotes axonal growth and recovery after spinal cord injury. J. Cell Biol. *173*, 47-58.

7. Hattori, M., Osterfield, M., and Flanagan, J.G. (2000). Regulated cleavage of a contact-mediated axon repellent. Science. 289:1360-5.
8. Kalus, I., Schnegelsberg, B., Seidah, N.G., Kleene, R., and Schachner, M. (2003). The proprotein convertase PC5A and a metalloprotease are involved in the proteolytic processing of the neural adhesion molecule L1. J. Biol. Chem. *278*,10381-8.
9. Muramatsu, R., Kubo T, Mori M, Nakamura Y, Fujita Y, Akutsu T, Okuno T, Taniguchi J, Kumanogoh A, Yoshida M, et al. (2011). RGMa modulates T cell responses and is involved in autoimmune encephalomyelitis. Nat. Med. *4*, 488-94.

10. Matsunaga, E., Tauszig-Delamasure, S., Monnier, P.P., Mueller, B.K., Strittmatter, S.M., Mehlen P., and Chédotal, A. (2004). RGM and its receptor Neogenin regulate neuronal survival. Nature Cell Biol. *6*, 749-755.

11. Matsunaga, E., Nakamura, H., and Chedotal, A. (2006). Repulsive Guidance Molecule plays multiple roles in neuronal differentiation and axon guidance. J. Neurosci. *26*, 6082-6088.
12. Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, et al. (2001) Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat. Genet. *28*, 241-249.
13. M onnier, P.P., Sierra, A., Macchi, P., Deitinghoff, L., Andersen, J.S., Mann, M., Flad, M., Hornberger, M.R., Stahl, B., Bonhoeffer, F., and Mueller, B.K. (2002). RGM is a repulsive guidance molecule for retinal axons. Nature *419*, 392-395.

14. Nohra, R., et al. (2010). RGMA and IL21R show association with experimental inflammation and multiple sclerosis. Genes Immun. *11*, 279-293.

15. Oertle, T., van der Haar, M.E., Bandtlow, C.E., Robeva, A., Burfeind, P., Buss, A., Huber, A.B., Simonen, M., Schnell, L., Brösamle, et al. (2003). Nogo-A Inhibits Neurite Outgrowth and Cell Spreading with Three Discrete Regions. J. Neurosci. *23*, 5393-5406.

16. Papanikolaou, G., M.E. Samuela, E.H. Ludwig, and Y.P. Goldberg. (2003). Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemachromatosis. Nature genetics *36*, 77-82.

17. Pasquato, A., Pullikotil, P., Asselin, M.C., Vacatello, M., Paolillo, L., Ghezzo, F., Basso, F., Di Bello, C., Dettin, M., Seidah, N.G. (2006). The Proprotein Convertase SKI-1/S1P: *in vitro* analysis of lassa virus glycoprotein-derived substrates and ex vivo validation of irreversible peptide inhibitors. J. Biol. Chem. *281*, 23471-23481.

18. Pasquato, A et al (2011). Arenavirus envelope glycoproteins mimic autoprocessing sites of the cellulr proprotie convertase subtilisin kexin isozyme-1/site-1 protease Virology 417:18-26.

19. Pasquato, A et al.(2012). Evaluation of the anti-arenaviral activity of the subtilisin kexin isozyme/site 1 protease inhibitor PF-429242, Virology 423, 14-22.

20. Phan, J., Zdanov, A., Evdokimov, A.G., Tropea, J.E., Peters, H.K. 3rd, Kapust, R.B., Li, M., Wlodawer, A., and Waugh, D.S. (2002). Structural basis for the substrate specificity of tobacco etch virus protease. J. Biol. Chem. *277*, 50564-72.

21. Pullikotil, P., Vincent, M., Nichol, S.T., and Seidah, N.G. (2004). Development of protein-based inhibitors of the proprotein of convertase SKI-1/S1P: processing of SREBP-2, ATF6, and a viral glycoprotein. J. Biol. Chem. *279*, 17338-17347.

22. Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., Mueller, B.K., and Strittmatter, S.M. (2004). Neogenin mediates the action of repulsive guidance molecule. Nat. Cell Biol. *8*,756-762.

23. Roebroek, A.J., Umans, L., Pauli I.G., Robertson, E.J., van Leuven, F., Van de Ven, W.J., and Constam, D.B. (1998). Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. Development *125*, 4863-4876.

24. Schwab J.M., Monnier P.P., Schluesener H.J., Conrad S., Chen L., Meyermann R., Mueller B.K. (2005). Central Nervous System injury-induced Repulsive Guidance Molecule expression in the adult brain. Arch. Neurol. 62: 1561-1568.

25. Schwab J.M., Conrad S., Monnier P.P., Julien S., Mueller B.K., Schluesener H.J. (2005). Spinal cord injury-induced lesional expression of the repulsive guidance molecule (RGM). Eur. Journal of Neurosci. 21: 1569-1576.

26. Seidah, N.G., and Chretien, M. (1999). Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res. *848*, 45-62.

27. Seidah, N.G., Mowla,S., Hameli, J., Mamarbachi, A.M., Benjannet, S., Toure B.B., Basak, A., Munzer J.S., Marcinkiewicz, J., Zhong, M. et al. (1999). Mamalian Subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with unique specificity and cellular localization. Proc. Natl. Acad. Sci. *96*, 1321-1326.

28. Seiradake, E., von Philipsborn, A.C., Henry, M., Fritz, M., Lortat-Jacob, H., Jamin, M., Hemrika, W., Bastmeyer, M., Cusack, S., and McCarthy, A.A. (2009). Structure and functional relevance of the Slit2 homodimerization domain. EMBO Rep. *10*, 736-41.

29. Tassew, N.G., Chestopolava, L., Beecroft, R., Matsunaga, E., Teng, H., Chedotal, A., and Monnier, P.P. (2008). Intraretinal RGMa is involved in retino-tectal pathfinding. Mol. Cell. Neurosci. *37*, 761-770.

30. Tassew, N.G., Charish, J., Chestopalova, L., and Monnier, P.P. (2009). Sustained in vivo inhibition of protein domains using scFv recombinant antibodies and its application to dissect RGMa activity on retinal axon outgrowth. J. Neurosci. *29*, 1126-1131.

31. Tessier-Lavigne, M., and C.S. Goodman. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

32. Thanos, S., and Mey, J. (2001). Development of the visual system of the chick. II. Mechanisms of axonal guidance. Brain Res. Brain Res. Rev. *35*, 205-45.

33. Zhang G, Zhang JH, Feng J, Li Q, Wu X, Qin X. (2011) Electrical stimulation of olfactory bulb downregulates RGMa expression after ischemia/reperfusion injury in rats. Brain Res Bull. 2011 Oct 10;86(3-4):254-61.

34. Zhong, M., Munzer, J.S., Basak, A., Benjannet, S., Mowla, S.J., Decroly, E., Chretien, M., and Seidah, N.G. (1999). The prosegments of furin and PC7 as potent inhibitors of proprotein convertases. In vitro and ex vivo assessment of their efficacy and selectivity. J. Biol Chem. *274*, 33913-33920.

35. Zhou, Z., Xie, J., Lee, D., Liu, Y., Jung, J., Zhou, L., Xiong, S., Mei, L., and Xiong, W.C. (2010). Neogenin regulation of BMP-induced canonical Smad signaling and endochondral bone formation. Dev. Cell *19*, 90-102.

36. Zisman, S., Marom, K., Avraham, O., Rinsky-Halivni, L., Gai, U., Kligun, G., Tzarfaty-Majar,V., Suzuki, T., and Klar, A. (2007). Proteolysis and membrane capture of F-spondin generates combinatorial guidance cues from a single molecule. J. Cell Biol. *178*, 1237-49.

37. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986;17:472–6.

38. Wang CX, Yang T, Shuaib A. An improved version of embolic model of brain ischemic injury in the rat.J Neurosci Methods. 2001 Aug 30;109(2):147-51.

Claims:

1. A method of inhibiting RGMa cleavage comprising contacting the RGMa with a proprotein convertase (PPC) inhibitor, optionally a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor and/or a furin inhibitor.

2. The method of claim 1 for inhibiting RGMa cleavage at RTFT↓D (amino acid 175 in human) comprising contacting the RGMa with a Subtilisin Kexin Isoenzyme-1 (SKI-1) inhibitor.

3. The method of claim 11, wherein inhibition of RGMa cleavage at RTFT↓D (amino acid 175 in human) inhibits formation of RGMa37.

4. The method of any one of claims 1 to 3, wherein the SKI-1 inhibitor is a serine protease inhibitor, optionally a membrane permeable serine protease inhibitor.

5. The method of claim 4, wherein the RGMa is attached to a cell and the serine protease inhibitor is a membrane permeable serine protease inhibitor.

6. The method of any one of claims 1 to 5 for inhibiting soluble NRGMa or NNRGMa release from a cell comprising contacting the cell with a SKI-1 inhibitor and/or a furin inhibitor.

7. The method of claim 6, wherein the SKI-inhibitor is a membrane permeable serine protease inhibitor.

8. The method of any one of claims 1 to 7, wherein PPC inhibitor is selected from a membrane permeable serine protease inhibitor, optionally AEBSF, an ER permeable serine protease inhibitor and RVKR peptide.

9. The method of any one of claims 1 to 8, wherein the SKI-1 inhibitor is selected from RRLL peptide (SEQ ID NO:4), PF-429242, peptide CIYISRRLLC (SEQ ID NO:5; optionally with terminal "C" residues cyclized) and/or prosegment inhibitor R134E

10. The method of any one of claims 1 to 9 for inducing neuron survival, axon growth and/or promoting neuron axon regeneration of a neural cell in a subject in need thereof, the method comprising: providing neural tissue with a PPC inhibitor, optionally a SKI-1 inhibitor and/or a furin inhibitor.

11. The method of claim 10, wherein the furin inhibitor is a furin prosegment inhibitor.

12. The method of claim 10 or 11, wherein the method is for inducing long range neuron survival, axon growth and/or regeneration.

