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[Continued on next page]

(54) Title: ANTI-RYK ANTIBODIES AND METHODS OF USING THE SAME

(57) Abstract: Methods for inhibiting degeneration of a neuron, methods of treating a neurological/neurodegenerative disease, methods of modulating the directional growth of a neuron, and methods of interfering with the interaction of Wnt and Ryk are provided herein. Also provided are isolated anti-Ryk antibodies and antibody fragments that specifically bind to a binding domain of Wnt.

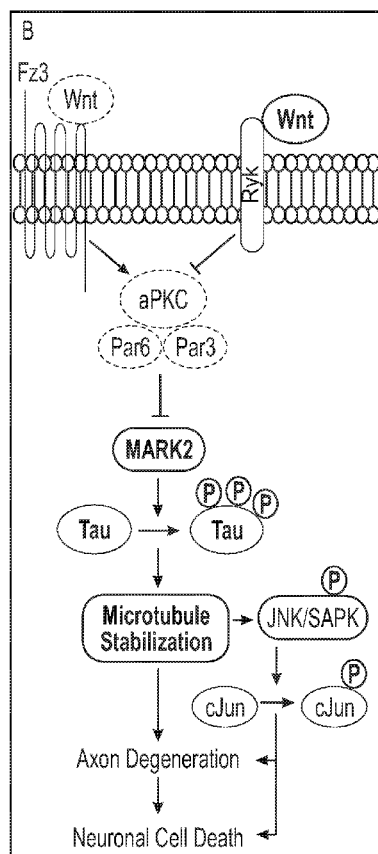


FIG. 7B



RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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ANTI-RYK ANTIBODIES AND METHODS OF USING THE SAME**CROSS REFERENCE TO RELATED APPLICATION(S)**

[0001] This application claims the benefit of priority under 35 U.S.C. § 119(e) of U.S. Serial No. 62/314,025, filed March 28, 2016, the entire content of which is incorporated herein by reference.

GRANT INFORMATION

[0002] This invention was made with government support under Grant Nos. NS047484 and NS081738 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 15, 2017, is named 20378-201389_SL.txt and is 14,104 bytes in size.

BACKGROUND OF THE INVENTION**FIELD OF THE INVENTION**

[0004] The invention relates generally to antibodies and more specifically to use of an anti-Ryk antibody or antibody fragment that specifically binds to a binding domain of Wnt to inhibit Wnt-Ryk signaling.

BACKGROUND INFORMATION

[0005] The central nervous system (CNS) is connected by ascending sensory pathways and descending motor or regulatory pathways. In the CNS, somatosensory pathways ascend to the brain centers, and motor pathways controlling body movement descend from the brain to the spinal cord. Unlike the peripheral nervous system, damage to the central nervous system axons, such as spinal cord axons cannot be repaired, causing permanent impairment of neural function, such as in paralysis. The spinal cord serves important functions in the central nervous system. One such function is to allow communication of the body and the brain. The nerve fibers within the spinal cord carry messages to and from

the brain to other parts of the body. In general, sensory information from the body travels along the spinal cord up to the brain and instruction from the brain, such as motor command, travels along the spinal cord down from the brain.

[0006] The term “spinal cord injury” refers to any injury of the neurons within the spinal canal. Spinal cord injury can occur from either trauma or disease to the vertebral column or the spinal cord itself. Most spinal cord injuries are the result of trauma to the vertebral column causing a fracture of the bone, or tearing of the ligaments with displacement of the bony column producing a pinching of the spinal cord. The majority of broken necks and broken backs, or vertebral fractures, do not cause any spinal cord damage; however, in 10-14% of the cases where a vertebral trauma has occurred, the damage is of such severity it results in damage to the spinal cord. It is estimated that the annual incidence of spinal cord injury (SCI), not including those who die at the scene of the accident, is approximately 40 cases per million population in the U.S., or approximately 11,000 new cases each year. The number of people in the U.S. who are alive today and who have SCI has been estimated to be between 721 and 906 per million population. This corresponds to between 183,000 and 230,000 persons. Treatment options for patients with spinal cord injuries are limited. Often, patients with SCI are left with severe, permanent disabilities.

[0007] In recent years, increasing evidence suggests that Wnts, which have been better known as morphogens in early development, are conserved axon guidance molecules during nervous system wiring both in vertebrates and invertebrates (Zou, Y. 2004. *Trends Neurosci* 27:528-32; Fradkin, et al. 2005. *J Neurosci* 25:10376-8; Zou & Lyuksyutova. 2007. *Curr Opin Neurobiol* 17:22-8; Salinas, et al. 2008. *Annu Rev Neurosci* 31:339-358). Wnts are secreted glycoproteins, which bind to three classes of receptors, the Frizzleds, Ryk and ROR2 (Gordon & Nusse. 2006. *J Biol Chem* 281:22429-33; Logan & Nusse. 2004. *Annu Rev Cell Dev Biol* 20:781-810). It has also been shown that the Wnt family proteins are essential guidance cues along the A-P axis of the spinal cord and topographic map formation in the retinotectal projections in development and may play important roles in regulating adult CNS axon regeneration after spinal cord injury (Lyuksyutova et al. 2003. *Science* 302:1984-8; Liu, et al. 2005. *Nat Neurosci* 8:1151-9; Schmitt et al. 2006. *Nature* 439:31-7; Wolf et al. 2008. *J Neurosci* 28:3456-67; Liu et al. 2008. *The Journal of Neuroscience* 28:8376-8382).

[0008] Commissural axons, which originate from the dorsal spinal cord, first project along the dorsal-ventral axis to grow towards the ventral midline, the floor plate. The ventrally directed growth of commissural axons are guided by repulsive cues, the BMPs emanating from the dorsal midline, the roof plate, and attractive cues, Netrin-1 and Sonic Hedgehog, secreted from the floor plate (Zou et al. 2007. *Curr Opin Neurobiol* 17:22-8). Once these commissural axons cross the midline, they lose responsiveness to midline attractants and gain sensitivity to chemorepellents, the Slits and Semaphorins, emanating from the floor plate and the neighboring ventral gray matter, forcing them to make a 90° turn into their longitudinal trajectory (Zou et al. 2000. *Cell* 102:363-75). The dorsal populations of rodent commissural axons all turn anterior and project towards the brain. The anterior turning requires Wnt-Frizzled signaling. Several Wnts, including Wnt4, Wnt7b, Wnt7a and Wnt5a, are expressed in an anterior-posterior decreasing gradient along the spinal cord at the ventral midline and attract post-crossing commissural axons, which have crossed the midline to turn anteriorly. When the Wnt gradient was disrupted by adding Wnt inhibitors, secreted Frizzled-related proteins (sFRPs) or positioning Wnt4-secreting cell aggregates in "open-book" explant culture, commissural axons show specific A-P randomize growth after midline crossing. In Frizzled3 mutant embryos, spinal cord commissural axons lose A-P directionality *in vivo* (Lyuksyutova et al. 2003. *Science* 302:1984-8).

[0009] Studies in *Drosophila* midline axon pathfinding independently showed that DWnt5 is a chemorepellent and repels a subset of commissural axons via a receptor called, Derailed (Yoshikawa et al. 2003. *Nature* 422:583-8). The vertebrate homologue of Derailed, Ryk, is also a repulsive Wnt receptor and an anterior-high posterior-low Wnt gradient created by differential expression of Wnt1 and Wnt5a, is required for the posterior growth of corticospinal tract axons in the spinal cord (Liu et al. 2005. *Nat Neurosci* 8:1151-9). Therefore, Wnts control A-P guidance of both ascending and descending axons in the spinal cord by attractive and repulsive guidance mechanisms. Wnt-Ryk signaling was also found to regulate the pathfinding of corpus callosum in the mammalian forebrain by a repulsive mechanism (Keeble et al. 2006. *J Neurosci* 26:5840-8). Studies in *C. elegans* showed that Wnt signaling control anterior-posterior directionality of the pathfinding of a number of axons and migration of neuroblasts (Pan et al. 2006. *Dev Cell* 10:367-77;

Hilliard et al. 2006. *Dev Cell* 10:379-90; Prasad & Clark. 2006. *Development* 133:1757-66). Therefore, the A-P guidance mechanisms appear to be highly conserved in animal kingdom (Zou. 2006. *Neuron* 49:787-9.).

[0010] In addition to the role of Wnts in axon pathfinding, Wnt3 is also a positional cue for topographic mapping in the retinotectal system, acting as a laterally-directing mapping force for retinal ganglion cell axons, opposing the medially-directed force created by ephrinB1 gradient in the optic tectum (Schmitt et al. 2006. *Nature* 439:31-7). Wnt3 is expressed in a medial-high to lateral-low gradient in the optic tectum. Ryk is expressed in a dorsal-ventral (D-V) increasing gradient in the retinal ganglion cells. Therefore, the more ventral RGC axon branches are more strongly repelled by Wnt3 and Wnt3-Ryk signaling drives the interstitial branches to grow towards the lateral tectum. In the meantime, ephrinB1 is expressed in the same graded fashion and EphBs are expressed at higher levels in ventral RGCs. EphBs mediate attraction to ephrinB1. Therefore, the more ventral RGC axon branches are more attracted by ephrinB1 towards the medial tectum. The balancing act between the medial (ephrinB1) and lateral (Wnt3) mapping forces ensures RGC axons to terminate at correct topographic positions. Remarkably, Wnt-Frizzled signaling is also required for proper dorsal-ventral retinotopic mapping in the *Drosophila* visual system (Sato et al. 2006. *Nat Neurosci* 9:67-75; Zou & Lyuksyutova. 2007. *Curr Opin Neurobiol* 17:22-8). Therefore, Wnts are conserved topographic mapping cues along the D-V axis.

[0011] Commissural axons of the developing spinal cord are guided to the ventral midline by a collaboration of chemoattractants (Netrin-1 and Sonic Hedgehog (Shh)) and chemorepellents (Bone Morphogenetic Proteins (BMPs)) secreted by midline floor plate and roof plate cells, respectively. Once these axons reach the floor plate they switch off their responsiveness to chemoattractants from the floor plate and become responsive to chemorepulsive cues also expressed by the floor plate cells and the surrounding ventral gray matter, including members of the Class 3 Semaphorins (Sema3B and Sema3F) and the Slit family proteins (Serafini, T., et al. *Cell* 78, 409-424, 1994; Kennedy, T. E., et al. *Cell* 78, 425-435, 1994; Serafini, T., et al. *Cell* 87, 1001-1014, 1996; Zou, Y., et al. *Cell* 102, 363-375, 2000; Charron, F., et al. *Cell* 113, 11-23, 2003; Long, H., et al. *Neuron* 42, 213-223, 2004). Neuropilin-2 mutant embryos showed severe guidance defects including stalling in

the midline, overshooting to the contralateral side of the spinal cord and randomly projecting along the anterior-posterior axis (Zou, Y., et al. Cell 102, 363-375, 2000).

[0012] The identification of modulators of neuronal growth and regeneration following SCI could be applied in new forms of treatment of patients with this debilitating condition. The identification of modulators of neuronal growth and regeneration could also be applied in the treatment of patients with other disorders involving neuronal dysfunction, such as neurological/neurodegenerative diseases or disorders. Agents that can promote axonal growth along the A-P axis following injury to the spinal cord may be applied to help prevent the permanent paralysis that is often associated with SCI. Therefore, there is a need for better treatments of SCI, and a greater understanding of modulators of neuronal growth and regeneration might lead to improved methods of treatment of such neurological/neurodegenerative diseases or disorders.

SUMMARY OF THE INVENTION

[0013] The present invention is based on the finding that an anti-Ryk antibody or antibody fragment that specifically binds to a binding domain of Wnt inhibits Wnt-Ryk signaling. As such, the anti-Ryk antibody or antibody fragment may be used to modulate the directional growth of a mammalian neuron when the spinal cord has been damaged, as well as to inhibit degeneration of a neuron and to treat a neurodegenerative disease. Accordingly, in one aspect, the invention provides an isolated anti-Ryk antibody or antibody fragment that specifically binds to a WnT binding domain on Ryk or specifically binds to an epitope within a region of the ectodomain of Ryk, amino-acids 90-183 of Ryk, the antibody or antibody fragment comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 8 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 4.

[0014] In another aspect, the present disclosure provides an isolated anti-Ryk antibody or antibody fragment that specifically binds to a binding domain of Wnt or specifically binds to the same epitope on Wnt as does a reference antibody or antibody fragment, or cross-competes for specific binding to Wnt with a reference antibody or antibody fragment. In various embodiments, the antibody or antibody fragment and/or the reference antibody or antibody fragment includes a heavy chain variable region comprising the CDR sequences set forth in

SEQ ID NOs: 5-7 or SEQ ID NOs: 5, 11, and 12; and/or a light chain variable region comprising the CDR sequences set forth in SEQ ID NOs: 1-3 or SEQ ID NOs: 9, 2, and 3. In various embodiments, the antibody or antibody fragment specifically binds to an epitope within amino acid residues 90-183 of Wnt. In various embodiments, the heavy chain variable region of the antibody or antibody fragment includes an amino acid sequence comprising at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to SEQ ID NOs: 8 or 13. In various embodiments, the light chain variable region of the antibody or antibody fragment includes an amino acid sequence comprising at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity to SEQ ID NOs: 4 or 10. In various embodiments, the antibody or antibody fragment is formulated in a pharmaceutically acceptable carrier or excipient.

[0015] In another aspect, the present disclosure provides a nucleic acid sequence encoding the isolated antibody or antibody fragment described herein. Also provided is a vector, such as an expression vector, that includes the nucleic acid sequence. Also provided is a host cell, such as a mammalian host cell that includes the vector.

[0016] In another aspect, the present disclosure provides an immunoconjugate of the isolated anti-Ryk antibody or antibody fragment linked to a therapeutic agent such as a cytotoxin or a radioactive isotope. In another aspect, the invention provides a bispecific molecule, *e.g.*, a bispecific antibody, which includes the isolated anti-Ryk antibody or antibody fragment linked to a second functional moiety having a different binding specificity than the isolated anti-Ryk antibody or antibody fragment.

[0017] In another aspect, the present disclosure provides a method of interfering with interaction of Wnt and Ryk. The method includes contacting a sample that includes Wnt and Ryk with the isolated antibody or antibody fragment described herein, thereby interfering with the interaction of Wnt and Ryk.

[0018] In another aspect, the present disclosure provides a method of inhibiting degeneration of a neuron. The method includes contacting the neuron with the isolated antibody or antibody fragment described herein, thereby inhibiting degeneration of the neuron. In various embodiments, degeneration of an axon of the neuron is inhibited or degeneration of a cell body of the neuron is inhibited. In various embodiments, the axon is a spinal cord

commissural axon, an upper motor neuron axon, or a central nervous system axon. In various embodiments, the neuron is a damaged spinal cord neuron, a sensory neuron, a motor neuron, a cerebellar granule neuron, a dorsal root ganglion neuron, a cortical neuron, a sympathetic neuron, or a hippocampal neuron. In various embodiments, the neuron forms part of a nerve graft or a nerve transplant. In various embodiments, the neuron is *ex vivo* or *in vitro*. In various embodiments, the nerve graft or the nerve transplant forms part of an organism, such as a mammal or human.

[0019] In another aspect, the present disclosure provides a method of treating a neurological disease or disorder, *e.g.*, a neurodegenerative disease or disorder in a subject having or being at risk of developing the neurological disease or disorder, *e.g.*, a neurodegenerative disease or disorder. The method includes administering to the subject the isolated antibody or antibody fragment described herein, thereby treating the neurological disease or disorder, *e.g.*, a neurodegenerative disease or disorder, in the subject. In various embodiments, the neurodegenerative disease is amyotrophic lateral sclerosis, Alzheimer's disease or Parkinson's disease.

[0020] In another aspect, the present disclosure provides a method for modulating the directional growth of a mammalian neuron. The method includes contacting the neuron with the isolated antibody or antibody fragment described herein, thereby modulating the directional growth of the neuron, a spinal cord commissural axon, an upper motor neuron axon, or a central nervous system axon. In various embodiments, the neuron is a damaged spinal cord neuron, a sensory neuron, a motor neuron, a cerebellar granule neuron, a dorsal root ganglion neuron, a cortical neuron, a sympathetic neuron, or a hippocampal neuron. In various embodiments, the neuron forms part of a nerve graft or a nerve transplant. In various embodiments, the neuron is *ex vivo* or *in vitro*. In various embodiments, the directional growth of the neuron facilitates regeneration of the neuron.

[0021] In another aspect, the present disclosure provides a transgenic non-human mammal such as a mouse whose genome comprises a heterozygous or homozygous deletion, inactivation or knock-out of the Ryk gene. In various embodiments, the mouse has the phenotype *Frizzled3^{-/-} Ryk^{+/-}*. In various embodiments, the mouse contains a conditional disruption of the Ryk gene, *e.g.*, a corticospinal tract (CST)-specific disruption of the Ryk gene. In various embodiments, the disrupted Ryk gene includes a recombinant Ryk allele, a

selectable marker, frt sites flanking the selectable marker, and loxP sites flanking a portion of the allele. The marker may be PGK Neo and the loxP sites may flank exons 3-6 of the allele. Thus, the invention also provides an isolated cell derived from the transgenic non-human mammal. Also provided is a vector for making the transgenic non-human mammal whose genome comprises a heterozygous or homozygous deletion, inactivation or knock-out of the Ryk gene. An exemplary vector can include a portion of a Ryk gene, wherein exons 3-6 of the Ryk gene are flanked by 3' and 5' loxP sites, a selectable marker between exon 6 and the 5' loxP site, and frt sites flanking the selectable marker.

[0022] In another aspect, the present disclosure provides a method of producing a knockout mouse with a conditional disruption of the Ryk gene, *e.g.*, a CST-targeted disruption in a Ryk gene. The method includes transfecting the vector described above into a population of murine embryonic stem (ES) cells, selecting a transfected ES cell which expresses said selectable marker, introducing said transfected ES cell into an embryo of an ancestor of said mouse, allowing said embryo to develop to term to produce a chimeric mouse with a conditional knock-out construct in its germ line, breeding said chimeric mouse to produce a heterozygous mouse with a conditionally disruptable Ryk gene, and breeding said heterozygous mouse with a mouse containing a loxP-flanked stop cassette preventing tdTomato expression only in corticospinal axons to produce a mouse with a conditional disruption of the Ryk gene, *e.g.*, a CST-specific disruption in the Ryk gene.

[0023] In another aspect, the present disclosure provides a method of screening for an agent that modulates the amount, level and/or activity of atypical protein kinases C (aPKC) or MARK2. For example, provided herein is a method of screening for a therapeutic agent for treating a neurological disease or disorder. The method includes administering a test agent to the transgenic non-human mammal described herein and evaluating the effect of the test agent on at least one of: the amount of atypical protein kinases C (aPKC) or MARK2 protein, the level of aPKC or MARK2 activity or the level of aPKC or MARK2 in at least one disease-relevant tissue of the transgenic non-human mammal, wherein at least one of: a decrease in the amount of aPKC protein, an increase in the amount of MARK2 protein, a decrease in the level of aPKC activity, an increase in the level of MARK2 activity, a reduction in the level of aPKC, or an increase in the level of MARK2 in at least one disease-relevant tissue relative to a similar transgenic non-human mammal that does not receive the test agent indicates the test agent is therapeutic for the neurological disease or disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figures 1A-1F are pictorial and graphical diagrams showing that inhibition of aPKC induces neurite degeneration and neuronal apoptosis. Figure 1A shows that endogenous aPKC is localized in cortical neuron cell bodies (E16.5) (arrows) and in SMI-312+ axons (arrowheads). Figures 1B-1F show overexpression of PKC ζ -WT, PKC ζ -KD or PKC ζ -T410A in cerebral cortical neurons (pCIG2-EGFP was used as control). Figure 1B shows that recombinant PKC ζ -WT protein is localized in both neuronal cell bodies (arrows) and processes (arrowheads) while PKC ζ -KD and PKC ζ -T410A proteins are absent in neurites. Figure 1C shows a higher magnification image showing intact neurites (plain arrows) in a PKC ζ -WT transfected neuron and fragmented neurites in a PKC ζ -KD transfected cell (dotted arrows) labeled for EGFP. Figure 1D shows aCasp3 immunostaining in neurons transfected with the indicated plasmid. Figure 1E shows the number of transfected neurons with degenerative neurites (dotted arrows in B) counted over the total number of EGFP+ neurons and expressed as the % of the control. Figure 1F shows the number of apoptotic transfected neurons (aCasp3+ EGFP+) counted over the total number of EGFP+ neurons. Data represent mean $\pm$ SEM (n= 5 experiments in Figure 1E, n=3 experiments in Figure 1F. * p<0.05, ** p<0.01, ANOVA with Bonferroni post-test. Scale bars: 200 μ m (Figure 1B), 100 μ m (Figure 1D), 50 μ m (Figures 1A and 1C).

[0025] Figures 2A-2H are pictorial and graphical diagrams showing that aPKC inhibition induces rapid axon degeneration that precedes neuronal cell body death. Figures 2A and 2B show axon degeneration in E16.5 cerebral cortical neurons treated with 10 μ M myristoylated aPKC PS for 2h. The proportion of neurons with degenerative axons was counted over the total number of neurons (SMI-312+ cells.). Plain arrows indicate neurons with intact axons, dotted arrows neurons with beading axons and arrowheads neurons without axons. Figures 2C and 2D show aPKC PS induced axon fragmentation in a dose- (Figure 2C) and time- (Figure 2D) dependent manner. Figure 2E shows aPKC PS treatment reduced phosphorylation of PKC ζ -T410. Cell cultures were fixed and immunolabeled with anti-phospho-PKC ζ -T410 antibody. P-PKC ζ -T410 immunoreactivity was measured in neuronal cell bodies (arrows) in neuronal cell cultures incubated with water (Control) or 10 μ M aPKC PS for 2h. Figures 2F-2I show axon degeneration induced by aPKC inhibition precedes cell body death. Cortical neurons incubated with water (Control) or 10 μ M aPKC

PS for 2h, 4h or 24h, fixed and double-labeled for SMI-312 and TUNEL (Figure 2F) or activated caspase-3. Plain arrows indicate dead neuronal cell bodies labeled for TUNEL in control neurons and neurons treated with aPKC PS. Dotted arrows indicate neurons with beading axons that are not labeled for TUNEL. Graphs represent the proportion of neurons with degenerative axons (Figure 2G) and of dead neurons (TUNEL+ (Figure 2H) or aCasp3+ (Figure 2I)) counted after 2h, 4h or 24h of aPKC PS treatment in SMI-312+ neurons. Data represent mean $\pm$ SEM (n=3 experiments). * p<0.05, ** p<0.01, ***p<0.001. Figures 2B-2D: ANOVA with Bonferroni post-test; Figures 2E and 2G-2I: unpaired Student's t test. Scale bars: 50 μ m (Figures 2A and 2F), 25 μ m (Figure 2E).

[0026] Figures 3A-3I are pictorial and graphical diagrams showing that inhibition of aPKC destabilizes microtubules through regulation of MARK2 activity and Tau phosphorylation. Figures 3A and 3B show axon degeneration induced by aPKC is partially prevented by stabilizing microtubules with taxol. Cortical neurons were incubated with 10 μ M taxol for 2 hours prior to and during 2h treatment of 7 μ M aPKC PS. Cells were fixed and immunolabeled with SMI-312 antibody. The proportion of neurons with intact axons was counted in SMI-312+ neurons (Figure 3B). Plain arrows indicate neurons with intact axons. Dotted arrows indicate neurons with beading axon. Arrowheads indicate neurons without axons. Figures 3C-3F show the results from Western blottings performed with proteins lysates from E16.5 cerebral cortical cell cultures incubated with water (Control) or 10 μ M aPKC PS for 2 hours. Phospho-protein levels were assessed by densitometric analysis and normalized with the corresponding total protein and actin or GAPDH (Figures 3C-3E). The level of Glu-tubulin protein was normalized with GAPDH (Figure 3F). Lanes 1-3 and lanes 4-6 represent triplicates for control and for aPKC PS respectively. Figures 3G-3I show that Tau-S262A overexpression protects neurons from neurite degeneration induced by PKC ζ -KD overexpression. E16.5 cortical cells cultured for 3 days transfected with the indicated plasmids were fixed 2 days after transfection and immunolabeled for EGFP and aCasp3 and counterstained with DAPI. Figure 3H shows the number of transfected neurons with degenerative neurites counted over the total number of EGFP+ neurons. Figure 3I shows the number of apoptotic transfected neurons (aCasp3+ EGFP+) counted over the total number of EGFP+ neurons. Data represent mean $\pm$ SEM. Figures 3B-3F: n=3 experiments; Figures 3H and 3I: n= 4 experiments. * p<0.05, ** p<0.01, ***

$p < 0.001$, Figures 3B-3F: Unpaired Student's t test; H,I: ANOVA with Bonferroni post-test. Scale bars: 50 μ m (Figure 3A), 100 μ m (Figure 3G).

[0027] Figures 4A-4E are pictorial and graphical diagrams showing that aPKC inhibition activates the JNK-cJun signaling pathway. Figures 4A-4C show that JNK phosphorylation is increased upon aPKC inhibition. Western blotting were performed with proteins lysates from E16.5 cortical neurons cultured for 3 days incubated with water (Control) or 10 μ M aPKC PS for 1h or 2h. Figures 4B and 4C show phospho-protein levels assessed by densitometric analysis. Levels of phospho-proteins were normalized with the corresponding total protein and GAPDH. Figures 4D and 4E show that cortical neuronal cultures incubated with water (Control) or 10 μ M aPKC PS for 1h were immunolabeled for P-JNK T183/T185 (D) or P-c-Jun S63 (Figure 4E). Arrows indicate increased immunoreactivity in neurons treated with aPKC PS compared to control neurons. Data represent mean $\pm$ SEM. $n=3$ experiments/group, * $p < 0.05$, ** $p < 0.01$, unpaired Student's t test. Scale bar: 100 μ m.

[0028] Figures 5A-5F are pictorial and graphical diagrams showing that Ryk promotes axonal degeneration induced by aPKC inhibition. Figures 5A and 5B show that aPKC activity is increased in Ryk KO mouse cortical neuronal cell cultures. Western blotting were performed with proteins lysates from cortical neurons from E16.5 Ryk wild type, heterozygous and KO embryos cultured for 3 days *in vitro*. Levels of P-PKC ζ -T410 were assessed by densitometric analysis and normalized with total PKC ζ and GAPDH. Figures 5C and 5D show that monoclonal Ryk antibody blocks degeneration. E16.5 cortical neurons were incubated with monoclonal mouse anti-Ryk antibody (50 μ g/ml or 100 μ g/ml) or normal mouse IgG (100 μ g/ml) for 2 hours prior to and during 2h treatment of 7 μ M aPKC PS. Cells were fixed and immunolabeled for SMI-312. Figure 5D shows the proportion of SMI-312+ neurons with degenerative axons. Figures 5E and 5F show reduced degeneration with Ryk knockout mouse. Cortical neurons individually dissociated from E16.5 WT (Ryk^{+/+}) and Ryk KO embryos (Ryk^{-/-}) were incubated with 7 μ M aPKC PS for 2h. Cells were fixed and immunolabeled for SMI-312. Figure 5F shows the proportion of SMI-312+ neurons with degenerative axons in Ryk^{+/+} and Ryk^{-/-} neurons treated with aPKC PS or water (control). Plain arrows indicate neurons with intact axons, dotted arrows neurons with beading axons and arrowheads neurons without axons. Data represent mean $\pm$ SEM (Figure 5A: Ryk^{+/+}: $n=3$ embryos, Ryk^{+/+} $n=3$ embryos, Ryk^{-/-}: $n=4$ embryos; D: $n=5$

experiments; Figure 5F: Ryk^{+/+}: n=4 embryos, Ryk^{-/-}: n=6 embryos). * p<0.05, ** p<0.001, *** p<0.001; Figures 5B and 5F: Unpaired Student's t test, D: ANOVA with Bonferroni post-test. Scale bars: 100µm.

[0029] Figures 6A-6E are pictorial and graphical diagrams showing genetic interaction between Ryk and Frizzled3 in neuronal cell death. Figure 6A shows a lower magnification of a WT brain section (Ryk^{+/+}) showing the localization of the retrosplenial (RSP) cortex. Figures 6B and 6C show that aCasp3⁺ cell number is decreased in Ryk^{-/-} embryos compared to Ryk^{+/+} mice. Figures 6D and 6E show that aCasp3⁺ cell number is increased in the RSP of Frizzled3^{-/-} embryos compared to Frizzled3^{+/+} and Frizzled3^{+/-} mice. Ryk knock-down attenuates cell death in the RSP of Frizzled3KO mice. Data represent mean±SEM (Ryk^{+/+}: n=6 embryos, Ryk^{+/-}: n=4 embryos, Ryk^{-/-}: n=5 embryos, Frizzled3^{+/+}: n=4 embryos, Frizzled3^{+/-}: n=6 embryos, Frizzled3^{-/-}: n=8 embryos, Frizzled3^{-/-} Ryk^{+/+}: n=5 embryos). * p<0.05, ** p<0.01, *** p<0.001. Figure 6C: Student's t test, E: ANOVA with Bonferroni post-test. HIPP: hippocampus, LV: lateral ventricle, RSP: retrosplenial cortex. Scale bars: 500µm (Figure 6A), 200µm (Figures 6B and 6D).

[0030] Figures 7A and 7B are pictorial diagrams showing a model of aPKC action on axonal integrity and neuronal cell survival. Figure 7A shows that in normal conditions when aPKC is expressed and active, aPKC inhibits MARK2 activity through phosphorylation on T585. Phosphorylation state of Tau is low and Tau binds to and stabilizes microtubules, maintaining axon integrity and neuronal cell survival. Figure 7B shows that when aPKC kinase activity is reduced or inhibited, MARK2 activity increases and phosphorylates Tau in its microtubule binding domain on S262. Hyperphosphorylated Tau detach from microtubules which destabilizes microtubules. Disruption of microtubules activates the stress kinase JNK/SAPK pathway, leading to axonal degeneration and neuronal cell death.

[0031] Figures 8A-8F are pictorial and graphical diagrams showing that *Ryk* conditional deletion enhances motor function recovery from spinal cord injury. Figure 8A shows a timeline outlining experimental details of bilateral cervical level S (CS) dorsal column lesion. Figures 8B-8C show generation of *Ryk* conditional allele. Figure 8B shows that exons 3-6 were flanked with loxP sites. Figure 8C shows the results of a Western blot of

postnatal day 7 motor cortex extract from mice infected at postnatal day 0 with AAV2/1 synapsin Cre. Full-length blot presented in Figs. 23A and 23B. Figures 8D and 8E show a schematic showing the level of the CS lesion in relation to motor neuron pools for distinct forelimb muscle groups (adapted from McKenna, Prusky, and Whishaw, 2000). Figure 8F shows behavioral performance on forelimb reach skilled food-pellet retrieval task shows enhanced recovery after *Ryk* conditional deletion in bilateral motor cortex (n=2S mice (control), 17 mice (*Ryk* cKO), from 21 litters, repeated measures ANOVA $P=0.0003$, $F(1,40)=16.0102$). Data presented as mean $\pm$ s.e.m.

[0032] Figures 9A-9H are pictorial and graphical diagrams showing *Ryk* conditional deletion enhances corticospinal axon sprouting after spinal cord injury. Figures 9A and 9B are representative images of tdTomato-labeled CST axons from eight serial sagittal cryosections spaced 140 μ m apart superimposed over GFAP astroglial staining at the center of injury (1 experiment, n=12 mice/group, from 11 litters; compass showing dorsal (D), ventral (V), rostral (R), and caudal (C)). Mice with *Ryk* conditional deletion had greater levels of collateralization both rostral and caudal to the lesion than control mice (one-tailed t-test $*P<0.05$). Figures 9C and 9D show higher magnification of single confocal planes from boxed regions (1.5mm caudal to the lesion site) indicated in Figure 9A and 9B, respectively. Figure 9E shows the sum of tdTomato labeled axons (normalized to pyramidal labeling) over 3mm rostral to lesion relative to control in the dorsal columns (n=12 mice/group, one-tailed t-test $P=0.12$, $t(21)=1.198$) and in the gray matter. Mice with *Ryk* conditional deletion had greater levels of collateralization both rostral and caudal to the lesion than control mice (n=12 mice/group, one-tailed t-test $*P<0.05$: rostral $P=0.0499$ $t(19)=1.730$, caudal $P=0.0397$ $t(19)=1.855$). Figures 9F-9H show the distribution of corticospinal axons (axon index is thresholded pixels at every 0.411 μ m in 8 total sagittal spinal cord cryosections divided by thresholded pixels in transverse pyramids) within the dorsal columns (Figure 9F) or spinal gray matter (Figures 9G and 9H). Figure 9H is a magnified view of rostral collaterals from Figure 9G. CS injury site is at 0 μ m, rostral is represented with negative numbers, caudal with positive. Data in Figure 9E is presented as median with inter-quartile range, data in Figures 9F-9H is presented as mean $\pm$ s.e.m.

[0033] Figures 10A-10D are pictorial and graphical diagrams showing changes of corticospinal connectivity after CS dorsal column lesion. Figures 10A and 10B show

media-lateral distribution of corticospinal axons shows highest increase in collaterals proximal to the main, dorsal corticospinal tract (regions I and II) after *Ryk* conditional deletion (n=12 mice/group, one-tailed t-test *P<0.05: II rostral P=0.0225 t(19)=2.146, II caudal P=0.0295 t(21)=1.996, I caudal P=0.0059 t(17)=2.819). Figure 10C shows both control and *Ryk* conditional deletion mice showed pre-synaptic densities (vGlut1 colocalization with tdTomato-labeled corticospinal axons) at 600µm rostral to the CS injury site (1 experiment, n=9mice/group). Figure 10D shows media-lateral distribution of corticospinal innervation at 600µm rostral to CS injury site. All data presented as median and inter-quartile range.

[0034] Figures 11A-11F are pictorial and graphical diagrams showing that secondary injury at cervical level 3 eliminates enhanced recovery. Figure 11A is a timeline outlining experimental details of secondary C3 lesion experiments following recovery from bilateral CS dorsal column lesion. Figure 11B is a schematic of secondary C3 injury, above the level of increased pre-synaptic density shown in (Figs. 10C-10E). Figure 11C shows behavioral performance on forelimb reach skilled food-pellet retrieval task shows elimination of enhanced recovery after *Ryk* conditional deletion by second C3 dorsal column lesion (1 experiment, n=8 (control sham), 7 (control C3), 6 (*Ryk* cKO sham & C3) mice, mice from 14 litters, ANOVA P=0.0102 F(3)=4.7432, Bonferroni corrected t-test *P<0.05: 1. *Ryk* cKO sham v. control sham P=0.0106, 2. *Ryk* cKO sham v. *Ryk* cKO C3 P=0.0092). Figures 11D-11F show secondary C3 dorsal column lesion eliminated enhanced levels of collateralization in *Ryk* conditional deleted mice. Figure 11D shows representative images of tdTomato-labeled CST axons from 8 serial sagittal cryosections spaced 140µm apart superimposed over GFAP astroglial staining at the center of injury (1 experiment, 7 control, 6 *Ryk* cKO mice). Figures 11E and 11F show distribution of corticospinal axons (axon index as described above) within the dorsal columns (Figure 11E) or spinal gray matter (Figure 11F). C3 injury site is at 0 µm, rostral is represented with negative numbers, caudal with positive. Data in Figure 11C presented as median and inter-quartile range, data in Figures 11E and 11F are presented as mean ± s.e.m.

[0035] Figures 12A-12I are pictorial and graphical diagrams showing that monoclonal *Ryk* antibody infusion promotes functional recovery from spinal cord injury. Figure 12A is a schematic showing antibody infusion by intrathecal catheterization. Figure 12B shows

behavioral performance on forelimb reach skilled food-pellet retrieval task shows enhanced recovery in rats infused with Ryk monoclonal antibody for 28 days starting at time of injury (n=6 rats (IgG control), 5 rats (Ryk monoclonal), repeated measures ANOVA $P=0.0354$, $F(1,9)=6.113$). Figure 12C shows behavioral performance on skilled locomotor grid crossing task was not affected by Ryk monoclonal antibody infusion. Figure 12D shows that Ryk monoclonal recognizes full-length Ryk protein expressed in transfected COS-7 cells by Western and immunocytochemistry. Figures 12E-12H show that BDA-labeled corticospinal axons in rats infused with Ryk monoclonal antibody had greater levels of collateralization than control mouse IgG infused rats. Figures 12E and 12F are images of BDA-labeled CST axons from 6 serial sagittal cryosections spaced 280 μ m apart superimposed over GFAP astroglial and NG2 staining at the center of injury. Figures 12G and 12H show a distribution of corticospinal axons (axon index is thresholded pixels at every 0.741 μ m in 6 total sagittal spinal cord cryosections divided by thresholded pixels in transverse pyramids) within the dorsal columns (Figure 12G) or spinal gray matter (Figure 12H). CS injury site is at 0 μ m, rostral is represented with negative numbers, caudal with positive. Figure 12I shows the sum of normalized axon collaterals over 5mm relative to control. Rats infused with Ryk monoclonal antibody had greater levels of collateralization both rostral and caudal to the lesion than control mouse IgG infused rats (n=6 rats (IgG control), 5 rats (Ryk monoclonal), one-tailed t-test 585 * $P<0.05$: rostral $P=0.0446$ $t(6)=2.000$, caudal $P=0.0196$ $t(6)=2.594$). Data in Figures 12B, 12C, 12G, and 12H is presented as mean $\pm$ s.e.m., data in Figure 12I is presented as median and inter-quartile range.