13. The method of any one of claims 10 to 12 wherein the neural cell is provided with the SKI-1 inhibitor and/or the furin inhibitor by administering the inhibitor to the subject.

14. The method of claim 13 wherein the subject has Multiple sclerosis.

15. The method of claim 13 wherein the subject has a nerve injury.

16. The method of claim 13 wherein the nerve injury is a spinal cord injury.

17. The method of claim 13 or 15, whrein the subject is suffering or has suffered an ischemic neuronal injury.

18. The method of claim 13, wherein the subject has glaucoma, Alzheimer's disease, Parkinson's disease and/or a CNS immune response.

19. The method of any one of claims 1 to 18, for stimulating neuron survival, axon growth and/or regeneration in a subject in need thereof, the method comprising administering to the subject a PPC inhibitor, optionally a SKI-1 and/or Furin inhibitor, in sufficient amount to promote neuron survival, axon growth and/or regeneration of the neuron.

20. The method of claim 19 for treating nerve damage comprising administering to a subject in need thereof a composition comprising a PPC inhibitor such as a SKI-1 inhibitor and/or a Furin inhibitor.

21. The method of claim 20, wherein the nerve damage is a neurodegenerative disease.

22. The method of claim 21 wherein the neurodegenerative disease is multiple sclerosis or glaucoma.

23. The method of claim 20, wherein the nerve damage is a spinal cord injury, ischemic injury such as a stroke or CNS immune injury.

24. The method of claim 20, wherein the damaged nerve comprises a sensory neuron or a motor neuron.

25. The method of any one of claims 1 to 18 for inhibiting an immune response in CNS tissues, e.g. brain and spinal cord in multiple sclerosis patients comprising administering a PPC inhibitor such as a SKI-1 inhibitor and/or a furin inhibitor.

26. The method of any one of claims 1 to 18 for inhibiting demyelination in a subject in need thereof comprising administering a SKI-1 inhibitor and/or a furin inhibitor.

27. The method of any one of claims 19 to 26, wherein the SKI-1 inhibitor and/or the Furin inhibitor is administered to the lesion site.

28. The method of any one of claims 19 to 26, wherein PPC inhibitor is selected from a membrane permeable serine protease inhibitor, optionally AEBSF, an ER permeable serine protease inhibitor and RVKR peptide.

29. The method of any one of claims 19 to 26, wherein the SKI-1 inhibitor is selected from RRLL peptide (SEQ ID NO: 4), PF-429242, peptide CIYISRRLLC (SEQ ID NO: 5; optionally with terminal "C" residues cyclized) and/or prosegment inhibitor R134E.

30. The method of any one of claims 19 to 29, wherein the inhibitor(s) and/or composition is/are administered by intravenous, intraspinal and/or intracranial infusion. In another embodiment he inhibitor(s) and/or composition is/are administered by Intraperitoneal and/or intrathecal application.

31. An isolated polypeptide comprising: a RGMa fragment selected from or corresponding to:

w) a RGMa fragment no longer than amino acid 1 to 168 of human RGMa ($RGMa_{1-168}$), optionally no longer than amino acid 47 to 168,

x) a RGMa fragment no longer than amino acid 1 to 127 of human RGMa ($RGMa_{1-127}$), optionally no longer than amino acid 47 to 127,

y) a RGMa fragment no longer than amino acid 1 to 133 of human RGMa ($RGMa_{1-133}$), optionally no longer than amino acid 47 to 133,

z) a RGMa fragment no longer than amino acid 1 to 175 of human RGMa ($RGMa_{1-175}$), optionally no longer than amino acid 47 to 175;

aa) a RGMa fragment no longer than amino acid 128 to 175 of human RGMa ($RGMa_{128-175}$)

bb) a RGMa fragment no longer than amino acid 169 to 450 of human RGMa ($RGMa_{169-450}$),

cc) a RGMa fragment no longer than amino acid 176 to 450 of human RGMa ($RGMa_{176-450}$);

dd) a RGMa fragment no longer than amino acid 128 to 450 of human RGMa ($RGMa_{128-450}$)

ee) a RGMa fragment no longer than amino acid 201-290 of human RGMa($RGMa_{201-290}$),

ff) corresponding species fragments of anyone of a) to i), and/or

gg) conservative variants of any one of a) to j).

32. The isolated polypeptide of claim 31 wherein $RGMa_{1-168}$ consists of: residues 1 to 168 of SEQ ID NO: 1 or residues 47 to 168 of SEQ ID NO: 1; $RGMa_{1-175}$ consists of: residues 1 to 175 of SEQ ID NO: 1 or residues 47 to 175 of SEQ ID NO: 1 or fragments thereof, $RGMa_{1-133}$ consists of residues 1 to 133 of SEQ ID NO: 1 or residues 47 to 133 of SEQ ID NO: 1; $RGMa_{169-450}$ consists of residues 169 to 450 of SEQ ID NO: 1; $RGMa_{176-450}$ consists of residues 176 to 450 of SEQ ID NO: 1; $RGMa_{128-450}$ consists of residues 128to 450 of SEQ ID NO: 1; or $RGMa_{201-290}$ consists of residues 201 to 290 of SEQ ID NO: 1, and/or a conservative variant thereof.

33. The isolated polypeptide of claim 31, wherein the fragment is $RGMa_{1-168}$, $RGMa_{1-133}$ and/or $RGMa_{201-290}$ and/or a corresponding species fragment and/or a conservative variant thereof, wherein the conservative variant retains the ability to bind neogenin.

34. The isolated polypeptide of claim 31 comprising RGMa128-175 in combination with a second fragment of RGMa, optionally no longer than amino acids 169 to 450 of human RGMa (RGMa169-450) wherein the isolated polypeptide is combined with the second fragment via a disulphide bridge forming a species that is approximately 37kDa when separated electrophoretically on a non-reducing agarose gel.

35. The isolated polypeptide of any one of claims 31 to 34, wherein the isolated polypeptide is glycosylated and/or further comprises a GPI anchor.

36. The isolated polypeptide of any one of claims 31 to 35, wherein the isolated polypeptide comprises one or more of a linker peptide and a tag such as a FLAG®-tag.

37. An antibody that specifically binds an epitope comprising amino acid 175 of SEQ ID NO: 1 and/or blocks cleavage of RGMa at amino acid 175 by SKI-1 or minds a D168 or H170 mutant.

38. A substrate comprising the isolated peptide of any one of claims 31 to 36 or the antibody of claim 37.

39. A composition comprising an isolated polypeptide of anyone of claims 31 to 36 or the antibody of claim 37.

Figure 1

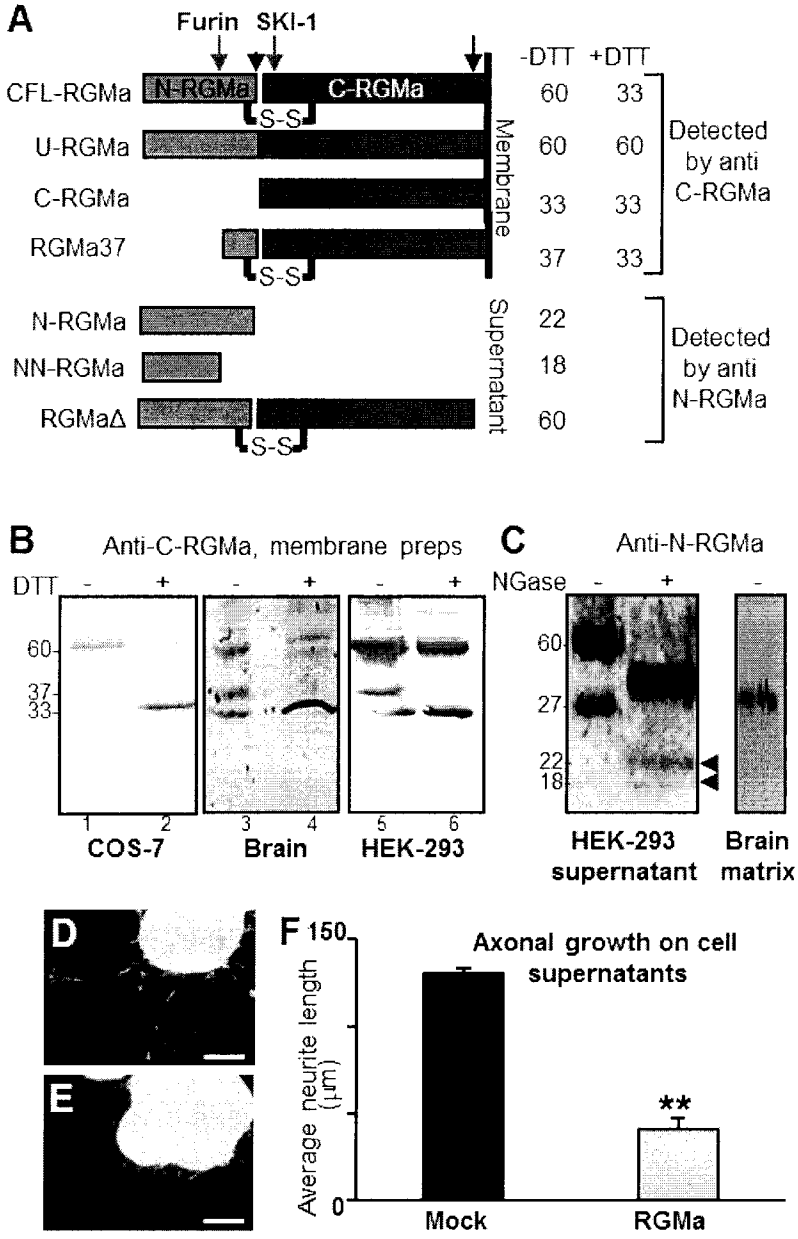


Figure 2

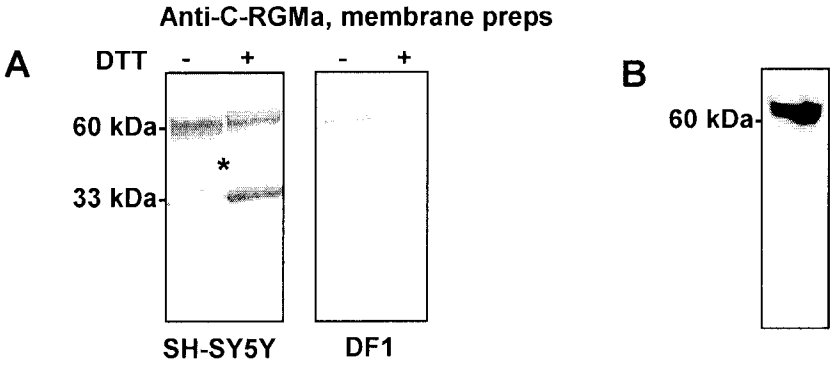


Figure 3

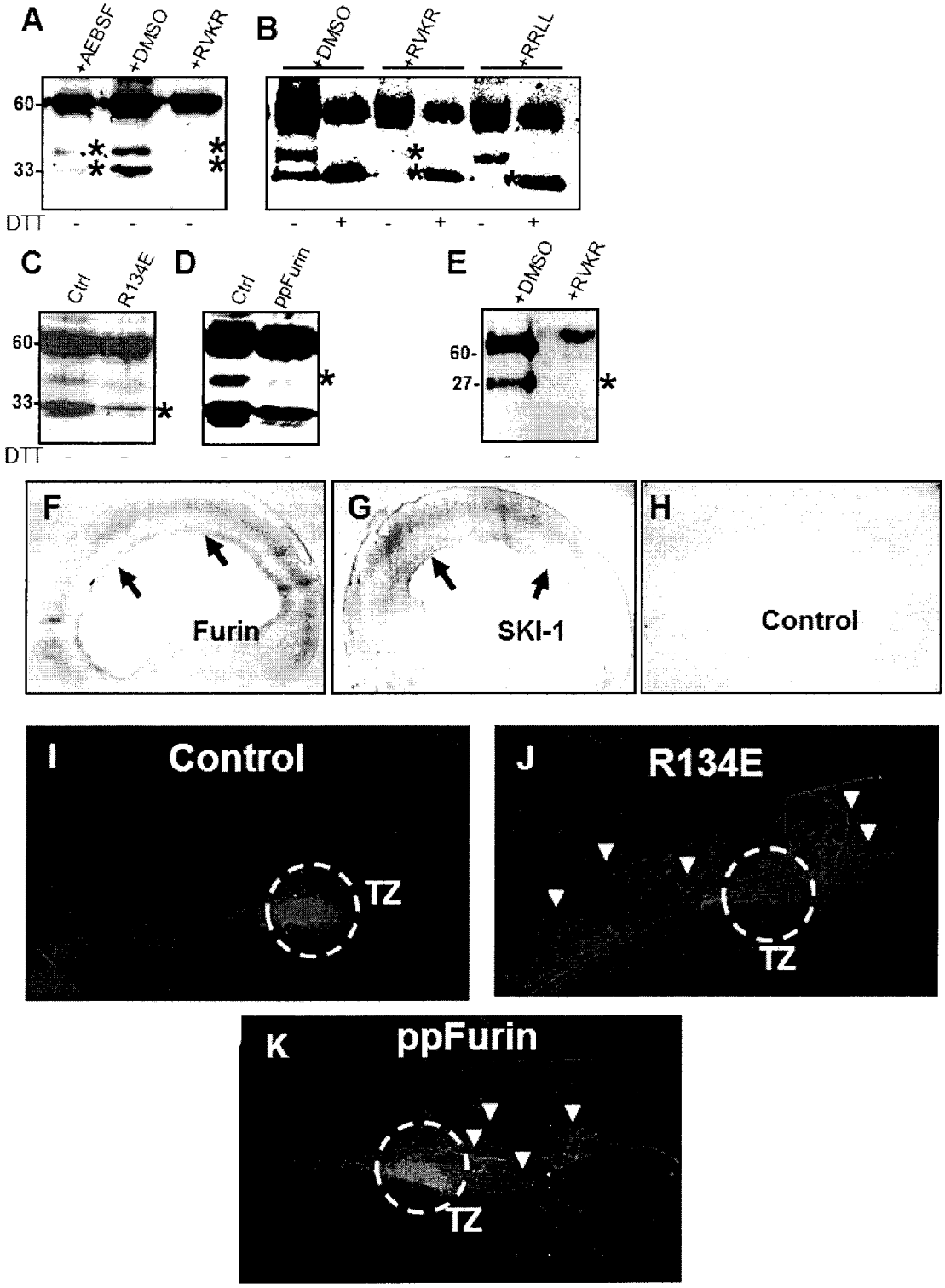

A
+AEBSF
+DMSO
+RVKR
60
33
DTT
B
+DMSO
+RVKR
+RRLL
C
Ctrl
R134E
D
Ctrl
ppFurin
E
+DMSO
+RVKR
60
27
F
Furin
G
SKI-1
H
Control
I
Control
TZ
J
R134E
TZ
K
ppFurin
TZ

Figure 4

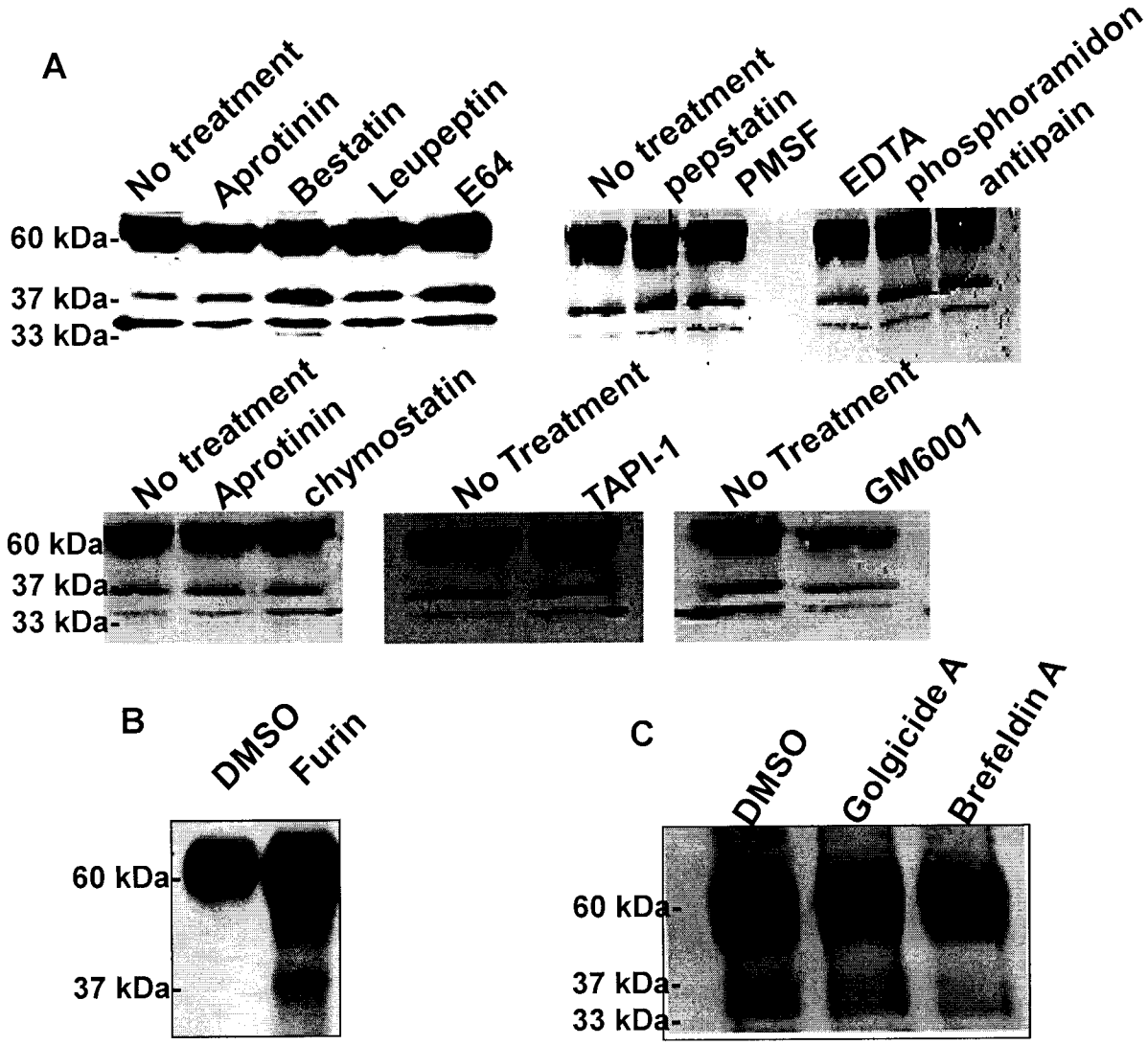

A
No treatment
Aprotinin
Bestatin
Leupeptin
E64
60 kDa-
37 kDa-
33 kDa-
No treatment
pepstatin
PMSF
EDTA
phosphoramidon
antipain
No treatment
Aprotinin
chymostatin
60 kDa
37 kDa
33 kDa-
No Treatment
TAPI-1
No Treatment
GM6001
B
DMSO
Furin
60 kDa-
37 kDa-
C
DMSO
Golgicide A
Brefeldin A
60 kDa-
37 kDa-
33 kDa-