[0036] Figures 13A-13C are graphical diagrams showing cortical map re-organization during recovery from spinal cord injury. Figure 13A is a timeline outlining experimental details of optogenetic mapping with weekly behavioral testing following bilateral CS dorsal column lesion. Figure 13B is a topographic representation of elbow flexor and extensor activation, relative to bregma (*) prior to and 3 days, 4 weeks, and 8 weeks after CS dorsal column lesion. Data presented as total number of mice responding with evoked movements at each location, lighter color indicates a larger number of mice are responsive at a given location. Each tic mark represents 300 μ m. Figure 13C shows that subsequent C3 dorsal column lesion disrupts remodeled circuitry, while subsequent pyramidotomy eliminates unilateral evoked motor output. Both measured at 3 days after injury.

[0037] Figures 14A-14F are graphical and pictorial diagrams showing forelimb motor map representations infiltrate quiescent former hindlimb cortical areas. Figure 14A is a cortical map re-organization from spinal cord injury in mice that received weekly training demonstrates how map centroids shift after injury. Size of the marker is proportional to the percentage of mice with evoked motor movements of a given muscle group. Figures 14B and 14C show elbow extensor motor maps shift caudal and medial towards cortex originally occupied by hindlimb representations. Figures 14D and 14E show that mice with *Ryk* conditional deletion have a greater proportion devoted to elbow flexor activation at 4 weeks post-CS lesion (Figure 14D) and conversely a smaller proportion of the motor cortex devoted to extensor activation (Figure 14E) (n=10 (control) 11 (*Ryk* cKO) mice, one-tailed t-test *P<0.05: elbow flexion P=0.0347 t(19)=1.925, elbow extension P=0.0460 t(16)= -1.791, data presented as mean $\pm$ s.e.m.). Figure 14F is a model for recruitment of ectopic cortical motor regions mediated by axon plasticity. After dorsal column injury, the immediate expansion of forelimb regions above the level of injury is likely mediated by lateral connectivity within the motor cortex. Increased axonal plasticity and connectivity after *Ryk* conditional deletion likely drives the formation of novel, ectopic areas of forelimb motor cortex.

[0038] Figures 15A-15D are graphical diagrams showing that cortical map re-organization and functional recovery from spinal cord injury are dependent upon rehabilitative training. Figure 15A is a timeline outlining experimental details of optogenetic mapping with only terminal behavioral testing at 8 weeks post-injury. Figure 15B is a topographic representation of elbow flexor and extensor activation, relative to bregma (*) prior to and 3 days, 4 weeks, and 8 weeks after CS dorsal column lesion in the absence of weekly behavioral testing. Figure 15C shows that at 8 weeks after CS dorsal column lesion, mice with weekly behavioral testing, both *Ryk* cKO and controls, performed better than those only tested at 8 weeks (n=10 (control weekly testing), 11 (*Ryk* cKO weekly testing), 5 (control & *Ryk* cKO 8wk only testing) mice, ANOVA P = 0.0037 F(3) = 5.7157, Bonferroni corrected t-test *P<0.05: 1. *Ryk* cKO weekly v. 8 week only testing P = 0.0277, 2. control weekly v. 8 week only testing P = 0.0346, data presented as median and inter-quartile range). Figure 15D shows that in animals with weekly behavioral testing (black Xs), there was a strong correlation of wrist movement and skilled forelimb reach performance, regardless of injury or genotype (n=84 measurements (4 time points, 21

mice), bivariate Pearson correlation P , $P < 0.0001$ $p = 0.665$). Light blue is density ellipse ($a=0.95$). Mice with no weekly behavioral testing are shown in red.

[0039] Figure 16 is a pictorial diagram showing a series of sample frames from a forelimb reach video. This example at 13 weeks after C5 dorsal column lesion (1 week after C3 sham operation) demonstrates the recovery of skilled forelimb grasp. Successful reach requires the use of a grasping motion as a sweeping motion would result in the pellet being dropped in either the gap between the food pellet platform (black) and the main enclosure, or through the wire-frame floor of the enclosure.

[0040] Figures 17A and 17B are pictorial diagrams showing Ryk monoclonal antibody infusion in rats. Figure 17A shows the specificity of Ryk monoclonal antibody. Full-length blot presented in Figs. 23A and 23B. Figure 17B is a timeline outlining experimental details of Ryk monoclonal antibody infusion after bilateral C5 dorsal column lesion in rats.

[0041] Figure 18 is a graphical diagram showing that axon collateralization increased after Ryk monoclonal antibody infusion caudal to the injury. Rats infused for 28 days with Ryk monoclonal antibody had greater levels of collateralization caudal to the lesion than control IgG infused rats ($n = 6$ (IgG control) 5 (Ryk monoclonal) rats, one-tailed t-test $*P = 0.0196$ $P = 0.0196$ $t(6) = 2.594$, data presented as mean $\pm$ s.e.m.). Injury site is at 0 μm , caudal is represented with positive numbers. Axon index is thresholded pixels in sagittal spinal cord divided by thresholded pixels in transverse pyramids.

[0042] Figure 19 is a pictorial diagram showing an optogenetic mapping example. Sedated mice with unilateral cranial windows were stimulated with 470nm LED by fiber optic cable to evoke muscle movements. Two examples of motor maps from one animal, pre- and 3 days post-C5 dorsal column lesion are shown.

[0043] Figure 20 is a graphical diagram showing the proportion of motor cortex occupied by characterized motor output changes over time in response to weekly training and Ryk conditional deletion

[0044] Figure 21 is a graphical diagram showing recovery of skilled forelimb reach for mice used in cortical mapping experiments. Behavioral performance on skilled forelimb reach task shows enhanced recovery after Ryk conditional deletion in contralateral motor

cortex (weeks 1-8 after C5 lesion, n = 10 (control) 11 (*Ryk* cKO) mice, repeated measures ANOVA $P=0.0304$ $F(1,19)=5.472$). Secondary C3 eliminates enhanced recovery after *Ryk* conditional deletion (n = 9 (control) 8 (*Ryk* cKO)), while unilateral pyramidotomy (n = 8 (control) 7 (*Ryk* cKO)) completely ablates the ability of mice to perform the task. Data presented as mean $\pm$ s.e.m.

[0045] Figure 22 is a graphical diagram showing that proportions of motor cortex occupied by characterized motor output are relatively stable in the absence of training after injury.

[0046] Figures 23A and 23B are pictorial diagrams showing the results of full Western blots from Figure 8C and Figure 17A. Figure 23A shows the specificity of *Ryk* monoclonal antibody from Figure 17A. Figure 23B shows a Western blot of postnatal day 7 motor cortex extract from mice infected at postnatal day 0 with AAV2/1 synapsin Cre from Figure 8C. E18.5 cortex from two separate *Ryk* KO embryonic mouse cortices as control in right two lanes. GAPDH loading control from same blot.

DETAILED DESCRIPTION OF THE INVENTION

[0047] The present invention is based on the finding that an anti-*Ryk* antibody or antibody fragment that specifically binds to a binding domain of Wnt inhibits Wnt-*Ryk* signaling. As such, the present invention provides methods for modulating neuron degeneration and neuron guidance using the anti-*Ryk* antibody or antibody fragment. Thus, the anti-*Ryk* antibody or antibody fragment can be used to treat a neurological disease or disorder, *e.g.*, a neurodegenerative disease or disorder, in a subject having or being at risk of developing the neurological disease or disorder, *e.g.*, a neurodegenerative disease or disorder, and/or to treat spinal cord injury (SCI) in a subject.

[0048] Before the present compositions and methods are described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0049] As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, references to “the method” includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0050] The term “comprising,” which is used interchangeably with “including,” “containing,” or “characterized by,” is inclusive or open-ended language and does not exclude additional, unrecited elements or method steps. The phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. The present disclosure contemplates embodiments of the invention compositions and methods corresponding to the scope of each of these phrases. Thus, a composition or method comprising recited elements or steps contemplates particular embodiments in which the composition or method consists essentially of or consists of those elements or steps.

[0051] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0052] As used herein, “treatment” or “treating,” or “palliating” or “ameliorating” are used interchangeably herein. These terms refer to an approach for obtaining beneficial or desired results including but not limited to therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the patient, notwithstanding that the patient may still be afflicted with the underlying disorder. For prophylactic benefit, the compositions may be administered to a patient at risk of developing a particular disease, or to a patient reporting one or more of the physiological symptoms of a disease, even though a diagnosis of this disease may not have been made. Treatment includes preventing the disease, that is,

causing the clinical symptoms of the disease not to develop by administration of a protective composition prior to the induction of the disease; suppressing the disease, that is, causing the clinical symptoms of the disease not to develop by administration of a protective composition after the inductive event but prior to the clinical appearance or reappearance of the disease; inhibiting the disease, that is, arresting the development of clinical symptoms by administration of a protective composition after their initial appearance; preventing re-occurring of the disease and/or relieving the disease, that is, causing the regression of clinical symptoms by administration of a protective composition after their initial appearance.

[0053] The term "effective amount" or "therapeutically effective amount" refers to the amount of an active agent sufficient to induce a desired biological result. That result may be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. The term "therapeutically effective amount" is used herein to denote any amount of the formulation which causes a substantial improvement in a disease condition when applied to the affected areas repeatedly over a period of time. The amount will vary with the condition being treated, the stage of advancement of the condition, and the type and concentration of formulation applied. Appropriate amounts in any given instance will be readily apparent to those skilled in the art or capable of determination by routine experimentation.

[0054] The term "pharmaceutically acceptable salt" refers to salts derived from a variety of organic and inorganic counter ions well known in the art and include, by way of example only, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, and the like; and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, hydrobromide, tartrate, mesylate, acetate, maleate, oxalate and the like.

[0055] A "subject," "individual," or "patient," is used interchangeably herein, which refers to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained *in vitro* or cultured *in vitro* are also encompassed.

[0056] As used herein, "promote" or "increase," or "promoting" or "increasing" are used interchangeably herein. These terms refer to the increase in a measured parameter (*e.g.*, activity, expression, signal transduction, neuron degeneration) in a treated cell (tissue or subject) in comparison to an untreated cell (tissue or subject). A comparison can also be made of the same cell or tissue or subject between before and after treatment. The increase is sufficient to be detectable. In some embodiments, the increase in the treated cell is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 1-fold, 2-fold, 3-fold, 4-fold or more in comparison to an untreated cell.

[0057] As used herein, "inhibit," "prevent" or "reduce," or "inhibiting," "preventing" or "reducing" are used interchangeably herein. These terms refer to the decrease in a measured parameter (*e.g.*, activity, expression, signal transduction, neuron degeneration) in a treated cell (tissue or subject) in comparison to an untreated cell (tissue or subject). A comparison can also be made of the same cell or tissue or subject between before and after treatment. The decrease is sufficient to be detectable. In some embodiments, the decrease in the treated cell is at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or completely inhibited in comparison to an untreated cell. In some embodiments the measured parameter is undetectable (*i.e.*, completely inhibited) in the treated cell in comparison to the untreated cell.

[0058] The term "selective inhibition" or "selectively inhibit" as referred to a biologically active agent refers to the agent's ability to preferentially reduce the target signaling activity as compared to off-target signaling activity, via direct or indirect interaction with the target.

[0059] The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

[0060] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -

carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Naturally encoded amino acids are the 20 common amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) and pyrrolysine and selenocysteine.

[0061] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0062] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known

in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0063] The following eight groups each contain amino acids that are conservative substitutions for one another:

[0064] 1) Alanine (A), Glycine (G);

[0065] 2) Aspartic acid (D), Glutamic acid (E);

[0066] 3) Asparagine (N), Glutamine (Q);

[0067] 4) Arginine (R), Lysine (K);

[0068] 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);

[0069] 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);

[0070] 7) Serine (S), Threonine (T); and

[0071] 8) Cysteine (C), Methionine (M) (see, *e.g.*, Creighton, Proteins (1984)).

[0072] A conservative substitution may include substitution such as basic for basic, acidic for acidic, polar for polar, etc. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram (Livingstone C. D. and Barton G. J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" Comput. Appl Biosci. 9: 745-756; Taylor W. R. (1986) "The classification of amino acid conservation" J. Theor. Biol. 119; 205-218). Conservative substitutions may be made, for example, according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

TABLE 1: Grouping of amino acids

Characteristic	Set	Characteristic	Sub-set
Hydrophobic	F W Y H K M I L V A G C	Aromatic Aliphatic	F W Y H I L V
Polar	W Y H K R E D C S T N Q	Charged Positive	H K R E D H K R
		Charged Negative	E D
Small	V C A G S P T N D	Tiny	A G S

[0073] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (*e.g.*, a polypeptide of the invention), which does not comprise additions or deletions, for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0074] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. The invention provides polypeptides that are substantially identical to the polypeptides, respectively, exemplified herein, as well as uses thereof including, but not limited to, use for treating or preventing neurological diseases or disorders, *e.g.*, neurodegenerative diseases or disorders, and/or treating SCI. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length, or the entire length of the reference sequence.

[0075] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0076] A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, *e.g.*, Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

[0077] Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) Nuc. Acids Res. 25:3389-3402, and Altschul et al. (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of

either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) Proc. Natl. Acad. Sci. USA 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0078] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0079] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, and complements thereof. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0080] As used herein, the term "dominant negative mutant" of a protein refers to a mutant polypeptide or nucleic acid, which lacks wild-type activity and which, once expressed in a cell wherein a wild-type of the same protein is also expressed, dominates the wild-type protein and effectively competes with wild type proteins for substrates, ligands, etc., and thereby inhibits the activity of the wild type molecule. The dominant negative mutant can be a polypeptide having an amino acid sequence substantially similar (*i.e.*, at least about 75%, about 80%, about 85%, about 90%, about 95% similar) to the wild type protein. The dominant negative mutant can also be a polypeptide comprising a fragment of

the wild type protein, *e.g.*, the C-domain of the wild-type protein. The dominant negative mutant can be a truncated form of the wild type protein.

[0081] Mouse Model

[0082] As used herein, “transgenic organism” refers to an animal in which exogenous DNA has been introduced while the animal is still in its embryonic stage. In most cases, the transgenic approach aims at specific modifications of the genome, *e.g.*, by introducing whole transcriptional units into the genome, or by up- or down-regulating or mutating pre-existing cellular genes. The targeted character of certain of these procedures sets transgenic technologies apart from experimental methods in which random mutations are conferred to the germline, such as administration of chemical mutagens or treatment with ionizing solution. A transgenic organism can include an organism which has a gene knockout or may result for inducing a genetic mutation.

[0083] A “genetic knock out” refers to partial or complete suppression of the expression of a protein encoded by an endogenous DNA sequence in a cell. The “knockout” can be affected by targeted deletion of the whole or part of a gene encoding a protein. Alternatively, the transgenic organism can be obtained by the targeted mutation of a functional protein in an embryonic stem cell. As a result, the deletion or mutation may prevent or reduce the expression of the protein in any cell in the whole animal in which it is normally expressed, or results in the expression of a mutant protein having a biological function different than the normal/wild-type protein.

[0084] The term “knockout animal” and “transgenic animal”, refer to a transgenic animal wherein a given gene has been suppressed or mutated by recombination with a targeting vector. It is to be emphasized that the term is intended to include all progeny generations. Thus, the founder animal and all F1, F2, F3, and so on, progeny thereof are included.

[0085] As used herein, the phrase “conditional knockout,” or “cKO,” when used to describe a non-human transgenic mammal such as a mouse, refers to mice containing a knock-out of a specific gene in a certain tissue. The creation of a genetically engineered cKO mouse involves inserting specific DNA sequences, such as a knock-out construct/vector, into the mouse DNA. The inserted sequences are recognized by two DNA specific enzymes, *frt* recombinase (also known as flippase) and Cre recombinase, not

normally present in mice. Cre recombinase recognition sites are termed loxP sites and flippase recognition sites are termed frt sites. Each of these enzymes can cut and remove a DNA sequence that is flanked by its recognitions sites. This can lead to disruption of gene function if a functional DNA sequence of the gene of interest is removed. In addition, a selectable marker gene is inserted into the mouse, the introduction of which allows selection of embryonic mouse cells (stem cells) that contain the Cre recombination or flippase recognition sites. The resultant mouse is a conditional knockout mouse.

[0086] A knock-out construct is a nucleic acid sequence, such as a DNA construct, which, when introduced into a cell, results in suppression (partial or complete) of expression of a polypeptide or protein encoded by endogenous DNA in the cell. An exemplary knock-out construct is provided herein. This construct contains a loxP site 5' to exon 3 and 3' to exon 6 of the Ryk gene, a selectable marker cassette and a loxP site 3' to the selectable marker cassette. The selectable marker cassette comprises frt sites 5' and 3' to the selectable marker and is between the 3' frt site and the selectable marker gene. Suitable selectable markers include, but are not limited to, neomycin, puromycin and hygromycin.

[0087] Animals containing more than one transgenic construct and/or more than one transgene expression construct may be prepared in any of several ways. An exemplary manner of preparation is to generate a series of animals, each containing one of the desired transgenic phenotypes. Such animals are bred together through a series of crosses, backcrosses and selections, to ultimately generate a single animal containing all desired transgenic traits and/or expression constructs, where the animal is otherwise congenic (genetically identical) to the wild type except for the presence of the construct(s) and/or transgene(s).

[0088] Embryonic stem (ES) cells are typically selected for their ability to integrate into and become part of the germ line of a developing embryo so as to create germ line transmission of the transgene. Thus, any ES cell line that can do so is suitable for use herein. ES cells are generated and maintained using methods well known to the skilled artisan, such as those described by Doetschman et al. (1985) *J. Embryol. Exp. Mol. Biol.* 87:27-45). Any line of ES cells can be used, however, the line chosen is typically selected for the ability of the cells to integrate into and become part of the germ line of a developing

embryo so as to create germ line transmission of the transgenic/knockout construct. Thus, any ES cell line that is believed to have this capability is suitable for use herein. One mouse strain that is typically used for production of ES cells, is the 129J strain. Another ES cell line is murine cell line D3 (American Type Culture Collection, catalog no. CKL 1934). Still another ES cell line is the WW6 cell line (Ioffe et al. (1995) PNAS 92:7357-7361). The cells are cultured and prepared for knockout construct insertion using methods well known to the skilled artisan, such as those set forth by Robertson in: *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. IRL Press, Washington, D.C. (1987)); by Bradley et al. (1986) *Current Topics in Devel. Biol.* 20:357-371); and by Hogan et al. (*Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986)).