Figure 5

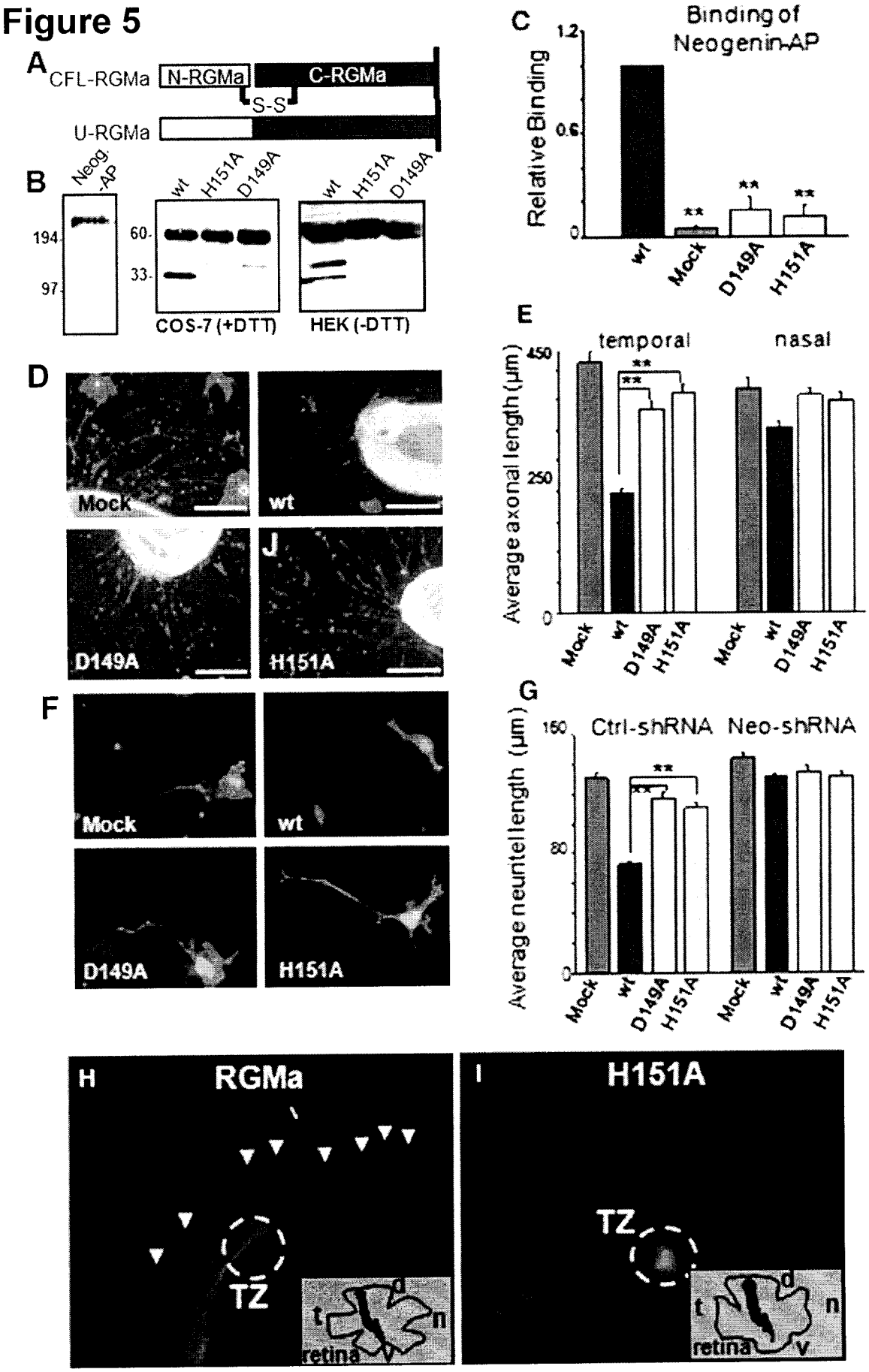

A
CFL-RGMa
N-RGMa
C-RGMa
S-S
U-RGMa
B
Neog.-AP
wt
H151A
D149A
194
97
60
33
COS-7 (+DTT)
HEK (-DTT)
C
Binding of Neogenin-AP
Relative Binding
1.2
0.6
0
Mock
D149A
H151A
D
Mock
wt
D149A
H151A
E
temporal
nasal
Average axonal length (μm)
450
250
0
F
Mock
wt
D149A
H151A
G
Ctrl-shRNA
Neo-shRNA
Average neuntel length (μm)
160
80
0
H
RGMa
TZ
t
n
retina
I
H151A
TZ
d
t
n
v
retina

Figure 6

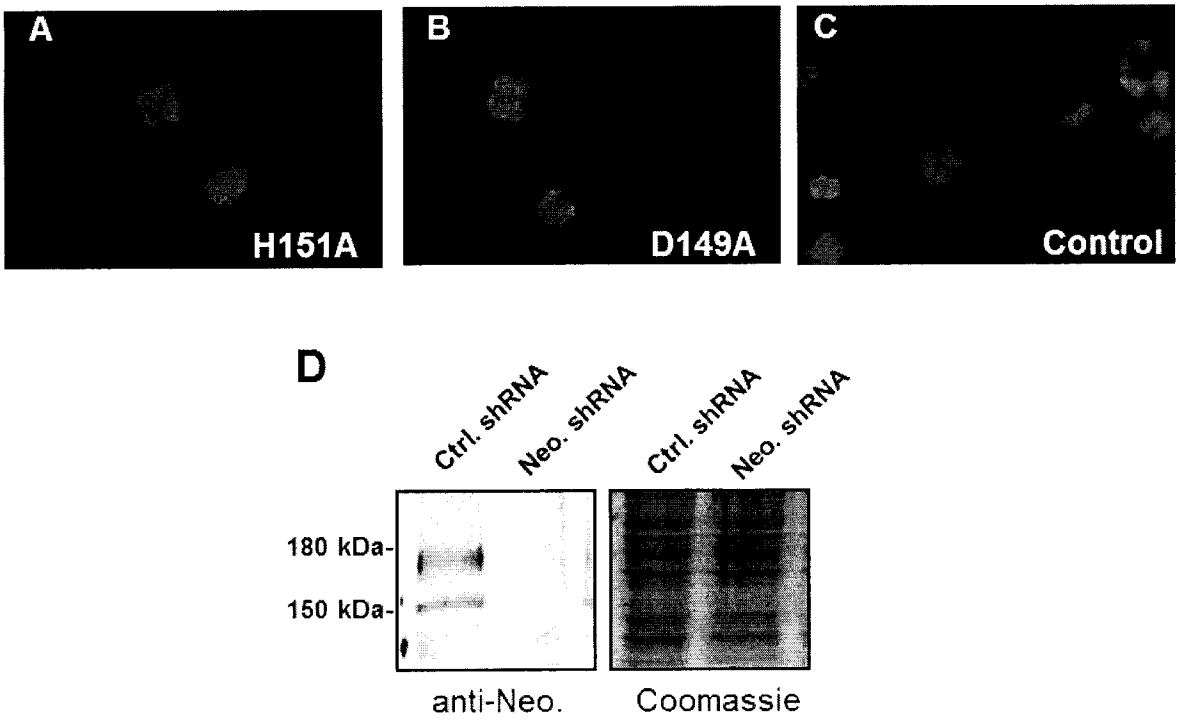

A
H151A
B
D149A
C
Control
D
Ctrl. shRNA
Neo. shRNA
Ctrl. shRNA
Neo. shRNA
180 kDa-
150 kDa-
anti-Neo.
Coomassie