[0089] Introduction of the knock-out construct into ES cells may be accomplished using a variety of methods well-known in the art, including, for example, electroporation, microinjection, and calcium phosphate treatment. For introduction of the DNA sequence, the knock-out construct DNA is added to the ES cells under appropriate conditions for the insertion method chosen. If the cells are to be electroporated, the ES cells and construct DNA are exposed to an electric pulse using an electroporation machine (electroporator) and following the manufacturer's guidelines for use. After electroporation, the cells are allowed to recover under suitable incubation conditions. The cells are then screened for the presence of the knockout construct. Screening for cells which contain the transgene (homologous recombinants) may be done using a variety of methods. For example, as described herein, cells can be processed as needed to render DNA in them available for screening with specific probes by polymerase chain reaction (PCR).

[0090] Once appropriate ES cells are identified, they are introduced into an embryo using standard methods. They can be introduced using microinjection, for example. Embryos at the proper stage of development for integration of the ES cell to occur are obtained, such as by perfusion of the uterus of pregnant females. For example, mouse embryos at 3-4 days development can be obtained and injected with ES cells using a micropipet. After introduction of the ES cell into the embryo, the embryo is introduced into the uterus of a pseudopregnant female mouse. The stage of the pseudopregnancy is selected

to enhance the chance of successful implantation. In mice, 2-3 days pseudopregnant females are appropriate.

[0091] Successful incorporation of ES cells into implanted embryos results in offspring termed chimeras. Chimeras capable of germline transmission of the mutant allele are identified by standard methods. Chimeras are bred and the resulting progeny are screened for the presence of the desired alteration (*e.g.*, the modified recombinant Ryk allele). This may be done, for example, on the basis of coat color or by obtaining DNA from offspring (*e.g.*, tail DNA) to assess for the transgene, using known methods (*e.g.*, Southern analysis, dot blot analysis, PCR analysis). Transgene expression may also be assessed (*e.g.*, to determine if a replacement construct is expressed) by known methods, such as northern analysis or PCR analysis. Southern hybridization or PCR analysis of progeny DNA (*e.g.*, tail DNA) may be conducted to identify desired genotypes. A suitable technique for obtaining completely ES cell derived transgenic non-human organisms is described in WO 98/06834, incorporated herein by reference.

[0092] In various embodiments, the cKO mice disclosed herein include at least three elements: (1) at least two enzyme-specific recognition sites flanking a critical portion of the target gene; (2) a gene encoding a selection marker such as, but not limited to neomycin; and (3) at least two enzyme-specific recognition sites flanking a selection marker gene for easy removal upon breeding with specific mouse strains. In a non-limiting example, exons 3-6 of the target gene has been designated as the critical portion. In one embodiment the enzyme-specific recognition sites flanking the critical portion of the target gene are loxP sites. In another embodiment, the enzyme-specific recognition sites flanking the selection marker gene are frt sites.

[0093] As mentioned above, the homologous recombination of the above described “knock-out” and/or “knock in” constructs is sometimes rare and such a construct can insert non-homologously into a random region of the genome where it has no effect on the gene which has been targeted for deletion, and where it can potentially recombine so as to disrupt another gene which was otherwise not intended to be altered. Such non-homologous recombination events can be selected against by modifying the above-mentioned targeting vectors so that they are flanked by negative selectable markers at either end (particularly

through the use of the diphtheria toxin gene, thymidine kinase gene, the polypeptide product of which can be selected against in expressing cell lines in an appropriate tissue culture medium well known in the art--*e.g.*, one containing a drug such as ganciclovir. Non-homologous recombination between the resulting targeting vector comprising the negative selectable marker and the genome will usually result in the stable integration of one or both of these negative selectable marker genes and hence cells which have undergone non-homologous recombination can be selected against by growth in the appropriate selective media (*e.g.*, media containing a drug such as ganciclovir). Simultaneous selection for the positive selectable marker and against the negative selectable marker will result in a vast enrichment for clones in which the construct has recombined homologously at the locus of the gene intended to be mutated. The presence of the predicted chromosomal alteration at the targeted gene locus in the resulting stem cell line can be confirmed by means of Southern blot analytical techniques which are well known to those familiar in the art. Alternatively, PCR can be used.

[0094] Other methods of making transgenic animals are also generally known. See, for example, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Recombinase dependent transgenic organisms can also be generated, *e.g.*, by homologous recombination to insert target sequences, such that tissue specific and/or temporal control of inactivation of a Ryk gene can be controlled by recombinase sequences.

[0095] Accordingly, in one aspect, the invention provides a transgenic non-human mammal such as a mouse whose genome comprises a heterozygous or homozygous deletion, inactivation or knock-out of the Ryk gene and methods of making the same. In various embodiments, the mouse has the phenotype *Frizzled3^{-/-} Ryk^{+/-}*. In various embodiments, the mouse contains a corticospinal tract (CST)-specific disruption of the Ryk gene. In various embodiments, the disrupted Ryk gene includes a recombinant Ryk allele, a selectable marker, *frt* sites flanking the selectable marker, and *loxP* sites flanking a portion of the allele. The marker may be PGK Neo and the *loxP* sites may flank exons 3-6 of the allele. Also provided is an isolated cell derived from the transgenic non-human mammal.

[0096] Neurons

[0097] As used herein, the term "neuron" include a neuron and a portion or portions thereof (*e.g.*, the neuron cell body, an axon, or a dendrite). The term "neuron" as used herein denotes nervous system cells that include a central cell body or soma, and two types of extensions or projections: dendrites, by which, in general, the majority of neuronal signals are conveyed to the cell body, and axons, by which, in general, the majority of neuronal signals are conveyed from the cell body to effector cells, such as target neurons or muscle. Neurons can convey information from tissues and organs into the central nervous system (afferent or sensory neurons) and transmit signals from the central nervous systems to effector cells (efferent or motor neurons). Other neurons, designated interneurons, connect neurons within the central nervous system (the brain and spinal column). Certain specific examples of neuron types that may be subject to treatment or methods according to the invention include cerebellar granule neurons, dorsal root ganglion neurons, and cortical neurons.

[0098] The term "neuronal degeneration" is used broadly and refers to any pathological changes in neuronal cells, including, without limitation, death or loss of neuronal cells, any changes that precede cell death, and any reduction or loss of an activity or a function of the neuronal cells. The pathological changes may be spontaneous or may be induced by any event and include, for example, pathological changes associated with apoptosis. The neurons may be any neurons, including without limitation sensory, sympathetic, parasympathetic, or enteric, *e.g.*, dorsal root ganglia neurons, motor neurons, and central neurons, *e.g.*, neurons from the spinal cord. Neuronal degeneration or cell loss is a characteristic of a variety of neurological diseases or disorders, *e.g.*, neurodegenerative diseases or disorders. In some embodiments, the neuron is a sensory neuron. In some embodiments, the neuron is a motor neuron. In some embodiments, the neuron is a damaged spinal cord neuron.

[0099] In some embodiments, degeneration occurs in a portion of the neuron such as the neuron cell body, an axon, or a dendrite. Accordingly, the degeneration can be inhibited in the degenerated portion or portions of the neuron. In some embodiments, the degeneration of an axon of the neuron is inhibited. In some embodiments, the degeneration of a cell body of the neuron is inhibited. The axon can be an axon of any neuron. For example, in some

embodiments, the axon is a spinal cord commissural axon, or an upper motor neuron axon, or a central nervous system axon.

[0100] As described herein, the disclosed methods can be carried out *in vivo*, such as in the treatment of neurodegenerative diseases, neurological disorders or injuries to the nervous system. The methods can also be carried out *in vitro* or *ex vivo*, such as in laboratory studies of neuron function and in the treatment of nerve grafts or transplants. Accordingly, in some embodiments, the neuron forms part of a nerve graft or a nerve transplant. In some embodiments, the neuron is *ex vivo* or *in vitro*. In some embodiments, the nerve graft or the nerve transplant forms part of an organism, human or non-human (*e.g.*, mammal, primate, rat, mouse, rabbit, bovine, dog, cat, pig, etc.).

[0101] Axon Degeneration

[0102] Axon degeneration is a common feature in many neurological and neurodegenerative diseases/disorders and in traumatic injuries. Studies indicate that it can occur independent of and before the death of neuronal cell bodies. However, the molecular and cellular mechanisms underlying axonal degeneration and protection are still unclear. Elucidating the degeneration pathways that are activated or the protection pathways that are inactivated during axon pathology will help develop specific therapeutic agents that preserve axon integrity and enhance regeneration.

[0103] During the development of the nervous system, axons respond to extracellular signals that promote the growth as well as those that inhibit their growth. Some extracellular cues attract axons to grow towards higher concentration and others repel axon away from higher concentration. The signaling pathways that regulate these opposite axon responses have profound effect on the extension and removal of axons, although their functions in mature axons have not been well characterized. Studies suggest that axon guidance molecules may play a role in neurological/neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS).

[0104] Atypical protein kinases C (aPKC), including PKC ζ and PKC ι/λ , play crucial roles in many cellular processes including cell polarization and survival. In neurons, aPKC has been involved in cell polarity (7-13), neurite differentiation (11-13) and axon guidance

(14) (15). aPKC mediates axon attraction to Wnts and anterior-posterior axon guidance of commissural axons via Wnt-Frizzled3 signaling (14) (15). Through its interaction with Par6 and Par3, aPKC is required for axon specification of hippocampal neurons by regulating the activity of the microtubule affinity regulating kinase MARK2 on microtubule-associated protein Tau phosphorylation and microtubule assembly (9). During neuronal polarization, aPKC is regulated by dishevelled (Dvl) which mediates Wnt signaling (11). aPKC has been also involved in prosurvival signaling in many different cell types including neural cells (16-23). However, the mechanisms underlying the prosurvival function of aPKC in neurons have not been elucidated. The present disclosure demonstrates that inhibiting aPKC using dominant negative constructs or a myristoylated atypical PKC pseudosubstrate promoted axonal degeneration and neuronal apoptosis. These biochemical studies showed that inhibition of aPKC led to microtubule destabilization by increasing MARK2 activity and Tau phosphorylation, resulting in axon degeneration, and eventually neuronal cell body death through the activation of the JNK-cJun pathway. This is the same signaling mechanism of how aPKC/Par6/Par3 is involved in neuronal polarity and promotes axon elongation (9). Together, these results indicate that aPKC is required for both axon elongation during initial axon development and maintenance by promoting microtubule assembly and stability once axons have formed.

[0105] Ryk is an atypical receptor tyrosine kinase that binds Wnts. Interestingly, Ryk mediates axon repulsion during development (24-27) and inhibits axon plasticity in adulthood after traumatic injury (28-33). Blocking Ryk signaling into injured dorsal spinal cord prevents axon retraction and can promote axon regrowth (32). Recently, it was found that Ryk expression was increased in motor neurons and axons of the ventral spinal cord in a mouse model of ALS, at early stage of the disease progression, suggesting that Ryk may be involved in early events that trigger neurodegeneration in ALS (6). In this study, it was first tested whether Ryk regulates aPKC. The findings described herein show that aPKC activity was increased in Ryk KO neurons, suggesting that Ryk might normally inhibit aPKC. This is a novel discovery of the interaction between Ryk and aPKC, which may also be relevant to understand how Ryk mediates axon repulsion.

[0106] It was then determined whether blocking Ryk signaling might prevent axonal degeneration induced by aPKC inhibition and neuronal death in Ryk knock-out (KO) mice

was analyzed. These studies indicate that inhibiting Wnt/Ryk signaling with specific antibodies or by Ryk KO decreased aPKC-induced axonal degeneration. Because both Ryk and Frizzled3 KO mice die at birth, brain areas that are undergoing neuronal death before birth were explored. It was found that an area of cortex shows clear evidence of neuronal death detectable by aCasp3 staining at E18.5, the retrosplenial cortex (RSP), localized between the neocortex and hippocampus.

[0107] Consistent with the proposed role of Ryk in promoting degeneration, apoptosis was decreased in E18.5 Ryk KO embryos. Furthermore, it was found that apoptosis was greatly increased in Frizzled3 KO embryos in the RSP at E18.5 and this increase was significantly attenuated in Ryk^{+/-} Frizzled3^{-/-} embryos, revealing a genetic interaction between Frizzled3 and Ryk. It should be noted that there are 10 Frizzleds in the mouse genome. The fact that only the RSP showed clear increase of aCasp3 immunoreactivity suggests significant functional redundancy among the Frizzled family members in cortical neuronal protection at this stage. It cannot be excluded that other areas of the brain would show increased cell death at later time points, including neonatal stage. In Frizzled3 KO embryos, only the RSP region of the cortex showed strong increase of death (5 fold), further suggesting this area being the most vulnerable before birth. Interestingly, when Ryk was reduced by half, the increase of death was significantly reduced.

[0108] This genetic interaction between the two opposite Wnt receptors which regulate aPKC in opposite ways further underlie the important role of aPKC and the complex function of Wnts in regulating neuronal survival. This is also consistent with a recent study showing that Frizzled3 is required for spinal cord motor axon survival after initial axon outgrowth (52). Another recent study also shows that planar cell polarity signaling pathway mediates axon guidance and aPKC is involved in amplifying planar cell polarity signaling in growth cone guidance (53) (15). Studies show that Ryk inhibits planar cell polarity signaling (54) (55). Here, the data shows that Ryk and aPKC play opposite roles in mediating axon and neuronal survival. These findings, therefore, will help untangle the intricate molecular signaling pathways that the nervous system uses to carefully assemble neural circuits and the disruption of which may underlie neurological/neurodegenerative disorders.

[0109] Method for Inhibiting Neuron Degeneration

[0110] Accordingly, the present invention provides methods and compositions for modulating growth of a nerve cell by contacting the neuron with an agent, thereby inhibiting degeneration of a neuron. In various embodiments, the agent may be an anti-Ryk monoclonal antibody or antibody fragment that specifically binds to a binding domain of Wnt affecting a Wnt signaling pathway. These methods and compositions can be used in a wide variety of therapeutic contexts where nerve growth and regeneration would be beneficial. For example, an anti-Ryk antibody or antibody fragment affecting a Wnt signaling pathway can be used to stimulate axonal growth of a damaged neuron along the A-P axis of a patient with SCI. Because it has also been observed that the Wnts are expressed in the several regions in the brain and the components of the Wnt signaling pathways are also present in axons of other central nervous system neurons, it is possible that the anti-Ryk antibody or antibody fragments described herein can be used to modulate growth and directional guidance of axons in the central nervous system.

[0111] In some embodiments, the methods as described herein result in at least a 10% decrease (*e.g.*, at least 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or even 100% decrease) in the degeneration of a population of neurons or in the degeneration of axons or cell bodies or dendrites of a neuron in a population of neurons as compared to a control population of neurons. In some embodiments, the methods as described herein result at least a 10% decrease (*e.g.*, at least 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% decrease) in the number of neurons (or neuron bodies, axons, or dendrites thereof) that degenerate in a subject compared to the number of neurons (or neuron bodies, axons, or dendrites thereof) that degenerate in a subject that is not administered the one or more of the agents described herein. In some embodiments, the methods as described herein result in at least a 10% decrease (*e.g.*, at least 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or even 100% decrease) in one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9) symptoms of a neurological/neurodegenerative disease or disorder and/or condition. In some embodiments, the methods as described herein result in at least a 10% decrease (*e.g.*, at least 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%,

90%, 95%, or even 100% decrease) in the likelihood of developing a neurological/neurodegenerative disease or disorder and/or condition.

[0112] The methods of inhibiting neuron degeneration include *in vitro*, *in vivo*, and/or *ex vivo* methods. In some embodiments, the methods are practiced *in vivo*, *i.e.*, the agent inhibiting neuron degeneration is administered to a subject. In some embodiments, the methods are practiced *ex vivo*, *i.e.*, neurons to be treated form part of a nerve graft or a nerve transplant in a subject. In some embodiments, the methods are practiced *in vitro*.

[0113] The methods of inhibiting neuron degeneration can be used to inhibit or prevent neuron degeneration in patients newly diagnosed as having a neurological/neurodegenerative disease or disorder or at risk of developing a new neurological/neurodegenerative disease or disorder. On the other hand, the methods of inhibiting neuron degeneration can also be used to inhibit or prevent further neuron degeneration in patients who are already suffering from, or have symptoms of, a neurological/neurodegenerative disease or disorder. Preventing neuron degeneration includes decreasing or inhibiting neuron degeneration, which may be characterized by complete or partial inhibition of neuron degeneration. This can be assessed, for example, by analysis of neurological function.

[0114] Spinal Cord Injury (SCI)

[0115] A large proportion of spinal cord injury patients have incomplete lesions, where parts of the spinal cord tissues remain intact. Due to the strong inhibitory environment in the injured adult spinal cord, especially in the glial scar, and reduced growth potential of adult axons, the original connections are usually not restored. Nonetheless, the complex circuitry can undergo remodeling to achieve variable levels of functional recovery with rehabilitative training.

[0116] Functional restoration of the corticospinal motor system after spinal cord injury is of principal importance since it is essential for recovery of voluntary motor control. In rodents, however, the role of the corticospinal tract (CST) is more limited, with little effect on locomotion and hindlimb usage. Nevertheless, the CST is crucial for skilled forelimb

motor control. Fine motor skills are lost after a dorsal column lesion of the main CST, with a varying extent of spontaneous recovery.

[0117] In order to understand how neural circuits reorganize to regain function after injury, functional, anatomical and behavioral analyses were performed. The present disclosure demonstrates that the motor cortex remaps such that the cortical areas are no longer used for the hindlimb are recruited to control the forelimb to achieve functional recovery after a dorsal column lesion and this reorganization requires continued training. The present disclosure also shows that removing Ryk, a receptor to axon guidance cues Wnts, results in greater CST axon plasticity and cortical circuit remodeling in conjunction with rehabilitative training, leading to maximal functional restoration. The more gradual and persistent changes in cortical control maps observed in *Ryk* conditional knockout injured mice are likely due to the enhanced changes in connectivity within the spinal cord and cortical circuits that occur in the absence of Wnt-Ryk signaling. It was previously found that Wnt-Ryk signaling controls topographic map formation in the developing visual system. The present disclosure reveals a novel function of Wnt-Ryk signaling in controlling motor cortex remapping after spinal cord injury in adulthood.

[0118] Previous work has demonstrated that the Wnt signaling, which regulates axon guidance in development, has a profound effect on axon plasticity after injury in the adult spinal cord. The motor output map was characterized after spinal cord injury and therefore forelimb motor maps spread into adjacent regions affected by the injury. It was observed that an expansion of flexor control area caudally and medially towards cortical regions originally responsible for hindlimb movements (Figures 7A, 7B, 13A and 13B). These changes are likely stereotypical, because wrist flexor representations exhibited a medial shift as they recovered, similar to observed shifts of digit representations in primates (Figure 7A, 13A and 13B).

[0119] To specifically test the function of Wnt-Ryk signaling in neurons, a conditional allele of *Ryk* was generated encoding a repulsive Wnt receptor, motor cortex specific knockout was performed and then the dorsal columns at cervical level 5 (CS) were lesioned. Following *Ryk* conditional knockout, mice recovered on a skilled forelimb reaching task to $81 \pm 7\%$ of peak pre-injury levels at 12 weeks after dorsal column lesion, compared to only

60 ± 5% in wild type control mice. This additional recovery depends on the segment of the main CST immediately rostral to the lesion (C3-CS), as a second dorsal column lesion at C3 reduces the functional recovery to control levels. Anatomical analyses showed significantly increased collateral sprouting of CST above and below the CS injury and with pre-synaptic puncta in these axon sprouts.

[0120] Using an optogenetic approach, the output map of the motor cortex was monitored. It was found that immediately after CS dorsal column lesion, forelimb elbow flexion can be activated by a much larger cortical area, whereas forelimb extension was lost. Over time, the area that activates forelimb flexion reduced back to the original size and a new area, which used to activate the hind limb, was recruited to activate forelimb extension. After the second lesion at C3, the control of forelimb flexion was lost but that of the new control of the forelimb extension was largely unaffected. In *Ryk* cKO, these changes are more gradual and persistent. Finally, mice that did not undergo weekly behavioral testing displayed only limited skilled forelimb recovery with performance similar to that of mice tested at one week after injury. In the absence of weekly testing, refinement of cortical motor maps was also impaired, irrespective of *Ryk* conditional deletion, highlighting the importance of targeted plasticity.

[0121] Alterations of motor output maps have been noted in spinal cord injury patients for many years but the neural circuit mechanisms remain unknown. It is known that naive transected hindlimb-projecting corticospinal neurons sprout into cervical spinal cord as early as one week post spinal cord injury. However, it is unlikely that early expansion of motor maps above the level of the injury, which was observed only 3 days later, was due to sprouting and establishment of new connectivity patterns; rather, without being bound by theory, it is likely due to a loss of lateral inhibition within the cortex. However, by 4 weeks after spinal cord injury cortical maps likely reflect the output to the remodeled corticospinal circuitry in the cervical spinal cord. It was observed that the greater CST axon collateral numbers induced following *Ryk* deletion leads to slight increase of connections with motor units distal to the injury site but robust increase with those rostral to the injury (Figures 2H and 3D). Therefore, the changes in connectivity above the level of injury could be the main source for the changes of cortical maps (Figure 7F). For example, the initial expansion of the biceps at 4 weeks post-injury, and subsequent reduction at 8 weeks, may result from an

initial sprouting of CST collaterals that normally project to forelimb motor units in the cervical spinal cord, followed by a subsequent pruning through Hebbian competition. The recruitment of the hindlimb cortical areas for triceps control may result from *de novo* connections from corticospinal neurons that originally controlled the hindlimb to the forelimb motor units of the cervical spinal cord. These sprouts may either directly contact motor units or form relays using propriospinal neurons. Conditional deletion of Ryk in corticospinal neurons enhances collateral sprouts and thus recruits more spinal cord circuitry, a process that likely underlies greater recovery of voluntary skilled forelimb control. The results of the antibody infusion experiments in the spinal cord suggest that circuit remodeling in the cervical spinal cord is sufficient to promote functional recovery. However, it is plausible that connectivity changes within the primary motor cortex also contribute to the remodeling of the entire circuit.

[0122] Other descending pathways are also involved in fine motor control and can partially compensate for the loss of CST input on a skilled forelimb reach task. Additionally, animals with incomplete lesion of the pyramids have been shown to exhibit similar success rates of skilled forelimb reach to intact control animals through compensatory forelimb movements, indicating that a small proportion of spared CST is capable of restoring full, if altered, function on the skilled forelimb reach task. In 59% of the *Ryk* cKO mice and 100% of Ryk monoclonal antibody-infused animals, recovery of skilled forelimb reach to levels at or above peak pre-injury levels was observed. This full recovery clearly requires the novel CST connections rostral to the lesion, as a second C3 lesion abolishes the enhanced recovery. While the CST is not the sole component mediating control of skilled forelimb reach, it is required for the recovered function as pyramidotomy in the mouse model described herein completely abolished the reaching and grasping behavior. These results suggest that restoring at least some CST function is a critical component in recovery of motor control after injury as compensatory plasticity of other tracts drives limited recovery in rodents, which are less dependent upon the CST for motor control than primates.

[0123] The present disclosure also demonstrates that a Ryk monoclonal antibody can be a therapeutic tool as blocking Ryk function after lesion leads to improved functional recovery. It has been shown here that maximal recovery of the forelimb can be achieved by

combining targeted plasticity for the forelimb function (continued reaching and grasping training) and molecular manipulation. Therefore, it is anticipated that combining targeted plasticity of other functions with molecular manipulation may allow recovery of other motor or sensory functions. A large proportion of patients have incomplete spinal cord injuries, providing a substrate for recovery. This disclosure illustrates that promoting circuit plasticity is a promising approach to restore maximal function following incomplete spinal cord injury.

[0124] Wnt Peptides

[0125] Wnts are secreted cysteine-rich glycosylated proteins that play a role in the development of a wide range of organisms. Wnts are thought to function in a variety of developmental and physiological processes since many diverse species have multiple conserved Wnt genes (McMahon, 1992; Nusse and Varmus, 1992). The Wnt growth factor family includes at least 19 genes identified in mammals, including Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt 6, Wnt7a, Wnt7b, Wnt8Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt16. Similar numbers of Wnt genes are present in other vertebrate species (see, *e.g.*, US Pub. No. 2011/0065645, incorporated herein by reference in its entirety). Of course, further Wnts may be discovered and/or characterized in the future, and those of skill will be able to employ any such Wnts in the context of the invention. Further, those of skill will be able to use the teachings herein to obtain and use Wnts of any species in the context of the invention.

[0126] Anti-Ryk Antibodies

[0127] Antibodies of the invention can be administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, and subcutaneous administration. In addition, antibodies can be administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, *e.g.*, by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

[0128] As used herein, the term “antibody” is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies. Antibodies are characterized, in part, in that they specifically bind to an antigen, particularly to one or more epitopes of an antigen. The term “binds specifically” or “specific binding activity” or the like, when used in reference to an antibody, means that an interaction of the antibody and a particular epitope has a dissociation constant of at least about 1×10^{-6} M, generally at least about 1×10^{-7} M, usually at least about 1×10^{-8} M, and particularly at least about 1×10^{-9} M or 1×10^{-10} M or less. As such, Fab, F(ab')₂, Fd and Fv fragments of an antibody that retain specific binding activity are included within the definition of an antibody.

[0129] The term “antibody” as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains (see Huse et al., *Science* 246:1275-1281, 1989, which is incorporated herein by reference). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known (Winter and Harris, *Immunol. Today* 14:243-246, 1993; Ward et al., *Nature* 341:544-546, 1989; Harlow and Lane, *Antibodies: A laboratory manual* (Cold Spring Harbor Laboratory Press, 1999); Hilyard et al., *Protein Engineering: A practical approach* (IRL Press 1992); Borrabeck, *Antibody Engineering*, 2d ed. (Oxford University Press 1995); each of which is incorporated herein by reference). In addition, modified or derivatized antibodies, or antigen binding fragments of antibodies, such as pegylated (polyethylene glycol modified) antibodies, can be useful for the present methods.

[0130] Antibodies can be tested for anti-target polypeptide activity using a variety of methods well-known in the art. Various techniques may be used for screening to identify antibodies having the desired specificity, including various immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), including direct and ligand-capture ELISAs, radioimmunoassays (RIAs), immunoblotting, and fluorescent activated cell sorting (FACS).

Numerous protocols for competitive binding or immunoradiometric assays, using either polyclonal or monoclonal antibodies with established specificities, are well known in the art. Such immunoassays typically involve the measurement of complex formation between the target polypeptide and a specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the target polypeptide is preferred, but other assays, such as a competitive binding assay, may also be employed. See, *e.g.*, Maddox et al, 1983, *J. Exp. Med.* 158:1211.

[0131] The location of the binding target of an antibody used in the invention can be taken into consideration in preparation and administration of the antibody. When the binding target is an intracellular molecule, certain embodiments of the invention provide for the antibody or antigen-binding fragment thereof to be introduced into the cell where the binding target is located. In one embodiment, an antibody of the invention can be expressed intracellularly as an intrabody. The term "intrabody," as used herein, refers to an antibody or antigen-binding portion thereof that is expressed intracellularly and that is capable of selectively binding to a target molecule, as described in Marasco, *Gene Therapy* 4:11-15, 1997; Kontermann, *Methods* 34:163-170, 2004; U.S. Pat. Nos. 6,004,940 and 6,329,173; U.S. Patent Application Publication No. 2003/0104402, and PCT Publication No. WO 03/077945. Intracellular expression of an intrabody is effected by introducing a nucleic acid encoding the desired antibody or antigen-binding portion thereof (lacking the wild-type leader sequence and secretory signals normally associated with the gene encoding that antibody or antigen-binding fragment) into a target cell. Any standard method of introducing nucleic acids into a cell may be used, including, but not limited to, microinjection, ballistic injection, electroporation, calcium phosphate precipitation, liposomes, and transfection with retroviral, adenoviral, adeno-associated viral and vaccinia vectors carrying the nucleic acid of interest.

[0132] In another embodiment, internalizing antibodies are provided. Antibodies can possess certain characteristics that enhance delivery of antibodies into cells, or can be modified to possess such characteristics. Techniques for achieving this are known in the art. For example, cationization of an antibody is known to facilitate its uptake into cells (see, *e.g.*, U.S. Pat. No. 6,703,019). Lipofections or liposomes can also be used to deliver the antibody into cells. Where antibody fragments are used, the smallest inhibitory fragment

that specifically binds to the binding domain of the target protein is generally advantageous. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology (see, *e.g.*, Marasco et al., Proc. Natl. Acad. Sci. U.S.A. 90:7889-7893, 1993).

[0133] Entry of modulator polypeptides into target cells can be enhanced by methods known in the art. For example, certain sequences, such as those derived from HIV Tat or the Antennapedia homeodomain protein are able to direct efficient uptake of heterologous proteins across cell membranes (see, *e.g.*, Chen et al., Proc. Natl. Acad. Sci. U.S.A. 96:4325-4329, 1999).

[0134] When the binding target is located in the brain, certain embodiments of the invention provide for the antibody or antigen-binding fragment thereof to traverse the blood-brain barrier. Certain neurological/neurodegenerative diseases are associated with an increase in permeability of the blood-brain barrier, such that the antibody or antigen-binding fragment can be readily introduced to the brain. When the blood-brain barrier remains intact, several art-known approaches exist for transporting molecules across it, including, but not limited to, physical methods, lipid-based methods, and receptor and channel-based methods.

[0135] Physical methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, circumventing the blood-brain barrier entirely, or by creating openings in the blood-brain barrier. Circumvention methods include, but are not limited to, direct injection into the brain (see, *e.g.*, Papanastassiou et al., Gene Therapy 9:398-406, 2002), interstitial infusion/convection-enhanced delivery (see, *e.g.*, Bobo et al., Proc. Natl. Acad. Sci. U.S.A. 91:2076-2080, 1994), and implanting a delivery device in the brain (see, *e.g.*, Gill et al., Nature Med. 9:589-595, 2003; and Gliadel Wafers™, Guildford Pharmaceutical). Methods of creating openings in the barrier include, but are not limited to, ultrasound (see, *e.g.*, U.S. Pub. No. 2002/0038086), osmotic pressure (*e.g.*, by administration of hypertonic mannitol (Neuwelt, E. A., Implication of the Blood-Brain Barrier and its Manipulation, Volumes 1 and 2, Plenum Press, N.Y., 1989)), permeabilization by, *e.g.*, bradykinin or permeabilizer A-7 (see, *e.g.*, U.S. Pat. Nos.

5,112,596, 5,268,164, 5,506,206, and 5,686,416), and transfection of neurons that straddle the blood-brain barrier with vectors containing genes encoding the antibody or antigen-binding fragment (see, *e.g.*, U.S. Pub. No. 2003/0083299).

[0136] Lipid-based methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, encapsulating the antibody or antigen-binding fragment in liposomes that are coupled to antibody binding fragments that bind to receptors on the vascular endothelium of the blood- brain barrier (see, *e.g.*, U.S. Pub. No. 2002/0025313), and coating the antibody or antigen-binding fragment in low-density lipoprotein particles (see, *e.g.*, U.S. Pub. No. 2004/0204354) or apolipoprotein E (see, *e.g.*, U.S. Pub. No. 2004/0131692).

[0137] Receptor and channel-based methods of transporting the antibody or antigen-binding fragment across the blood-brain barrier include, but are not limited to, using glucocorticoid blockers to increase permeability of the blood-brain barrier (see, *e.g.*, U.S. Pub. Nos. 2002/0065259, 2003/0162695, and 2005/0124533); activating potassium channels (see, *e.g.*, U.S. Pub. No. 2005/0089473), inhibiting ABC drug transporters (see, *e.g.*, U.S. Pub. No. 2003/0073713); coating antibodies with a transferrin and modulating activity of the one or more transferrin receptors (see, *e.g.*, U.S. Pub. No. 2003/0129186), and cationizing the antibodies (see, *e.g.*, U.S. Pat. No. 5,004,697).

[0138] Antibody compositions used in the methods of the invention are formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibodies of the invention present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described

herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

[0139] For the prevention or treatment of disease, the appropriate dosage of an antibody (when used alone or in combination with other agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 µg/kg to 15 mg/kg (*e.g.*, 0.1 mg/kg-10 mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg, or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, *e.g.*, every week or every three weeks (*e.g.*, such that the patient receives from about two to about twenty, or, *e.g.*, about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

[0140] In some embodiments, different antibody regions are illustrated by reference to IgG, which contains four amino acid chains - two longer length heavy chains and two shorter light chains that are inter-connected by disulfide bonds. The heavy and light chains each contain a constant region and a variable region. A heavy chain is comprised of a heavy chain variable region and a heavy chain constant region. A light chain is comprised of a light chain variable region and a light chain constant region. In various embodiments,

there are three hypervariable regions within the variable regions that are responsible for antigen specificity. In various embodiments, the hypervariable regions are referred to as complementarity determining regions (CDR) and are interposed between more conserved flanking regions referred to as framework regions (FW). In various embodiments, the variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

[0141] Accordingly, in one aspect, the invention provides an anti-Ryk antibody and functional fragments thereof that inhibit Wnt-Ryk signaling. In various embodiments, the antibody is an isolated monoclonal antibody that specifically binds to a binding domain of Wnt to inhibit Wnt-Ryk signaling. Sequence data of the key regions of antibodies of the invention are shown in Tables 2 and 3:

Table 2: Sequence Data of Ab5.5

SEQ ID NO:	DESCRIPTION	SEQUENCE
1	CDR of Ab5.5 antibody Light Chain Variable Region	QDINSY
2	CDR of Ab5.5 antibody Light Chain Variable Region	RAN
3	CDR of Ab5.5 antibody Light Chain Variable Region	LQYDEFPLT
4	Ab5.5 antibody Light Chain Variable Region	DIKMTQSPSSMYASLGERVTITCKASQDINSYLS WIQQKPGKSPKTLIYRANRLVDGVPSRFSGSGSG QDYSLTISSLEYEDMGIYYCLQYDEFPLTFGAGT KLELKRADAAPTVSIFPPSSEQLTSGGASVVCFLN NFYPKDINVKWKIDGSERQNGVLNSWTDQDSKD STYSMSSTLTLTKEYERHNSYTCEATHKTSTSPI VKSFNRNEC
5	CDR of Ab5.5 antibody Heavy	GFTFSSYT

	Chain Variable Region	
6	CDR of Ab5.5 antibody Heavy Chain Variable Region	ISNGGGGT
7	CDR of Ab5.5 antibody Heavy Chain Variable Region	HGDNGDYWGHGSTLTVSSAK
8	Ab5.5 antibody Heavy Chain Variable Region	EVKLVESGGDLVQPGGSLKLSCAASGFTFSSYTM SWIRQTPEKRLEWVAYISNGGGGGTYYPDTVKG FTISRDNAKNTLYLQMNSLKSEDTAMYYCTRHG <u>DNGDYWGHGSTLTVSSAK</u> TPPSVYPLAPGSAA QTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGV HTFPAVLQSDLYTLSSSVTVPSSTWPSETVTCNV AHPASSTKVDDKIVPRDCGCKPCICTVPEVSSVFI FPPKPKDVLITLTPKVTCVVVD ISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNST FRSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIE KTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTC MITDFFPEDITVEWQWNGQPAENYKNTQPIMDT DGSYFVYSKLVNQKSNWEAGNTFTCSVLHEGLH NHHTEKSLSHSPGK

Table 3: Sequence Data of Ab11.4

SEQ ID NO:	DESCRIPTION	SEQUENCE
9	CDR of Ab11.4 antibody Light Chain Variable Region	QDINRY
2	CDR of Ab11.4 antibody Light Chain Variable Region	RAN
3	CDR of Ab11.4 antibody Light Chain Variable Region	LQYDEFPLT

10	Ab11.4 antibody Light Chain Variable Region	DIKMTQSPSSMYASLGERVTITCKASQDINRYLS WFQQKPGKSPETLIYRANRLVDGVPSRFSGSGSG QDYSLTISSLEYEDMGIYYCLQYDEFPLTFGAGT KLELKRADAAPTVSIFPPSSEQLTSGGASVVCFLN NFYPKDINVKWKIDGSRQNGVLNSWTDQDSKD STYSMSSTLTTLTKDEYERHNSYTCEATHKTSTSPI VKSFNRNEC
5	CDR of Ab11.4 antibody Heavy Chain Variable Region	GFTFSSYT
11	CDR of Ab11.4 antibody Heavy Chain Variable Region	ISTGGGST
12	CDR of Ab11.4 antibody Heavy Chain Variable Region	HGEFNYWGQGLVTVSAAK
13	Ab11.4 antibody Heavy Chain Variable Region	EVKLVESGGGLVQPGGSLKLSCAASGFTFSSYTM SWVRQTPPEKRLWVAYISTGGGSTYYPDTVKGR FTISRDNAKNTLYLQMSSLKSEDTAMYYCARHG EFNYWGQGLVTVSAAKTPPSVYPLAPGSAAQ TNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVH TFPAVLQSDLYTLSSSVTPSSTWVPSSETVTCNVA HPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIF PPKPKDVLITLTPKVTCVVVDISKDDPEVQFSW FVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQ DWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKA PQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVE WQWNGQPAENYKNTQPIMDTDGSYFVYSKLVN QKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSP GK

[0142] Accordingly, the invention provides an antibody that specifically binds to a binding domain of Wnt to inhibit Wnt-Ryk signaling. Thus, in various embodiments, the anti-Ryk antibodies of the present invention include any polypeptide or protein having a binding domain which is, or is substantially identical to the set of CDRs within an antibody variable region described herein that is specific for binding to a Wnt binding domain (*e.g.*, at least about 80% identical, at least about 85% identical, at least about 90% identical, at

least about 95% identical, or at least 99% identical to those herein). In particular, the antibody has a light chain variable region having the CDR sequences set forth in SEQ ID NOs: 1-3 or SEQ ID NOs: 9, 2 and 3; and a heavy chain variable region having the CDR sequences set forth in SEQ ID NOs: 5-7 or SEQ ID NOs: 5, 11, and 12.