Figure 7

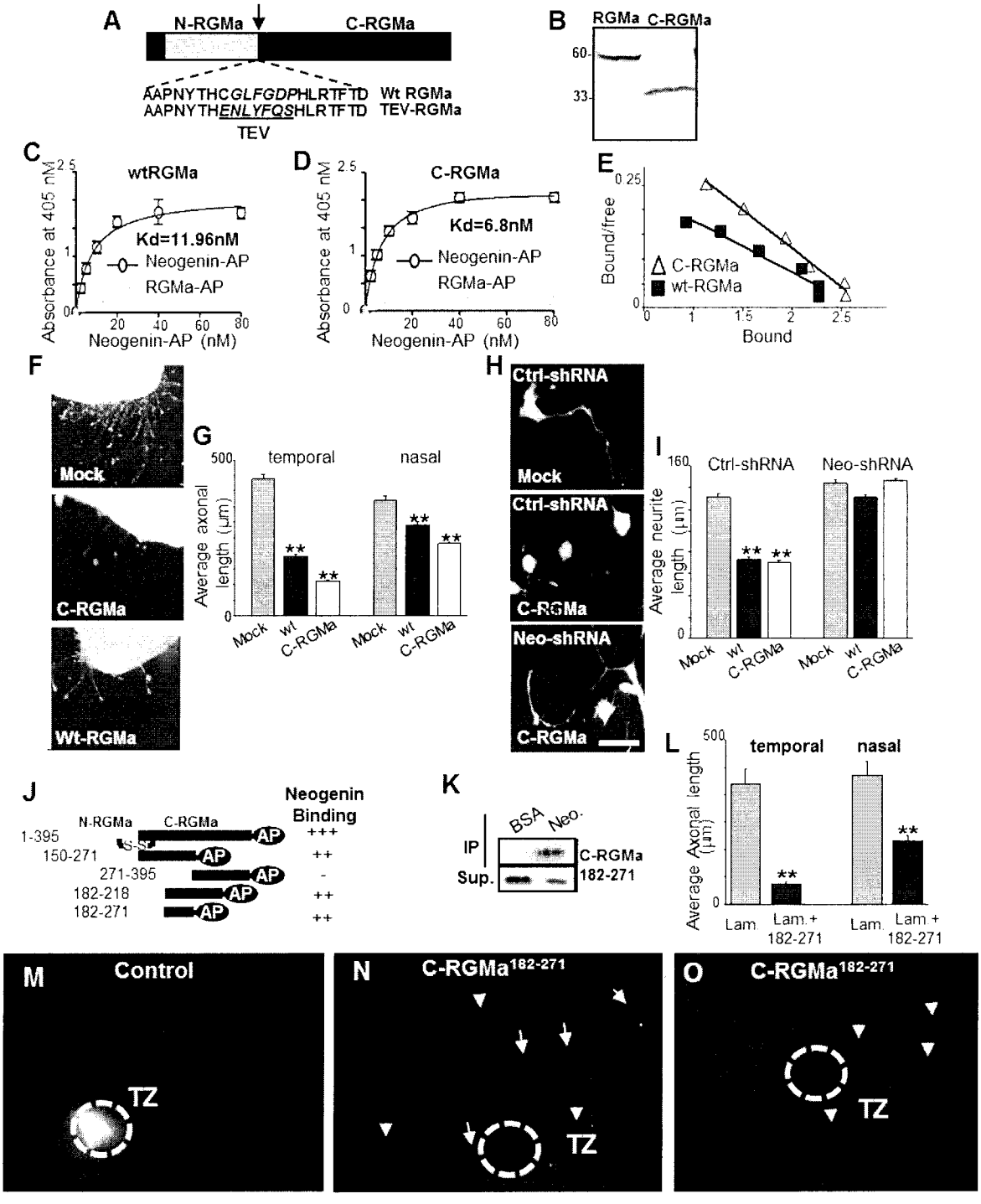

A
N-RGMa
C-RGMa
AAPNYTHCGLFGDPHLRTFTD Wt RGMa
AAPNYTHENLYFQSHLRTFTD TEV-RGMa
TEV
B
RGMa C-RGMa
60
33
C
wtRGMa
Kd=11.96nM
Neogenin-AP
RGMa-AP
Absorbance at 405 nM
Neogenin-AP (nM)
D
C-RGMa
Kd=6.8nM
Neogenin-AP
RGMa-AP
Absorbance at 405 nM
Neogenin-AP (nM)
E
C-RGMa
wt-RGMa
Bound/free
Bound
F
Mock
C-RGMa
Wt-RGMa
G
temporal
nasal
Average axonal length (μm)
Mock
wt
C-RGMa
H
Ctrl-shRNA
Mock
Ctrl-shRNA
C-RGMa
Neo-shRNA
C-RGMa
I
Ctrl-shRNA
Neo-shRNA
Average neurite length (μm)
J
Neogenin Binding
N-RGMa
C-RGMa
1-395 +++
150-271 ++
271-395 -
182-218 ++
182-271 ++
K
BSA
Neo.
IP
Sup.
C-RGMa 182-271
L
temporal
nasal
Average Axonal length (μm)
Lam.
Lam.+ 182-271
M
Control
TZ
N
C-RGMa182-271
TZ
O
C-RGMa182-271
TZ

Figure 8

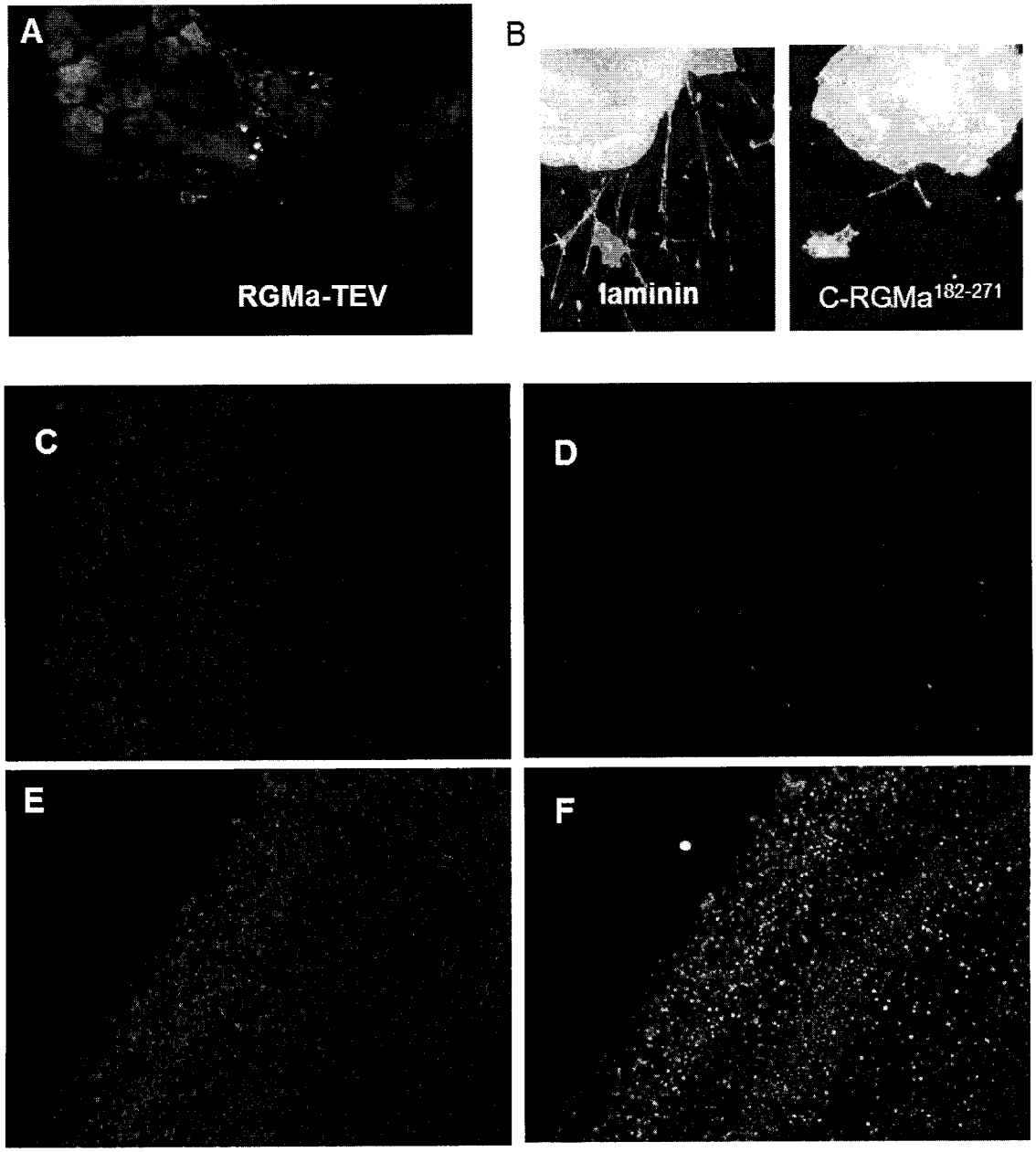


Figure 9

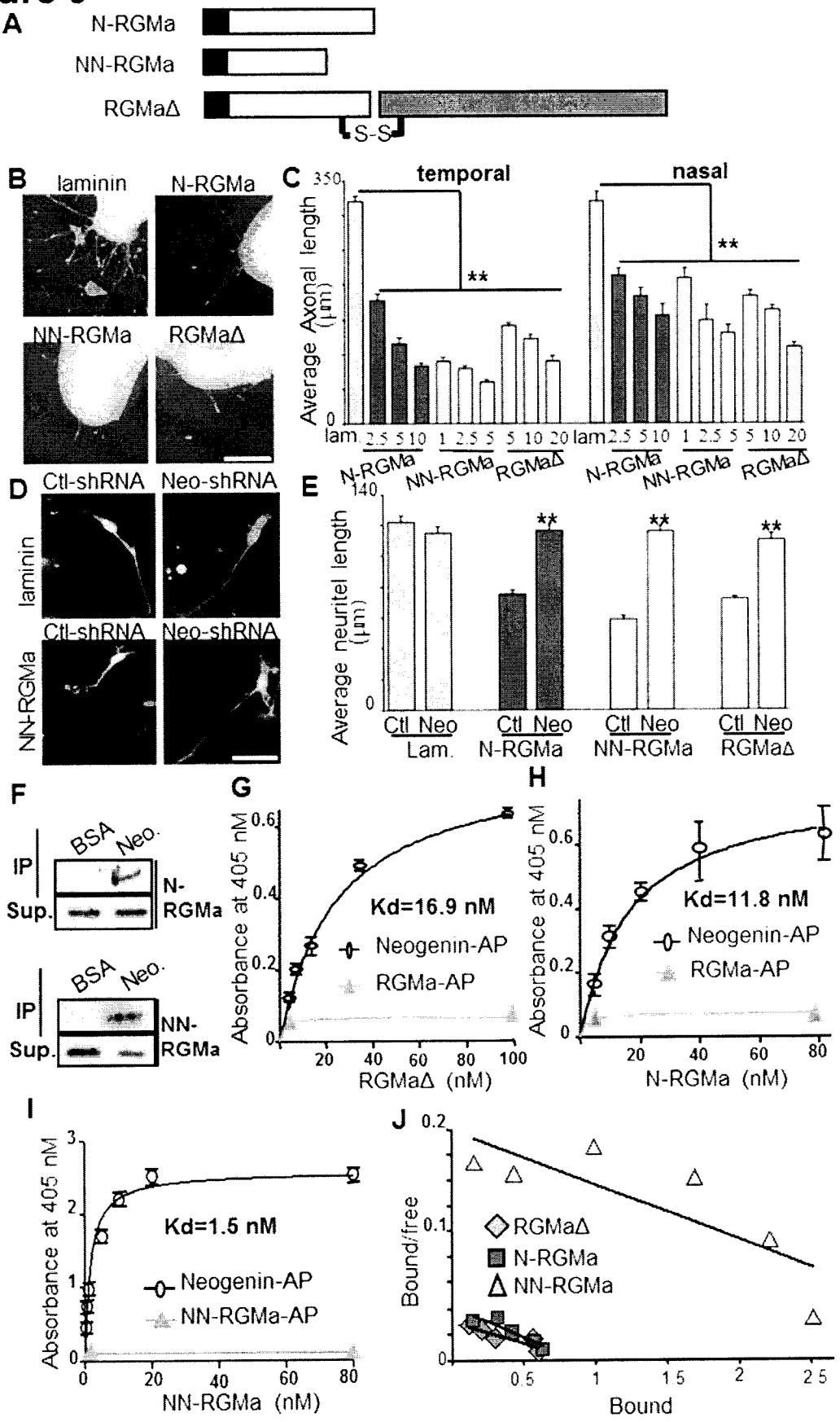
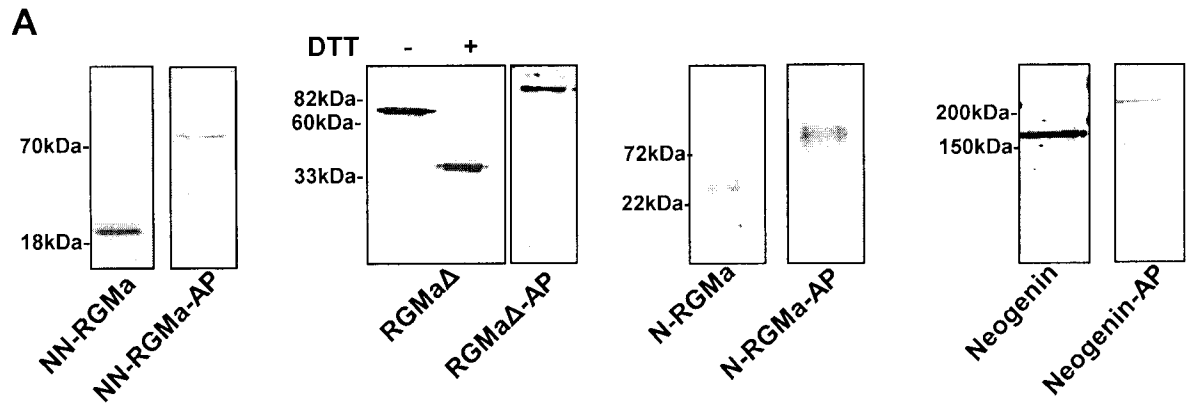
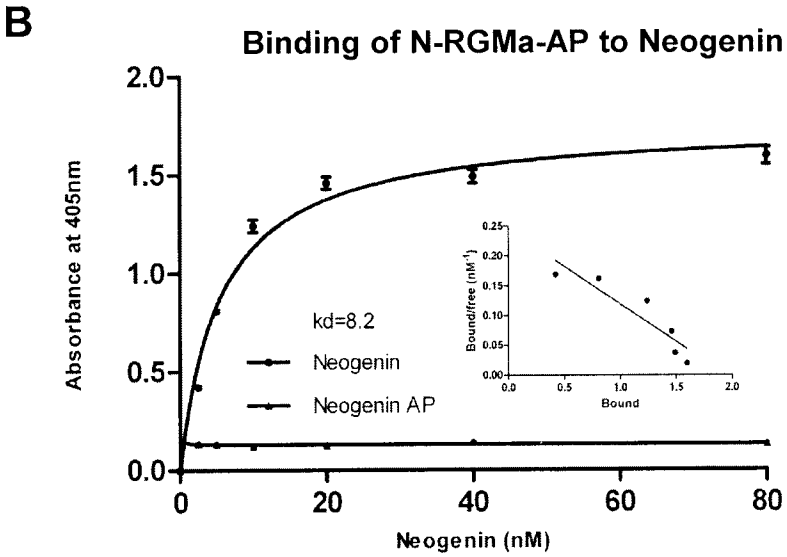