[0143] In various embodiments, the light chain variable region has an amino acid sequence with at least 85% sequence identity to SEQ ID NOs: 4 or 10, optionally at least 90% sequence identity to SEQ ID NOs: 4 or 10. In further embodiments, the light chain variable region has an amino acid sequence having at least 95% sequence identity to SEQ ID NOs: 4 or 10, optionally at least 99% sequence identity to SEQ ID NOs: 4 or 10. In further embodiments, the anti-Ryk antibody Ab5.5 has a light chain variable region with the amino acid sequence set forth in SEQ ID NO: 4. In further embodiments, the anti-Ryk antibody Ab11.4 has a light chain variable region with the amino acid sequence set forth in SEQ ID NO: 10.

[0144] In various embodiments, the heavy chain variable region has an amino acid sequence with at least 85% sequence identity to SEQ ID NOs: 8 or 13, optionally at least 90% sequence identity to SEQ ID NOs: 8 or 13. In further embodiments, the heavy chain variable region has an amino acid sequence having at least 95% sequence identity to SEQ ID NOs: 8 or 13, optionally at least 99% sequence identity to SEQ ID NOs: 8 or 13. In further embodiments, the anti-Ryk antibody Ab5.5 has a heavy chain variable region with the amino acid sequence set forth in SEQ ID NO: 8. In further embodiments, the anti-Ryk antibody Ab11.4 has a heavy chain variable region with the amino acid sequence set forth in SEQ ID NO: 13.

[0145] While the heavy chain variable region and light chain variable region may be combined with other light chain variable regions and other heavy chain variable regions respectively, so long as specific binding to a binding domain of Wnt can be maintained to inhibit Wnt-Ryk signaling binding, in some embodiments, the anti-Ryk antibody includes a heavy chain variable region having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO: 8, the heavy chain variable region further having three CDR sequences set forth in SEQ ID NOs: 5-7; and a light chain region variable region having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO:

4, the light chain variable region also having the three CDR sequences set forth in SEQ ID NOs: 1-3. In some embodiments, the anti-Ryk antibody includes a heavy chain variable region having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO: 13, the heavy chain variable region further having three CDR sequences set forth in SEQ ID NOs: 5, 11, and 12; and a light chain region variable region having at least 85% sequence identity to the amino acid sequence set forth in SEQ ID NO: 10, the light chain variable region also having the three CDR sequences set forth in SEQ ID NOs: 9, 2, and 3.

[0146] In further embodiments, the heavy chain variable region has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 8 and the light chain variable region has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 4. In still further embodiments, the heavy chain variable region has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 8 and the light chain variable region has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 4. In still further embodiments, the heavy chain variable region has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 8 and the light chain variable region has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 4. In the anti-Ryk antibody AB5.5, the heavy chain variable region has the amino acid sequence of SEQ ID NO: 8 and the light chain variable region has the amino acid sequence of SEQ ID NO: 4.

[0147] In further embodiments, the heavy chain variable region has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 13 and the light chain variable region has at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 10. In still further embodiments, the heavy chain variable region has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 13 and the light chain variable region has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 10. In still further embodiments, the heavy chain variable region has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 13 and the light chain variable region has at least 99% sequence identity to the amino acid sequence of SEQ ID NO: 10. In the anti-Ryk antibody AB11.4, the heavy chain variable region has the amino acid sequence of SEQ ID NO: 13 and the light chain variable region has the amino acid sequence of SEQ ID NO: 10.

[0148] As indicated above, the invention encompasses variations in sequence around the CDRs of the antibody within a percent sequence identity. The term "percent sequence identity", with respect to two amino acid or polynucleotide sequences, refers to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. Thus, 80% amino acid sequence identity means that 80% of the amino acids in two optimally aligned polypeptide sequences are identical. Likewise, 85% amino acid sequence identity means that 85% of the amino acids in two optimally aligned polypeptide sequences are identical; and 90%, 95%, and 99% amino acid sequence identity means that 90%, 95%, and 99% respectively of the amino acids in two optimally aligned polypeptide sequences are identical.

[0149] Variations in sequence identity are permitted outside of the CDRs because not all amino acid residues within the heavy and light variable regions are required for binding Ryk at a binding domain of Wnt. In particular regions outside of CDRs, such as framework regions, may be mutated without losing Ryk binding capability. Still further, framework regions may be further mutated and thus vary in sequence when adapting the CDRs for the treatment of different species. Mutations or variations can be described by use of the following nomenclature: position (#); substituted amino acid residue(s). According to this nomenclature, the substitution of, for instance, an alanine residue for a glycine residue at position can be indicated as A#G, where # represents the position. When an amino acid residue at a given position is substituted with two or more alternative amino acid residues, these residues are separated by a comma or a slash. For example, substitution of alanine with either glycine or glutamic acid can be indicated as #G/E, or #G, #E. The deletion of alanine in the same position can be shown as Ala#* or A#* or *#Ala or *#A, where # refers to the position of the amino acid. Multiple mutations are separated by a plus sign or a slash. For example, two mutations in positions (each indicated by "#") substituting alanine and glutamic acid for glycine and serine, respectively, are indicated as A#G+E#S or A#G/E#S. When an amino acid residue at a given position # is substituted with two or more alternative amino acid residues, these residues are separated by a comma or a slash. For example, substitution of alanine at a position # with either glycine or glutamic acid is indicated as A#G,E or A#G/E, or A#G, A#E. When a position # suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position.

Thus, for instance, when a modification of an alanine at a position # is mentioned but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid residue (*i.e.*, any one of R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V).

[0150] Referring back to SEQ ID NOs: 1-13, the invention can be defined as a set of CDR regions with anti-Ryk binding activity; however, in preferred embodiments the CDR peptides (each corresponding to a single CDR) are joined to form an anti-Ryk antibody having a variable region for specific binding to a Wnt binding domain. Again, the antibody variable region can be present in, for example, a complete antibody, an antibody fragment (*e.g.*, F(ab), F(ab')₂, scFv, minibody, tetrabody and others) or a recombinant derivative of an antibody or antibody fragment. In some aspects, the antibody variable region is present in a recombinant derivative. Examples of recombinant derivatives include single-chain antibodies, diabody, triabody, tetrabody, and miniantibody. In some embodiments, an anti-Ryk antibody also contains one or more variable regions recognizing the same or different epitopes.

[0151] In various embodiments, the anti-Ryk antibodies of the invention may contain additional components including, but not limited to, components other than variable regions or additional variable regions that provide, or help provide, useful and/or additional activities. Useful activities include, for example, antibody effector functions such as antibody-dependent cellular cytotoxicity, phagocytosis, complement-dependent cytotoxicity, and half-life/clearance rate. In some embodiments, antibody effector functions are mediated by different host components, such as Fcγ receptors, neonatal Fc receptor (FcRn), and C1q. In various embodiments, different types of antibody components or alterations are used to enhance effector functions. Examples of useful components or alterations include the use of non-fucosylated oligosaccharides, amino acids altered to have increased stability, amino acids with enhanced binding to FcRn, amino acid alterations with enhanced binding to a Fcγ receptor, and amino acid alterations with decreased binding affinity to a Fcγ receptor.

[0152] In various embodiments, the anti-Ryk antibodies of the invention may contain additional components to alter the physiochemical properties of the protein, providing pharmacological advantages. For example, the attachment of polyethylene glycol ("PEG")

to molecules, in some embodiments, improves safety by reducing toxicity and increasing efficiency of the molecules when used as therapeutics. Physiochemical alterations include, but are not limited to, changes in conformation, electrostatic binding, and hydrophobicity which can work together to increase systemic retention of a therapeutic agent. Additionally, by increasing the molecular weight of an anti-Ryk antibody or functional fragment thereof by attaching a PEG moiety, pharmacological advantages may include extended circulating life, increased stability, and enhanced protection from host proteases. PEG attachment can also influence binding affinity of the therapeutic moiety to cell receptors. PEG is a non-ionic polymer composed of repeating units ($\text{—O—CH}_2\text{—CH}_2\text{—}$) to make a range of molecular weight polymers from 400 to greater than 15,000 (*e.g.*, PEG polymers with molecular weights of up to 400,000 are commercially available). Methods for incorporating PEG or long chain polymers of PEG are well known in the art (described, for example, in Veronese, F. M., et al., *Drug Disc. Today* 10: 1451-8 (2005); Greenwald, R. B., et al., *Adv. Drug Deliv. Rev.* 55: 217-50 (2003); Roberts, M. J., et al., *Adv. Drug Deliv. Rev.*, 54: 459-76 (2002)), the contents of which is incorporated herein by reference. Other methods of polymer conjugations known in the art can also be used in the present invention.

[0153] Thus, the anti-Ryk antibody or functional fragment thereof may be derivatized, linked to or co-expressed with another functional molecule, *e.g.*, another peptide or protein (*e.g.*, a Fab fragment). For example, the antibody can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, to produce a bispecific or a multispecific antibody), a cytotoxin, cellular ligand or antigen (*e.g.*, to produce an immunoconjugate, such as an immunotoxin). The antibody also can be linked to other therapeutic moieties, *e.g.*, a radioisotope, a small molecule anti-cancer drug, an anti-inflammatory agent, or an immunosuppressive agent. Accordingly, the present invention encompasses a large variety of antibody conjugates, bispecific and multispecific molecules, and fusion proteins, all of which specifically bind to a binding domain of Wnt or specifically bind to the same epitope on Wnt as does a reference antibody or antibody fragment, or cross-competes for specific binding to Wnt with a reference antibody or antibody fragment, as described herein.

[0154] Nucleic Acids

[0155] Nucleic acid sequences encoding the anti-Ryk polypeptide sequences, which include any of SEQ ID NOs: 1-13 are also provided. Recombinant nucleic acids encoding anti-Ryk antibodies are particularly useful for expression in a host cell that in effect serves as a factory for the anti-Ryk antibodies. In various embodiments, nucleic acids are isolated when purified away from other cellular components or other contaminants (*e.g.*, other nucleic acids or proteins present in the cell) by standard techniques including, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well-known in the art. See *e.g.*, F. Ausubel, et al., ed. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York. In various embodiments, a nucleic acid is, for example, DNA or RNA and may or may not contain intronic sequences. In a preferred embodiment, the nucleic acid is a cDNA molecule. In various embodiments, a recombinant nucleic acid provides a recombinant gene encoding the anti-Ryk antibody that exists autonomously from a host cell genome or as part of the host cell genome.

[0156] In some embodiments, a recombinant gene contains nucleic acids encoding a protein along with regulatory elements for protein expression. Generally, the regulatory elements that are present in a recombinant gene include a transcriptional promoter, a ribosome binding site, a terminator, and an optionally present operator. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. Antibody associated introns may also be present. The degeneracy of the genetic code is such that, for all but two amino acids, more than a single codon encodes a particular amino acid. This allows for the construction of synthetic DNA that encodes a protein where the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequences disclosed herein, but still encodes such a protein. Such synthetic DNAs are intended to be within the scope of the present invention.

[0157] Diseases

[0158] The anti-Ryk antibodies or antibody fragments described herein can be used in methods for inhibiting neuron (*e.g.*, axon) degeneration. These antibodies or antibody fragments are, therefore, useful in the therapy of, for example, (i) disorders of the nervous system (*e.g.*, neurological/neurodegenerative diseases or disorders), (ii) conditions of the

nervous system that are secondary to a disease, condition, or therapy having a primary effect outside of the nervous system, (iii) injuries to the nervous system caused by physical, mechanical, or chemical trauma, (iv) pain, (v) ocular-related neurodegeneration, (vi) memory loss, and (vii) psychiatric disorders. Non-limiting examples of some of these diseases, conditions, and injuries are provided below.

[0159] Examples of neurological/neurodegenerative diseases and conditions that can be prevented or treated according to the invention include amyotrophic lateral sclerosis (ALS), trigeminal neuralgia, glossopharyngeal neuralgia, Bell's Palsy, myasthenia gravis, muscular dystrophy, progressive muscular atrophy, primary lateral sclerosis (PLS), pseudobulbar palsy, progressive bulbar palsy, spinal muscular atrophy, progressive bulbar palsy, inherited muscular atrophy, invertebrate disk syndromes (*e.g.*, herniated, ruptured, and prolapsed disk syndromes), cervical spondylosis, plexus disorders, thoracic outlet destruction syndromes, peripheral neuropathies, porphyria, mild cognitive impairment, Alzheimer's disease, Huntington's disease, Parkinson's disease, Parkinson's-plus diseases (*e.g.*, multiple system atrophy, progressive supranuclear palsy, and corticobasal degeneration), dementia with Lewy bodies, frontotemporal dementia, demyelinating diseases (*e.g.*, Guillain-Barre syndrome and multiple sclerosis), Charcot-Marie-Tooth disease (CMT; also known as Hereditary Motor and Sensory Neuropathy (HMSN), Hereditary Sensorimotor Neuropathy (HSMN), and Peroneal Muscular Atrophy), prion disease (*e.g.*, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and bovine spongiform encephalopathy (BSE, commonly known as mad cow disease)), Pick's disease, epilepsy, and AIDS demential complex (also known as HIV dementia, HIV encephalopathy, and HIV-associated dementia).

[0160] The methods of the invention can also be used in the prevention and treatment of ocular-related neurodegeneration and related diseases and conditions, such as glaucoma, lattice dystrophy, retinitis pigmentosa, age-related macular degeneration (AMD), photoreceptor degeneration associated with wet or dry AMD, other retinal degeneration, optic nerve drusen, optic neuropathy, and optic neuritis. Non-limiting examples of different types of glaucoma that can be prevented or treated according to the invention include primary glaucoma (also known as primary open-angle glaucoma, chronic open-angle glaucoma, chronic simple glaucoma, and glaucoma simplex), low-tension glaucoma,

primary angle-closure glaucoma (also known as primary closed- angle glaucoma, narrow-angle glaucoma, pupil-block glaucoma, and acute congestive glaucoma), acute angle-closure glaucoma, chronic angle-closure glaucoma, intermittent angle-closure glaucoma, chronic open-angle closure glaucoma, pigmentary glaucoma, exfoliation glaucoma (also known as pseudoexfoliative glaucoma or glaucoma capsulare), developmental glaucoma (*e.g.*, primary congenital glaucoma and infantile glaucoma), secondary glaucoma (*e.g.*, inflammatory glaucoma (*e.g.*, uveitis and Fuchs heterochromic iridocyclitis)), phacogenic glaucoma (*e.g.*, angle-closure glaucoma with mature cataract, phacoanaphylactic glaucoma secondary to rupture of lens capsule, phacolytic glaucoma due to phacotoxic meshwork blockage, and subluxation of lens), glaucoma secondary to intraocular hemorrhage (*e.g.*, hyphema and hemolytic glaucoma, also known as erythroclastic glaucoma), traumatic glaucoma (*e.g.*, angle recession glaucoma, traumatic recession on anterior chamber angle, postsurgical glaucoma, aphakic pupillary block, and ciliary block glaucoma), neovascular glaucoma, drug-induced glaucoma (*e.g.*, corticosteroid induced glaucoma and alpha-chymotrypsin glaucoma), toxic glaucoma, and glaucoma associated with intraocular tumors, retinal detachments, severe chemical burns of the eye, and iris atrophy.

[0161] Certain diseases and conditions having primary effects outside of the nervous system can lead to damage to the nervous system, which can be treated according to the methods of the present invention. Examples of such conditions include peripheral neuropathy and neuralgia caused by, for example, diabetes, cancer, AIDS, hepatitis, kidney dysfunction, Colorado tick fever, diphtheria, HIV infection, leprosy, lyme disease, polyarteritis nodosa, rheumatoid arthritis, sarcoidosis, Sjogren syndrome, syphilis, systemic lupus erythematosus, and amyloidosis.

[0162] In addition, the methods of the invention can be used in the treatment of nerve damage, such as peripheral neuropathy, which is caused by exposure to toxic compounds, including heavy metals (*e.g.*, lead, arsenic, and mercury) and industrial solvents, as well as drugs including chemotherapeutic agents (*e.g.*, vincristine and cisplatin), dapsone, HIV medications (*e.g.*, Zidovudine, Didanosine, Stavudine, Zalcitabine, Ritonavir, and Amprenavir), cholesterol lowering drugs (*e.g.*, Lovastatin, Indapamid, and Gemfibrozil), heart or blood pressure medications (*e.g.*, Amiodarone, Hydralazine, Perhexiline), and Metronidazole.

[0163] The methods of the invention can also be used to treat injury to the nervous system caused by physical, mechanical, or chemical trauma. Thus, the methods can be used in the treatment of peripheral nerve damage caused by physical injury (associated with, *e.g.*, burns, wounds, surgery, and accidents), ischemia, prolonged exposure to cold temperature (*e.g.*, frost-bite), as well as damage to the central nervous system due to, *e.g.*, stroke or intracranial hemorrhage (such as cerebral hemorrhage).

[0164] Further, the methods of the invention can be used in the prevention or treatment of memory loss such as, for example, age-related memory loss. Types of memory that can be affected by loss, and thus treated according to the invention, include episodic memory, semantic memory, short-term memory, and long-term memory. Examples of diseases and conditions associated with memory loss, which can be treated according to the present invention, include mild cognitive impairment, Alzheimer's disease, Parkinson's disease, Huntington's disease, chemotherapy, stress, stroke, and traumatic brain injury (*e.g.*, concussion).

[0165] The methods of the invention can also be used in the treatment of psychiatric disorders including, for example, schizophrenia, delusional disorder, schizoaffective disorder, schizophreniform, shared psychotic disorder, psychosis, paranoid personality disorder, schizoid personality disorder, borderline personality disorder, anti-social personality disorder, narcissistic personality disorder, obsessive-compulsive disorder, delirium, dementia, mood disorders, bipolar disorder, depression, stress disorder, panic disorder, agoraphobia, social phobia, post-traumatic stress disorder, anxiety disorder, and impulse control disorders (*e.g.*, kleptomania, pathological gambling, pyromania, and trichotillomania).

[0166] In addition to the *in vivo* methods described above, the methods of the invention can be used to treat nerves *ex vivo*, which may be helpful in the context of nerve grafts or nerve transplants. Thus, the compounds provided herein can be useful as components of culture media for use in culturing nerve cells *in vitro*.

[0167] The antibodies or antibody fragments described herein can be optionally combined with or administered in concert with each other or other agents known to be useful in the treatment of the relevant disease or condition. Thus, in the treatment of ALS,

for example, the compounds can be administered in combination with Riluzole (Rilutek), minocycline, insulin-like growth factor 1 (IGF-I), and/or methylcobalamin. In another example, in the treatment of Parkinson's disease, inhibitors can be administered with L-dopa, dopamine agonists (*e.g.*, bromocriptine, pergolide, pramipexole, ropinirole, cabergoline, apomorphine, and lisuride), dopa decarboxylase inhibitors (*e.g.*, levodopa, benserazide, and carbidopa), and/or MAO-B inhibitors (*e.g.*, selegiline and rasagiline). In a further example, in the treatment of Alzheimer's disease, inhibitors can be administered with acetylcholinesterase inhibitors (*e.g.*, donepezil, galantamine, and rivastigmine) and/or NMDA receptor antagonists (*e.g.*, memantine). The combination therapies can involve concurrent or sequential administration, by the same or different routes, as determined to be appropriate by those of skill in the art. The invention also includes pharmaceutical compositions and kits including combinations as described herein.

[0168] In the context of the invention, the terms "contact" or "contacting" are defined to mean any manner in which a compound is brought into a position where it can mediate, modulate, or inhibit the growth of a neuron. "Contacting" can comprise injecting a diffusable or non-diffusable substance into the neuron or an area adjacent a neuron. "Contacting" can comprise placing a nucleic acid encoding a compound into or close to a neuron or non-neuronal cell in a manner such that the nucleic acid is expressed to make the compound in a manner in which it can act upon the neuron. Those of skill in the art, following the teachings of this specification, will be able to contact neurons with substances in any manner.

[0169] The methods for modulating growth of a neuron may, in certain embodiments, be methods for stimulating growth of a neuron, methods for regenerating a damaged neuron, or methods for guiding growth of a neuron along the anterior-posterior axis. In other embodiments, the methods for modulating growth of a neuron are further defined as methods for directionally orienting axon growth of a neuron between the spinal cord and the brain.

[0170] In certain embodiments, the neuron is contacted with an anti-Ryk monoclonal antibody or antibody fragment that specifically binds to a binding domain of Wnt affecting a Wnt signaling pathway, and may further involve exposing the neuron to a gradient of the

anti-Ryk monoclonal antibody or antibody fragment that specifically binds to a binding domain of Wnt affecting a Wnt signaling pathway. The gradient may be in the spinal cord, such as a decreasing anterior-posterior gradient within the spinal cord. In other embodiments, exposing the neuron to the gradient involves stimulating directionally-oriented axon growth of the neuron along the anterior-posterior axis. Any direction of axon growth is contemplated by the present invention. In certain embodiments, the axon growth is directed from the spinal cord to the brain, such as in the growth of neurons in ascending somatosensory pathways. In other embodiments, the axon growth is directed from the brain to the spinal cord, such as in the growth of neurons in descending motor pathways or other regulatory pathways. In further embodiments, the axon growth is directed along the spinothalamic pathway.

[0171] The present invention also includes methods of modulating growth of a neuron in a subject, including: (a) providing a composition that includes an anti-Ryk antibody or antibody fragment that specifically binds to a binding domain of Wnt affecting a Wnt signaling pathway; and a pharmaceutical preparation suitable for delivery to the subject; and (b) administering the composition to the subject. The methods for modulating neuron growth of the present invention contemplate measurement of neuronal growth by any known means, as discussed above. For example, the method of modulating neuron growth may be defined as a method of promoting growth and regeneration of a neuron in a subject, a method of promoting axon growth and regeneration in a subject, or a method of promoting directionally-oriented axon growth in a subject. Directionally-oriented axon growth may be along the anterior-posterior axis such as from the spinal cord to the brain, or from the brain to the spinal cord.

[0172] In another aspect, the invention provides a composition comprising the antibody or antibody fragment of the invention, which can be prepared for administration to a subject by mixing the antibody or immunogenic peptide fragment with physiologically acceptable carriers or excipients. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the particular antibody with saline, buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrans, or chelating agents such as EDTA, glutathione

and other stabilizers and excipients. Such compositions can be in suspension, emulsion or lyophilized form and are formulated under conditions such that they are suitably prepared and approved for use in the desired application.

[0173] A physiologically acceptable carrier or excipient can be any material that, when combined with an immunogenic peptide or a polynucleotide of the invention, allows the ingredient to retain biological activity and does not undesirably disrupt a reaction with the subject's immune system. Examples include, but are not limited to, any of the standard physiologically acceptable carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline. Compositions comprising such carriers are formulated by well-known conventional methods (see, for example, Remington's Pharmaceutical Sciences, Chapter 43, 14th Ed., Mack Publishing Co., Easton PA 18042, USA).

[0174] For administration to a subject, a peptide, or an encoding polynucleotide, generally is formulated as a composition. Accordingly, the present invention provides a composition, which generally contains, in addition to the peptide or polynucleotide of the invention, a carrier into which the peptide or polynucleotide can be conveniently formulated for administration. For example, the carrier can be an aqueous solution such as physiologically buffered saline or other solvent or vehicle such as a glycol, glycerol, an oil such as olive oil or an injectable organic esters. A carrier also can include a physiologically acceptable compound that acts, for example, to stabilize the peptide or encoding polynucleotide or to increase its absorption. Physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. Similarly, a cell that has been treated in culture for purposes of the practicing the methods of the invention, for example, synovial fluid mononuclear cells, dendritic cells, or the like, also can be formulated in a composition when the cells are to be administered to a subject.

[0175] It will be recognized to the skilled clinician that choice of a carrier or excipient, including a physiologically acceptable compound, depends, for example, on the manner in

which the peptide or encoding polynucleotide is to be administered, as well as on the route of administration of the composition. Where the composition is administered under immunizing conditions, *i.e.*, as a vaccine, it generally is administered intramuscularly, intradermally, or subcutaneously, but also can be administered parenterally such as intravenously, and can be administered by injection, intubation, or other such method known in the art. Where the desired modulation of the immune system is tolerization, the composition preferably is administered orally, or can be administered as above.

[0176] Screening Methods

[0177] In another aspect, the invention provides a method of screening for a therapeutic/test/candidate agent for treating a neurological disease or disorder. The method includes administering a test agent to the transgenic non-human mammal described herein and evaluating the effect of the test agent on at least one of: the amount of atypical protein kinases C (aPKC) or MARK2 protein, the level of aPKC or MARK2 activity or the level of aPKC or MARK2 in at least one disease-relevant tissue of the transgenic non-human mammal, wherein at least one of: a decrease in the amount of aPKC protein, an increase in the amount of MARK2 protein, a decrease in the level of aPKC activity, an increase in the level of MARK2 activity, a reduction in the level of aPKC, or an increase in the level of MARK2 in at least one disease-relevant tissue relative to a similar transgenic non-human mammal that does not receive the test agent indicates the test agent is therapeutic for the neurological disease or disorder.

[0178] A “test agent” or “candidate agent” refers to an agent that is to be screened in one or more of the assays described herein. The agent can be virtually any chemical compound. It can exist as a single isolated compound or can be a member of a chemical (*e.g.*, combinatorial) library. In one embodiment, the test agent is a small organic molecule. The term small organic molecules refers to molecules of a size comparable to those organic molecules generally used in pharmaceuticals.

[0179] Conventionally, new chemical entities with useful properties are generated by identifying a chemical compound (called a “lead compound”) with some desirable property or activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds.

[0180] Any agent identified as being a potential therapeutic for treating a neurological disease or disorder may be brought into association with a pharmaceutically acceptable carrier, which constitutes one or more accessory ingredients. The term “pharmaceutically acceptable,” when used in reference to a carrier, is meant that the carrier, diluent or excipient must be compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

[0181] Pharmaceutically acceptable carriers useful for formulating an agent for administration to a subject are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the conjugate. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the physico-chemical characteristics of the therapeutic agent and on the route of administration of the composition, which can be, for example, orally, intranasally or any other such method known in the art. The pharmaceutical composition also can contain a second (or more) compound(s) such as a diagnostic reagent, nutritional substance, toxin, or therapeutic agent, for example, a cancer chemotherapeutic agent and/or vitamin(s).

[0182] The total amount of a compound or composition to be administered in practicing a method of the invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which multiple doses are administered over a prolonged period of time. One skilled in the art would know that the amount of the plasma expander used to treat blood loss in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose as necessary. In general, the formulation of the pharmaceutical composition and the

routes and frequency of administration are determined, initially, using Phase I and Phase II clinical trials.

[0183] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1

Methods

[0184] *Animals* – Time-mated pregnant CD1 mice rats were purchased from Charles River. Ryk-deficient mice were obtained from Steven Stacker (Ludwig Institute for Cancer Research, Melbourne, VIC, Australia) (50). Frizzled3-deficient and were obtained from Jeremy Nathans (John Hopkins University, Baltimore, MD) (51). Genotypes were determined by standard PCR. The day of vaginal plug detection was considered E0.5. Animals were housed in a temperature-controlled room with standard laboratory food and water provided *ad libitum*.

[0185] *Antibodies, Reagents and Plasmidic Constructs* – Myristoylated aPKC and cPKC pseudosubstrates were purchased from Enzo Life Sciences-Biomol (#BML-P-219 and #BML-P205 respectively).

[0186] Monoclonal mouse Ryk antibodies were generated against the ectodomain of Ryk, amino-acids 90-183, fused with maltose binding protein.

[0187] Commercial primary antibodies used in this study included P-PKC ζ T410 (Santa Cruz sc-101778, rabbit 1:1000), Total PKC ζ (Santa Cruz C-20 sc-216, rabbit, 1:2000), P-MARK2 T595 (Abcam, rabbit 1:1000), Total MARK2 (Abcam, goat 1:1000), SMI-312 (Covance, mouse, 1:1000), MAP2 (Abcam, rabbit 1:1500), β III-tubulin (Covance, mouse, 1:1000), Rhodamine-phalloidin (Invitrogen, 1:100), P-Tau S262 (Invitrogen, rabbit, 1:1000), Tau (Tau5, Millipore, mouse, 1:2000 for cell culture, 1:4000 for western blotting), P-JNK/SAPK T183/T185 (Cell Signaling, rabbit, 1:1000), Total JNK/SAPK (Cell Signaling, 1:1000), P-c-Jun S63 (Cell Signaling, rabbit 1:1000), Total c-Jun (Cell Signaling, rabbit 1:1000), GAPDH (Millipore-Chemicon, mouse, 1:50,000), actin (mouse, 1:5000), activated Caspase-3 (aCasp3, Cell signaling, rabbit, 1:1000 on cell cultures, 1:500 on tissue sections) and CTIP2 (Abcam, rat, 1:500).

[0188] cDNAs of PKC ζ -WT, PKC ζ -KD (K281W) and PKC ζ -T410A plasmidic constructs (56) were cloned into pCIG2-EGFP plasmid (14). pCIG2-Tau-WT and pCIG2-Tau-S262A plasmids were provided by Georges Mairret-Coello (The Scripps Research Institute, La Jolla, CA) (57).

[0189] *Cerebral cortical cell culture* – E16.5 mouse pregnant females were sacrificed by CO₂ asphyxia and cervical dislocation. Embryos were removed from uterine horns. Skin, skull, and meninges were removed from embryo heads. The cortex was dissected in L-15 medium (Sigma), digested with 0.025% trypsin/0.221 mM EDTA (Mediatech) for 20 min followed by incubation in 5% horse serum prepared in L-15 medium for 5 min to inhibit trypsin. Cortices were mechanically dissociated and cells were plated at 500 cells/mm² on poly-D-lysine (33.3 μ g/ml, Millipore) and laminin (3.3 μ g/ml, Invitrogen)-coated 12 mm glass coverslips in 24-well plates or at 1050 cells/mm² on poly-D-lysine (0.1mg/ml)-coated 6 well plates (35 mm dishes). Cells were incubated in Neurobasal (Gibco) medium supplemented with B27 (Gibco) containing Glutamax (Gibco), 40mM Glucose, 100U/ml penicillin and 1mg/ml streptomycin (Mediatech). Cultures were maintained in a humidified 5% CO₂ /air incubator at 37°C.

[0190] *Magnetofection* – For magnetofection, cortices from E17.5 embryos were dissociated in papain (Worthington) supplemented with DNase I (100mg/ml, Sigma) for 20 min at 37°C, washed three times and manually triturated in plating medium. Cells were plated at 565/mm² on 12 mm-glass bottom dishes coated with poly-D-lysine (1mg/ml, Sigma). Cells were cultured in neurobasal medium supplemented with 2.5% fetal bovine serum (Gemini), B27 (1X), L-glutamine (2mM) and penicillin (2.5U/ml)-streptomycin (2.5mg/ml) (Invitrogen). Neurons were transfected at DIV3 by magnetofection using NeuroMag (OZ Bioscience), according to manufacturer's instructions and as previously described (57). Briefly, 2 μ g cDNA was incubated with NeuroMag in neurobasal medium for 15 min at room temperature, and then the mixture was applied dropwise on culture cells. Cultures were placed on magnet for 20 min for transfection. Cotransfections were performed at a 1:1 ratio (w/w).

[0191] *Immunohistochemistry, Immunocytochemistry and TUNEL assay* – Cell cultures were fixed with 4% PFA for 30 min. Embryonic brains were dissected in cold PBS, fixed

in 4% PFA for 2h and incubated overnight in 30% sucrose for cryoprotection. Brains were embedded in a commercial embedding medium (Tissue-Tek, Sakura Finetek), quickly frozen on dry-ice and coronally sectioned at a thickness of 20 μ m using a cryostat-microtome (Leica). Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific) and stored at -20°C. Cells or sections were permeabilized in PBS containing 0.3% triton X-100 (PBS-T) for 10 min and incubated with the primary antibody overnight at 4°C. Monoclonal antibodies were diluted in PBS-T and polyclonal antibodies were diluted in a PBS-T solution containing 10% lactoproteins, 1% bovine serum albumin. Staining was visualized using Alexa Fluor 588 (1:500-1:1000), 594 (1:500-1:1000) or 649 (1:200-1:500) conjugated secondary antibodies (Jackson ImmunoResearch). For CTIP2 immunodetection, sections were submitted to antigen retrieval procedure in 10mM citrate buffer pH=6 at 90°C-95°C for 10 min. Sections were counterstained with DAPI (Thermo Fisher Scientific). TUNEL was performed using the In situ cell death detection kit TMR red (Roche) according to the manufacturer's instructions.

[0192] *Western-blotting* – Cortical cells cultured in 6 well plates were washed twice with PBS (pH 7.4), scrapped using a rubber policeman and lysed in lysis buffer containing 20 μ M Tris-HCl pH7.6, 150 μ M NaCl, 0.1% SDS, 1% triton X-100, protease inhibitors (cOmplete mini tablets, Roche Applied Science), phosphatase inhibitors (PhosSTOP, Roche Applied Science). Twenty micrograms of proteins were separated by SDS PAGE and then electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with blocking buffer containing 2% BSA or 5% fat-free dry milk in Tris-buffered saline solution and Tween 20 (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) and incubated overnight at 4°C with primary antibody diluted in blocking buffer. Incubations with HRP conjugated secondary antibodies (1:5000) were performed for 1h at room temperature and visualization was performed using chemiluminescence (ECL).

[0193] *Image acquisition and analysis* – All images were acquired on an inverted Zeiss LSM510 confocal microscope with LSM acquisition software (Carl Zeiss Microscopy). For quantification of P-PKC ζ T410 immunoreactivity in cell cultures, exposure time was adjusted to be under the saturation level for the highest immunofluorescence signal and was conserved between the different samples. Mean immunofluorescence intensity level in neuronal cell bodies was measured using FiJi-ImageJ software (NIH). All quantifications

were performed on 10 non-overlapping fields in cell cultures from at least three independent experiments and on four non-consecutive sections from at least three animals for *in vivo* experiments.

[0194] *Statistical Analysis* – Statistical analyses were performed using ANOVA with Bonferroni Posttest or Student's t test using GraphPad InStat 3.05 (GraphPad Software). All data are expressed as means or percents $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

EXAMPLE 2

Inhibition of aPKC Promotes Axonal Degeneration and Neuronal Apoptosis

[0195] A culture system was established to investigate the expression and role of aPKC in the survival of cerebral cortical neurons. E16.5 cortical cells were cultured for 3 days *in vitro* (DIV3) and double immunolabeled for the pan-axonal marker SMI-312 and the dendritic marker MAP2. At this stage, most neurons exhibit a long process labeled for SMI-312 corresponding to the axon and few short MAP2+ processes that will give rise to the dendrites. To evaluate the proportion of polarized neurons at this stage, the number of neurons with a long process labeled for SMI-312 and not labeled for MAP2 in its distal part was counted over the total number of SMI-312+ neurons. It was found that $84.2 \pm 3.45\%$ of SMI-312+ neurons were polarized in these culture conditions. Then, aPKC expression was investigated in cortical neurons by using an antibody that recognized all isoforms of aPKC family including PKC ζ , PKC λ and PKM ζ (34). These observations indicate that aPKC immunolabeling was localized in neuronal cell bodies and SMI-312+ axons (Fig. 1A).

[0196] To investigate the role of aPKC in neuronal survival, aPKC activity was inhibited by transfecting a kinase-defective PKC ζ (PKC ζ -KD) or a nonphosphorylatable mutant (PKC ζ -T410A) in cortical neuron cultures. aPKC inhibition by these constructs induced neurite degeneration (Figs. 1B, 1C, and 1E) after 2 days of transfection, as shown by the massive fragmented neurites of PKC ζ -KD and PKC ζ -T410A transfected neurons (Figs. 1B and 1C). PKC ζ -KD and PKC ζ -T410A overexpression also induced neuronal apoptosis as shown by the increase of the proportion of aCasp3+ neurons (Figs. 2D and 2F).

Overexpression of PKC ζ -WT protein did not affect neurite integrity or neuronal survival (Figs. 1B-1F). The massive axon fragmentation suggests that axons have grown extensively initially and underwent degeneration all at once, suggesting that aPKC is required for axon

maintenance. Interestingly, while overexpressed PKC ζ was localized in both neuronal cell bodies and neurites, overexpressed PKC ζ -KD and PKC ζ -T410A mutant proteins were excluded from the processes and were restricted to the cell bodies (Fig. 1B), suggesting that PKC ζ activity might regulate its own localization and/or the stability of the protein.

[0197] In order to test the role of aPKC in axon survival using an independent method and that allows biochemical analyses, a cell-permeable myristoylated pseudosubstrate specific to aPKC was used (14). As a control, a myristoylated pseudosubstrate specific to conventional PKC (14) was used. The IC₅₀ of aPKC and cPKC inhibitors are 10 μ M and 8 μ M respectively, so a starting dose of 10 μ M was used. At this dose, aPKC inhibitor but not cPKC inhibitor promotes rapid axonal degeneration in cortical neurons as shown by the beading aspect of SMI-312+ axons, 2h after aPKC pseudosubstrate treatment (Figs. 2A and 2B). Furthermore, aPKC pseudosubstrate promotes axonal degeneration in a dose- (Fig. 2C) and time-dependent manner (Fig. 2D), with an effect starting at 5 μ M and as early as 1h at a dose of 10 μ M. To verify the efficacy of aPKC pseudosubstrate, P-PKC ζ -T410 immunoreactivity was measured in neuronal cell bodies after 2h of 10 μ M aPKC PS treatment and it was found that it was significantly decreased (Fig. 2E), indicating that aPKC pseudosubstrate decreases (auto)-phosphorylation activity of aPKC.