A
N-RGMa
NN-RGMa
RGMaΔ
S-S
B
laminin
N-RGMa
NN-RGMa
RGMaΔ
C
temporal
nasal
Average Axonal length (μm)
350
0
**
**
lam. 2.5 5 10 1 2.5 5 5 10 20 lam 2.5 5 10 1 2.5 5 5 10 20
N-RGMa
NN-RGMa
RGMaΔ
N-RGMa
NN-RGMa
RGMaΔ
D
Ctl-shRNA
Neo-shRNA
laminin
NN-RGMa
Ctl-shRNA
Neo-shRNA
E
Average neuritel length (μm)
140
0
**
**
**
Ctl Neo
Lam.
Ctl Neo
N-RGMa
Ctl Neo
NN-RGMa
Ctl Neo
RGMaΔ
F
IP
Sup.
BSA
Neo.
N-RGMa
IP
Sup.
BSA
Neo.
NN-RGMa
G
Absorbance at 405 nM
0.6
0.4
0.2
0
Kd=16.9 nM
Neogenin-AP
RGMa-AP
20 40 60 80 100
RGMaΔ (nM)
H
Absorbance at 405 nM
0.6
0.4
0.2
0
Kd=11.8 nM
Neogenin-AP
RGMa-AP
20 40 60 80
N-RGMa (nM)
I
Absorbance at 405 nM
3
2
1
0
Kd=1.5 nM
Neogenin-AP
NN-RGMa-AP
0 20 40 60 80
NN-RGMa (nM)
J
Bound/free
0.2
0.1
0
RGMaΔ
N-RGMa
NN-RGMa
0.5 1 1.5 2 2.5
Bound

Figure 10


A
DTT - +
70kDa-
18kDa-
82kDa-
60kDa-
33kDa-
72kDa-
22kDa-
200kDa-
150kDa-
NN-RGMa
NN-RGMa-AP
RGMaΔ
RGMaΔ-AP
N-RGMa
N-RGMa-AP
Neogenin
Neogenin-AP


B
Binding of N-RGMa-AP to Neogenin
Absorbance at 405nm
Neogenin (nM)
kd=8.2
Neogenin
Neogenin AP
Bound/free (nM-1)
Bound

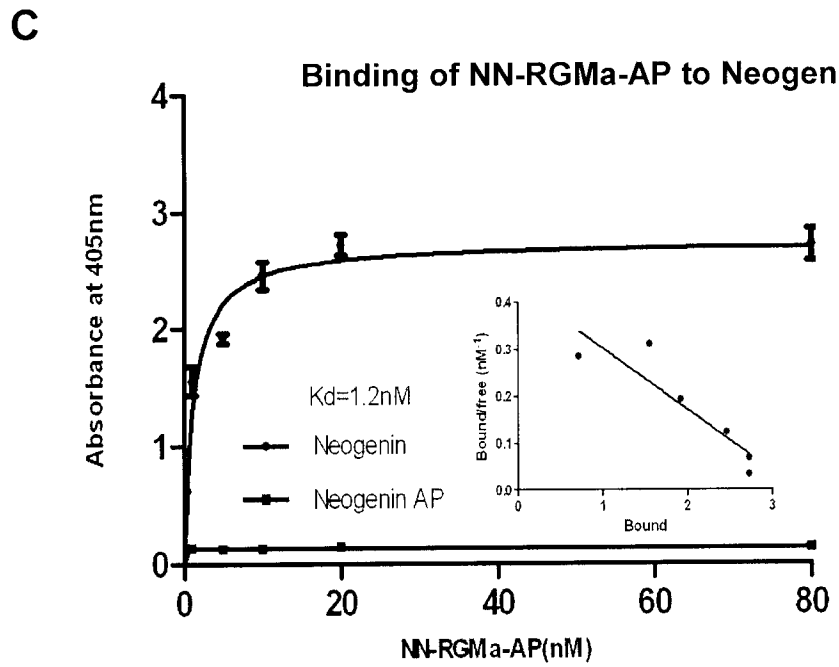
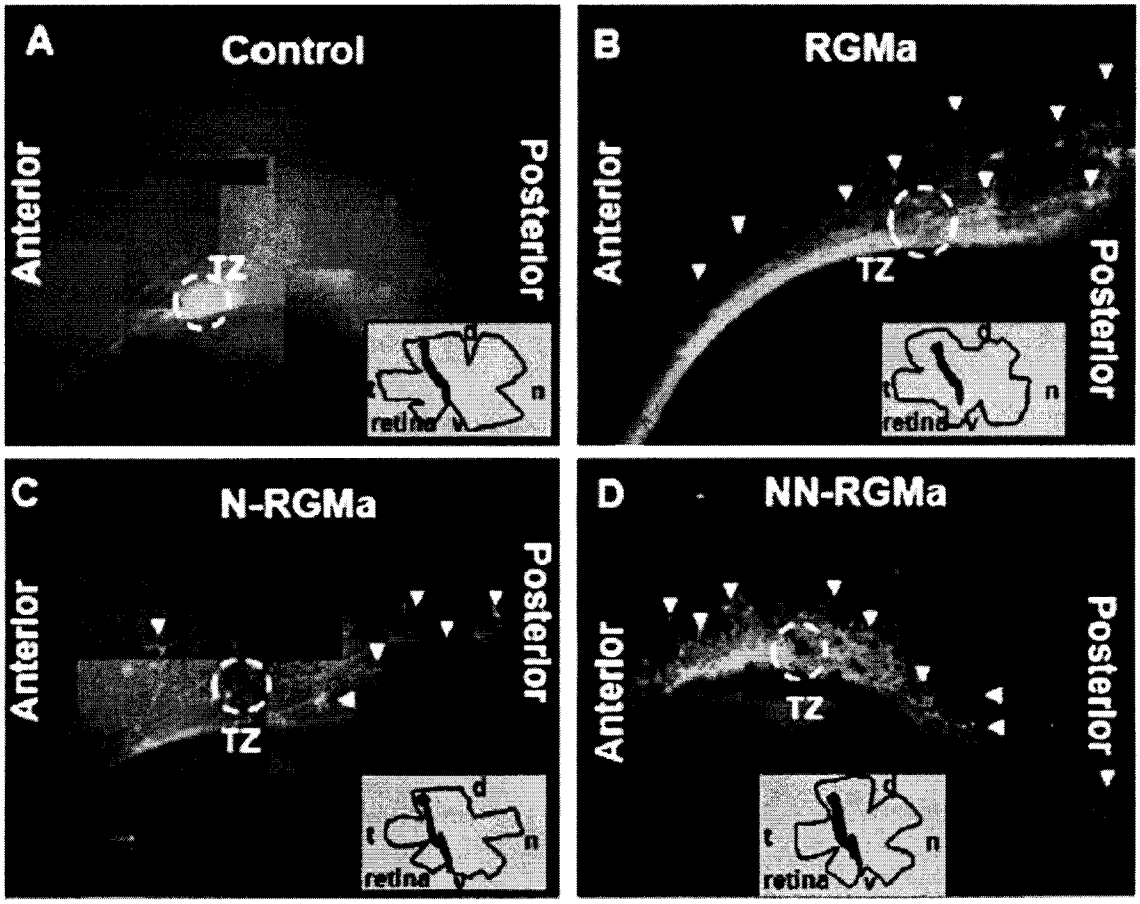

C
Binding of NN-RGMa-AP to Neogenin
Absorbance at 405nm
NN-RGMa-AP(nM)
Kd=1.2nM
Neogenin
Neogenin AP
Bound/free (nM-1)
Bound

Figure 11



E

	-TZ	AT	PT	Ab
Control (n=8)	0%	0%	12%	0%
RGMa (n=10)	60%	70%	70%	30%
N-RGMa (n=10)	40%	70%	60%	40%
NN-RGMa (n=12)	25%	42%	100%	58%

Figure 12

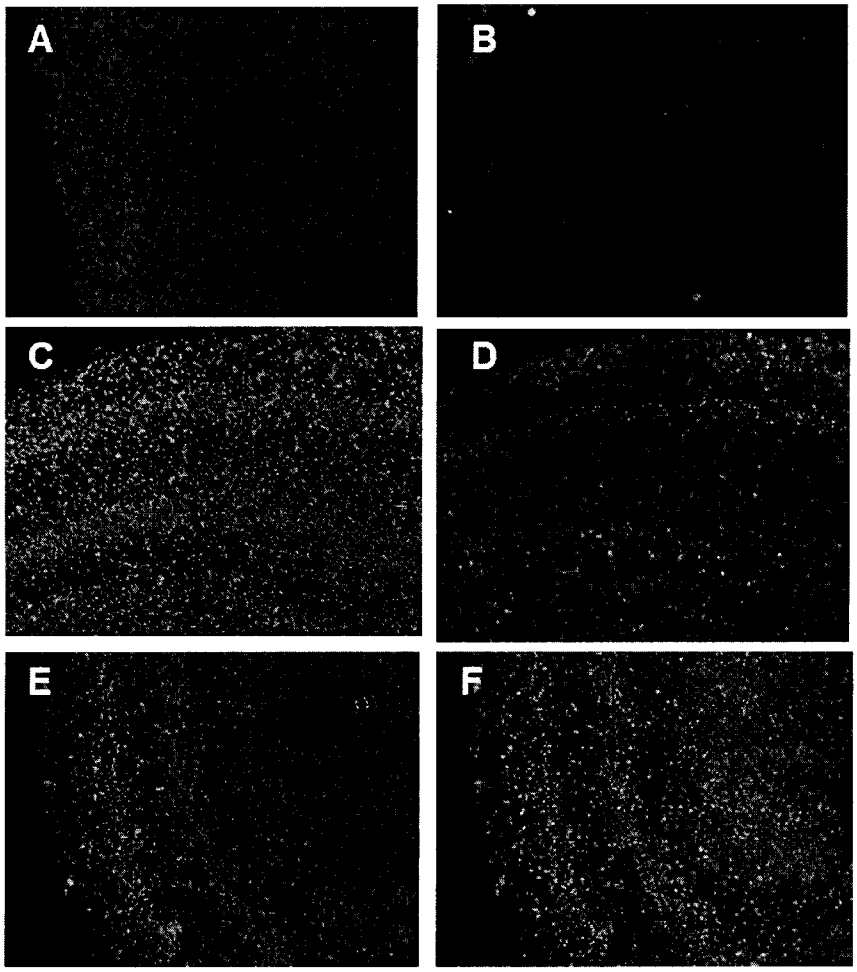


Figure 13

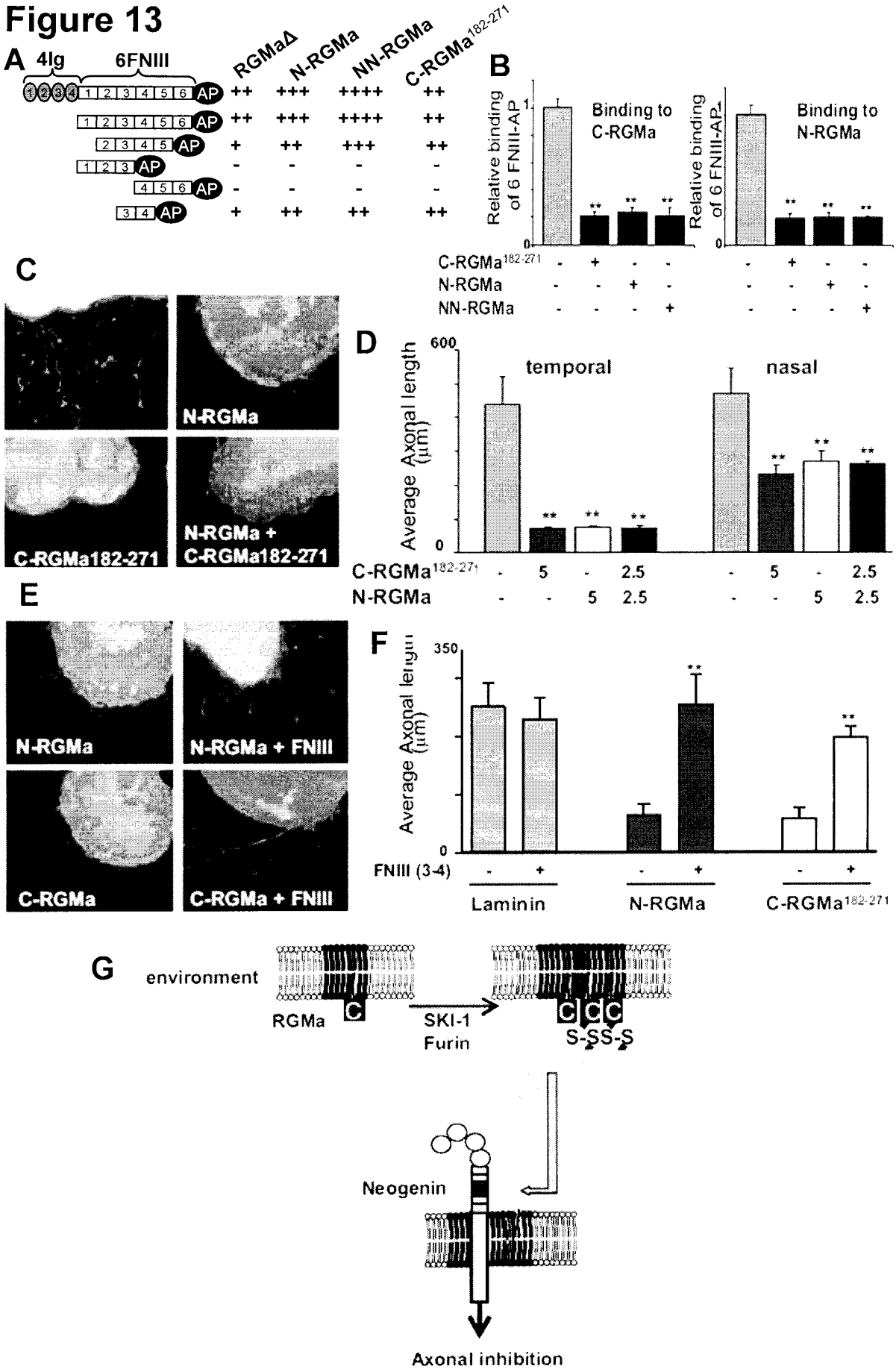

A
4Ig
6FNIII
RGMaΔ
N-RGMa
NN-RGMa
C-RGMa182-271
AP
++ +++ ++++ ++
+ ++ +++ ++
- - - -
B
Relative binding of 6 FNIII-AP
Binding to C-RGMa
Binding to N-RGMa
C-RGMa182-271
N-RGMa
NN-RGMa
C
N-RGMa
C-RGMa182-271
N-RGMa + C-RGMa182-271
D
Average Axonal length (µm)
temporal
nasal
C-RGMa182-271
N-RGMa
E
N-RGMa
N-RGMa + FNIII
C-RGMa
C-RGMa + FNIII
F
Average Axonal length (µm)
FNIII (3-4)
Laminin
N-RGMa
C-RGMa182-271
G
environment
RGMa
SKI-1
Furin
S-S
Neogenin
Axonal inhibition

Figure 14

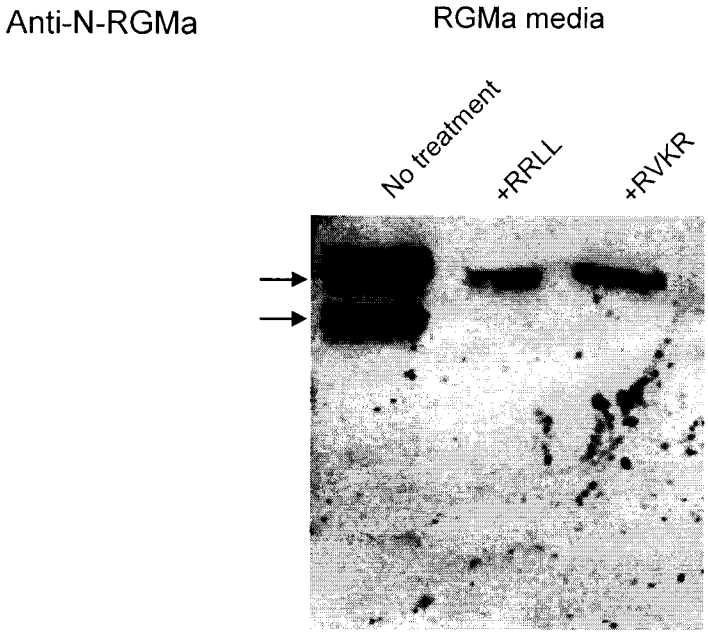


Figure 15

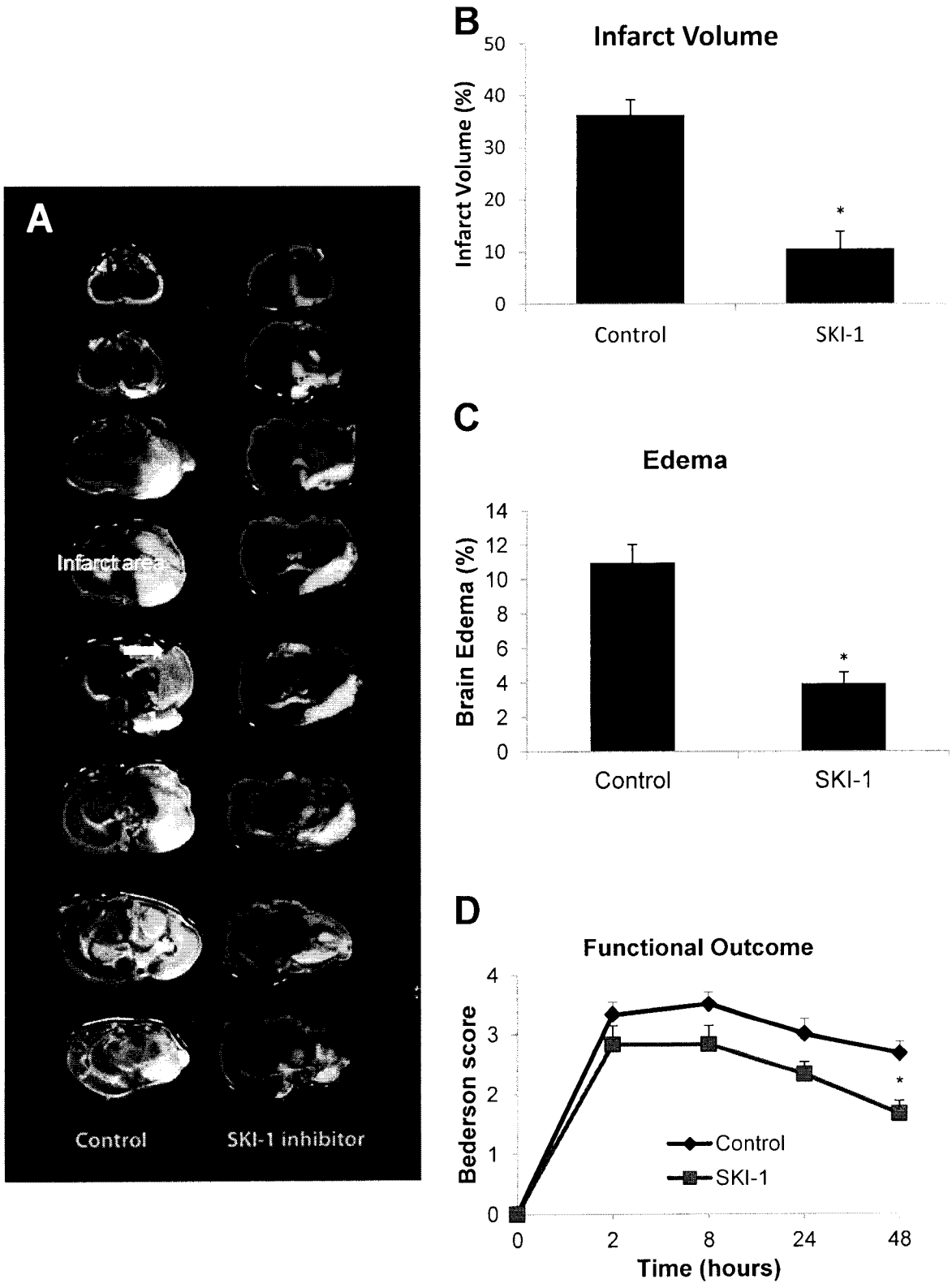
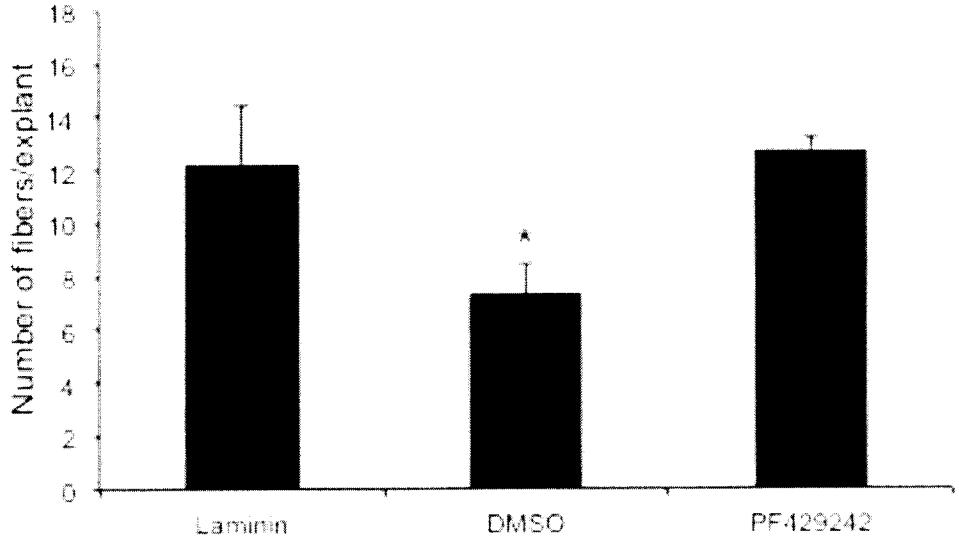
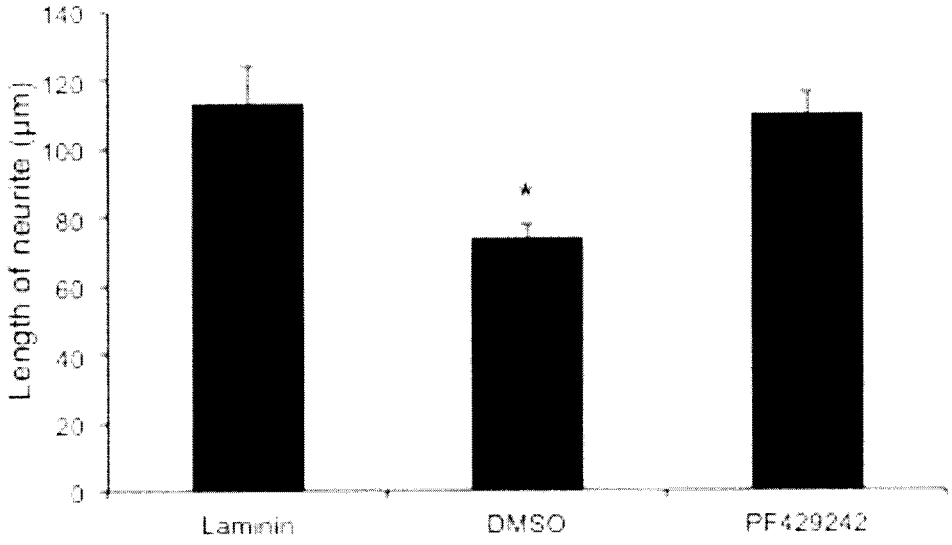

A
Infarct area
Control
SKI-1 inhibitor
B
Infarct Volume
Infarct Volume (%)
50
40
30
20
10
0
*
Control
SKI-1
C
Edema
Brain Edema (%)
14
12
10
8
6
4
2
0
*
Control
SKI-1
D
Functional Outcome
Bederson score
4
3
2
1
0
*
Control
SKI-1
0
2
8
24
48
Time (hours)

Figure 16

A


Number of fibers/explant
18
16
14
12
10
8
6
4
2
0
*
Laminin
DMSO
PF429242

B


Length of neurite (µm)
140
120
100
80
60
40
20
0
*
Laminin
DMSO
PF429242

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2013/000104

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07K 14/705*** (2006.01) , ***A61K 38/07*** (2006.01) , ***A61K 38/08*** (2006.01) , ***A61K 38/17*** (2006.01) , ***A61K 39/395*** (2006.01) , ***A61P 25/00*** (2006.01) (more IPCs on the last page)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: ***C07K 14/705*** (2006.01) , ***A61K 38/07*** (2006.01) , ***A61K 38/08*** (2006.01) , ***A61K 38/17*** (2006.01) , ***A61K 39/395*** (2006.01) , ***A61P 25/00*** (2006.01) (more IPCs on the last page)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases: TotalPat, Scopus, Medline, Canadian Patent Database, Genome Quest

Keywords: RGMa, furin, cleavage, subtilisin, proprotein convertase, PCSK*, PC* and SEQ ID Nos: 1, 4 and 5

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	TASSEW, N. G. et al. "SKI-1 and Furin Generate Multiple RGMa Fragments that Regulate Axonal Growth", DEVELOPMENTAL CELL. February 14, 2012 (14-02-2012), Vol. 22, pages 391-402, ISSN: 1534-5807. See whole document.	1-39
P, X	KEY, B et al. "Repulsive Guidance Molecule A", CELL ADHESION & MIGRATION. March/April 2012 (03/04-2012), Vol. 6, No. 2, pages 85-90, ISSN1933-6926. See whole document.	1-39
X	WO 2011/070045 A1 (ABBOTT GMBH & CO. KG) 16 June 2011 (16-06-2011) See SEQ ID No:2 and Examples 1 and 2.	31-33, 35, 36, 38 and 39

[ ] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2013 (31-05-2013)	06 June 2013 (06-06-2013)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Kristoffer Wilde (819) 953-0551

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2013/000104

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 1-30

because they relate to subject matter not required to be searched by this Authority, namely :

1-30 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 1-30.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2011070045A1	16 June 2011 (16-06-2011)	AU2010329955A1	24 May 2012 (24-05-2012)
		CA2780069A1	16 June 2011 (16-06-2011)
		CN102656190A	05 September 2012 (05-09-2012)
		EP2510001A1	17 October 2012 (17-10-2012)
		IL219766D0	31 July 2012 (31-07-2012)
		KR20120118002A	25 October 2012 (25-10-2012)
		MX2012006560A	05 October 2012 (05-10-2012)
		SG181563A1	30 July 2012 (30-07-2012)
		US2011135664A1	09 June 2011 (09-06-2011)

C07K 14/47 (2006.01) , ***C07K 16/18*** (2006.01) , ***C07K 16/28*** (2006.01) , ***C07K 17/00*** (2006.01)