[0198] Axon degeneration induced by aPKC pseudosubstrate occurred as early as 1h (Fig. 2D) and the time course analysis revealed that almost all neurons exhibited degenerating axon or were devoid of axon by 4h (Figs. 2F and 2G). In parallel to axon degeneration, neuronal cell body death was measured using TUNEL and activated Caspase-3 staining at 2h, 4h and 24h. It was found that cell death in SMI312+ neurons started at 4h of incubation with aPKC pseudosubstrate (Fig. 2H), when the quasi-totality of axons had already degenerated (Fig. 2G), suggesting that blocking aPKC signaling triggers a dying back mechanism. Interestingly, neurons started to show aCasp3 after 4h but all neurons did not express activated Caspase-3 by 24h (Fig. 2I) while all of them were TUNEL+ by 24 h (Fig. 2H), suggesting that aPKC inhibition may trigger both caspase-3-dependent and -independent cell death pathways.

EXAMPLE 3

aPKC Inhibition Destabilizes Microtubules by Regulating MARK2 Activity and Tau Phosphorylation

[0199] Microtubule degradation is an early cellular event in axon degeneration process, preceding axonal beading and neurofilament fragmentation (1, 35-37). It was hypothesized that inhibition of aPKC may promote rapid axon degeneration by destabilizing microtubules. To test this hypothesis, cortical neuronal cell cultures were first pre-incubated with taxol, a microtubule stabilizer (38), 2h prior to and during aPKC pseudosubstrate treatment. Axon degeneration induced by aPKC inhibition was reduced when neuronal cell cultures were pre-treated with taxol (Figs. 3A and 3B). Using specific markers for the different compartments of the neuronal cytoskeleton (Tau for microtubules, SMI-312 for neurofilaments and rhodamine-phalloidine for F-actin filaments), it was found that microtubules and neurofilaments, but not actin filaments, were disrupted in axons of neurons treated with aPKC pseudosubstrate using confocal microscopy. These observations suggest that aPKC is required for microtubule stability and that aPKC inhibition promotes both microtubule and neurofilament disruption, ultimately leading to axonal degeneration.

[0200] The signaling pathway involved in microtubule destabilization induced by aPKC inhibition was further investigated. First, the efficacy of aPKC pseudosubstrate on blocking aPKC (auto-)phosphorylation activity was confirmed by western blotting. aPKC pseudosubstrate decreased P-PKC ζ T410 level after 2h of incubation (Fig. 3C), in agreement with the immunocytochemical analyses (Fig. 2E). Then the focus was on MARK2 because (1) it belongs to the MARK/Par1 family of kinases that were originally discovered to trigger microtubule disassembly (39) and (2) it is inhibited when phosphorylated by aPKC on T595 (40). It was found that aPKC pseudosubstrate treatment reduced phosphorylation of MARK2 on T595 at 2h (Fig. 3D), indicating that MARK2 activity was increased when aPKC was inhibited. Furthermore, Tau phosphorylation in the microtubule binding domain at S262 (Fig. 3E) was increased at 2h while stable microtubules, revealed by Glu-Tubulin (Fig. 3F), were decreased. These observations are in agreement with previous studies showing that MARK family kinases destabilize microtubules by phosphorylating MAPs including Tau (9, 39, 41).

[0201] To further test the role of Tau phosphorylation in axonal degeneration induced by aPKC inhibition, cortical neurons were co-transfected with PKC ζ -KD and Tau-WT or a non phosphorylatable mutant Tau at S262 (Tau-S262A) plasmidic construct. Overexpression of Tau-WT, but not Tau-S262A, induced axonal degeneration and neuronal apoptosis in a

similar extent than PKC ζ -KD (Figs. 3G-3I). Co-transfection of PKC ζ -KD with Tau-S262A but not with Tau-WT prevented axonal degeneration and neuronal cell death induced by PKC ζ -KD overexpression (Figs. 3G-3I), suggesting that axonal degeneration induced by PKC ζ -KD is mediated by Tau phosphorylation at S262.

EXAMPLE 4

aPKC Inhibition activates the JNK-cJun Signaling Pathway

[0202] JNKs/SAPKs are a subfamily of MAPKs involved in a variety of physiological and pathological processes in the central and peripheral nervous system. In particular, JNKs mediate neuronal cell death in response to stress and injury and in some pathological conditions (42, 43). JNKs were also shown to induce, retrograde axonal degeneration and to limit motor recovery after spinal cord injury (44). It was therefore sought to determine whether JNKs might be involved in axonal degeneration induced by aPKC inhibition. Western-blotting experiments revealed that aPKC inhibition induced by aPKC PS leads to an increase of phosphorylation of JNKs at T183/T185. Increased phosphorylation occurred on both p54 and p46 protein isoforms at 1h (Figs. 4A and 4B). Phosphorylation of the transcription factor c-Jun was increased at S63, at 1h and 2h (Figs. 4A and 4C), indicating that c-Jun, a direct substrate of JNK (45), is quickly activated when aPKC activity is inhibited. Increase of P-JNK-T183/T185 and P-cJun-S63 was confirmed in the nucleus of neurons by immunocytochemistry (Figs. 4D and 4E). These results suggest that aPKC inhibition might promote axonal degeneration and neuronal cell death by activating the cell death JNK/c-Jun pathway.

EXAMPLE 5

Ryk Promotes Axonal Degeneration Induced by aPKC Inhibition

[0203] Previous studies showed that aPKC is a kinase required for Wnt-Frizzled3 mediated axon growth and attraction and also exerts a positive feedback function to amplify planar cell polarity signaling by increasing Frizzled3 endocytosis (46). Because Ryk is a repulsive Wnt receptor and inhibits planar cell polarity signaling, it was tested whether Ryk regulates aPKC. It was found that P-aPKC-T410 level measured by Western-Blotting was increased in Ryk KO cortical neurons cultured for 3 days (Figs. 5A and 5B), suggesting that Ryk normally inhibit aPKC activity.

[0204] It was previously shown that expressions of certain Wnts and of the Ryk receptor are re-induced after spinal cord injury and that blocking Wnt/Ryk signaling reduces the retraction of corticospinal axons from the injury site (32). These recent studies indicate that Ryk expression is increased in motor neurons and axons in the spinal cord of a mouse model of ALS at early stage of the disease progression, suggesting that Ryk is involved in the early steps of neurodegeneration in ALS (47). It was therefore hypothesized that Ryk may be also involved in axon degeneration and it was tested whether blocking Wnt/Ryk signaling could protect cortical axons from degeneration induced by aPKC inhibition.

[0205] To block Ryk signaling, a mouse monoclonal antibody against the ectodomain of mouse Ryk was generated (by the laboratory of Alex Kolodkin at John Hopkins University, Baltimore, MD). To test the specificity of this antibody, Western-Blotting analyses were performed on protein extracts from HEK cells transfected with a mouse Ryk expression vector. A signal was detected at approximately 70 KDa, the expected size for Ryk protein. A weaker band of similar size was detected endogenously in E14.5 embryonic wild type mouse tissue but not in Ryk KO tissue, suggesting that the antibody is specific to Ryk. Pre-incubation of cortical neuronal cell cultures with the mouse Ryk antibody but not with normal mouse IgG, 2h prior to and during the 2h of aPKC PS treatment, partially blocked axonal degeneration (Figs. 5C and 5D). Axonal degeneration induced by aPKC inhibition was also analyzed in Ryk KO mouse neuronal cell cultures. Axonal degeneration triggered by aPKC PS was reduced in Ryk KO cortical neurons compared to WT neurons (Figs. 5E and 5F).

EXAMPLE 6

Neuronal Cell Death is Reduced in the Retrosplenial Cortex of Developing Ryk KO Mice

[0206] The *in vitro* results suggest that Ryk may be involved in neuronal cell death. Previous studies showed that Ryk is expressed in the developing rodent forebrain including the isocortex, hippocampus and striatum (24, 48, 49). Apoptosis was examined in several regions of the forebrain in E18.5 Ryk deficient embryos, using aCasp3 immunostaining. Ryk KO mice exhibit craniofacial abnormalities including cleft palate and die at birth (50), so all analyses were performed prenatally during late gestation on E18.5 embryos. At E18.5, the overall architecture of Ryk^{-/-} brain appears roughly normal and undistinguishable

from Ryk^{-/-} and Ryk^{+/-} embryos (27). The number of aCasp3⁺ cells, although low in WT embryos was decreased by 45% in E18.5 RykKO mice in a restricted area of the cortex, the RSP, which corresponds to the posterior part of the cingulate cortex (Figs. 6A-6C). However, apoptosis was not changed in the dorsolateral cortex, hippocampus and striatum in Ryk KO embryos, suggesting that Ryk might regulate cell death in specific regions of the developing cortex.

EXAMPLE 7

Ryk Heterozygosity Attenuates Neuronal Cell Death in the Retrosplenial Cortex of Frizzled3 KO Embryos

[0207] Previous studies showed extensive cell death in the striatum of mouse embryos deficient for the Wnt receptor Frizzled3 and that Frizzled3 was strongly expressed in the developing striatum and isocortex (51). Cell death was examined in Frizzled3 KO mice, in the striatum and other regions of the forebrain including the RSP. These observations confirmed that apoptosis was strongly increased in the striatum. Interestingly, it was found that apoptosis was increased by 5 fold in the RSP of E18.5 Frizzled3^{-/-} embryos, compared to Frizzled3^{+/+} and Frizzled3^{+/-} mice (Figs. 6D and 6E). Double immunolabelings with neural precursor marker nestin and early neuronal marker β III-tubulin showed that aCasp3⁺ immunolabeling was localized in β III-tubulin⁺ neurons. More precisely, aCasp3 co-localized with CTIP2 which is expressed in neurons of cortical layer V during embryonic development. However, apoptosis was not significantly different in E18.5 Frizzled3^{-/-} compared to Frizzled3^{+/+} embryos in the dorsolateral part of the cortex and hippocampus.

[0208] Since cell death was regulated in an opposite manner in the RSP of Frizzled3 KO and Ryk KO embryos, Frizzled3^{+/-} mice were crossed with Ryk^{+/-} mice. Double heterozygous mice resulting from these crossings were crossed with Frizzled3^{+/-} mice for 6 generations to get the Frizzled3/Ryk mouse line into the Frizzled3 strain (Black6). Then, Frizzled3^{+/-} Ryk^{+/-} females were crossed with Frizzled3^{+/-} Ryk^{+/-} males. Over 52 embryos from 7 litters did not produce any embryos double knock-out for Frizzled3 and Ryk at E18.5, suggesting that Frizzled3^{-/-} Ryk^{-/-} embryos might not be viable at this developing stage. The analyses were therefore performed in Frizzled3^{-/-} Ryk^{+/-} embryos and it was found that knocking down Ryk expression by 50% attenuated apoptosis in the RSP of Frizzled3 KO embryos at E18.5, as shown by a 30% reduction of the number of activated

Caspase 3⁺ cells in the RSP of Frizzled3^{-/-} Ryk^{+/-} embryos compared to Frizzled3^{-/-} Ryk^{+/+} animals (Figs. 6D and 6E).

EXAMPLE 8

Additional Methods

[0209] Animals were housed on a 12hr light/dark cycle and behavioral analyses were done at consistent morning hours during the light cycle. Both mice and rats were group housed, except for mice with cranial windows which were singly housed after window implantation. Group sample sizes were chosen based upon previous studies and power analysis (25% effect size, $\alpha=0.05$, $639 \sim 0.2$, power of 80%). One mouse was excluded from the study due to evidence of incomplete CS lesion with labeled corticospinal axons present at and below the level of the injury and one mouse was excluded as it did not attempt to perform the behavioral task after injury. All procedures and methods (surgical procedures, behavioral assays, tissue processing, immunostaining, image analysis, COS-7 transfection, and cortical mapping) were performed by investigators blinded to genotype or treatment group.

[0210] *Generation of transgenic mice* – The target vector containing loxP-flanked exons 3-6 as well as the PKG-neo selection cassette was transfected into ES cells. Cells were screened by Southern blot and PCR for integration of the targeting vector. Chimeric mice were then generated. Ryk cKO mice were crossed with Ai14 B6.Cg mice containing a loxP-flanked stop cassette preventing tdTomato expression (The Jackson Laboratory, Bar Harbor, ME, RRID:IMSR_JAX:007914) and backcrossed into C57BL/6J for 6 generations. For cortical mapping experiments, Ryk cKO::tdTomato Ai14 mice were crossed with mice expressing channelrhodopsin (Chr2) behind the Thy1 promoter (B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J) (The Jackson Laboratory).

[0211] *Surgical Procedures* – Cortical AAV injection: Adult female C57BL/6J mice (6.1 $\pm$ 0.1 weeks old) were deeply anaesthetized with isoflurane until unresponsive to toe and tail pinch and the area over the skull was shaved and cleaned with povidone-iodine before incision. The skull was thinned bilaterally over the motor cortex and self-complementary AAV2/6 Cre-HA (Salk Institute for Biological Studies Gene Transfer, Targeting and Therapeutics Core, La Jolla, CA) (1.49×10^{11} genome copies/ml) was injected into 10 sites

per hemisphere (250nl/site) with a 36ga NanoFil needle (World Precision Instruments Inc., Sarasota, FL).

[0212] *Cranial window:* Adult female C57BL/6J mice (7.4 ± 0.2 weeks old) were deeply anaesthetized with isoflurane. The skin over the skull was removed, the skull surrounding the motor cortex contralateral to the dominant forelimb was thinned, the skull over the motor cortex was removed, and self-complementary AAV2/6 Cre-HA was injected to 10 sites, as above. The exposed cortex was covered with a 5mm #1 round glass coverslip (Warner Instruments, Hamden, CT) secured with VetBond (3M, St. Paul, MN). The exposed skull was covered with dental grip cement (Dentsply, York, PA).

[0213] *CS and C3 dorsal column lesion (mice):* Mice were deeply anaesthetized with isoflurane, spinal level CS (or C3) was exposed by laminectomy and the dorsal columns were lesioned at a depth of 1mm with Vannas spring scissors (Fine Science Tools, Foster City, CA). The dorsal musculature was sutured with 4-0 silk sutures and the skin was closed with wound clips. Mice were randomly selected for C3 lesion or C3 sham (laminectomy only) groups.

[0214] *Pyramidotomy:* Mice were deeply anesthetized with ketamine (120mg/kg) and xylazine (12mg/kg), an incision was made and the ventral musculature was pushed aside to expose the pyramids. The dura was opened and the pyramid ipsilateral to the craniotomy was lesioned by 15° microscalpel (Electron Microscopy Sciences, Hatfield, PA) as previously described.

[0215] *CS dorsal column lesion (rats):* Adult female Fischer 344 rats (120-135g) were deeply anaesthetized with 2ml/kg of ketamine cocktail (25 mg per ml ketamine, 1.3mg per ml xylazine and 0.25mg per ml acepromazine). Spinal level CS was exposed by laminectomy, the dura was punctured over the dorsal horn, and the dorsal columns were lesioned with 2 passes of a Scouten wire-knife (David Kopf Instruments, Tujunga, CA). The dorsal musculature was sutured with 4-0 silk sutures and the skin was closed with wound clips. Polyethylene intrathecal catheters (Durect Corp., Cupertino, CA) were pre-filled with either mouse IgG or Ryk monoclonal IgG (clone 25.5.5 generated against the ectodomain of Ryk, amino acid range 90-183, by Johns Hopkins Monoclonal Antibody Core, Baltimore, MD) (1 mg/ml) in artificial cerebrospinal fluid, threaded through magna

cisterna to the cervical spinal cord, secured with 4-0 silk sutures, and attached to model 2004 osmotic minipumps (Durect Corp.) filled with 200 μ l mouse IgG or Ryk IgG. Rats were randomly selected for mouse IgG or Ryk monoclonal IgG treatment. Osmotic minipumps were removed after 28 days. At 16 weeks after CS spinal cord injury, rats were injected bilaterally with 10% wt/vol 10,000 MW biotinylated dextran amine (BDA) in sterile phosphate buffered saline at 20 sites per hemisphere (250nl/site); animals were sacrificed 2 weeks later.

[0216] *Cortical mapping:* Mice were lightly anaesthetized with ketamine (100mg/kg) xylazine (10mg/kg) mixture, still responsive to toe and tail pinch, and maintained during the course of stimulation with ketamine/xylazine mixture. Mice were fixed in a stereotaxic frame (David Kopf Instruments) and a fiber optic cable and cannulae (200 μ m diameter) affixed to the stereotax arm were used to stimulate motor cortex locations relative to bregma that were unobstructed by skull or dental cement (maximum of 165 sites). Stimulation was 3 pulses of 470nm light, 250ms duration at 1 Hz from a single channel LED driver (Thorlabs, Newton, NJ). The intensity of stimulation was increased from 50mA up to 1000mA until movement was detected in 3 consecutive pulses. Sites with no evoked movements at 1000 mA were scored as unresponsive. Only contralateral forelimb movements were scored; occasional, weaker, ipsilateral movements were observed as previously described in rats.

[0217] *Ryk knockout in postnatal day 0 pups:* Pups were injected with 0.5 μ l AAV2/1-synapsin-Cre (Penn Vector Core, Philadelphia, PA) (1.99×10^{13} genome copies/ml) was injected into 2 sites in the motor cortex unilaterally with a 36ga NanoFil needle (World Precision Instruments Inc.). Mice were sacrificed 7 days later and the motor cortex was isolated and homogenized in lysis buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM β -glycerophosphate, 1 mM Na_3VO_4 , 0.5% wt/vol sodium dodecyl sulfate, 1% vol/vol TritonX-100, and complete protease inhibitor cocktail (Roche, Indianapolis, IN). Protein was analyzed by Western blot (40 μ g/well). Antibodies used for Western mouse anti-Ryk (20 μ g/ml) (Johns Hopkins Monoclonal Antibody Core), GAP DH (1:1000) (EMO Millipore, Billerica, MA, catalog #MAB374, RRID:AB_2107445).

[0218] *Sacrifice and Tissue Processing:* Animals were deeply anaesthetized with ketamine cocktail, transcardially perfused with ice-cold PBS followed by 4% wt/vol paraformaldehyde in PBS, and brains and spinal columns were post-fixed overnight at 4°C in 4% wt/vol paraformaldehyde. Tissue was cryoprotected in 30% wt/vol sucrose in PBS. Mouse spinal cords and brainstems, and rat brainstems were sectioned on a cryostat (Leica, Buffalo Grove, IL) at 20µm (sagittal spinal cords, transverse brainstems) and mounted directly on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Rat spinal cords were sectioned sagittally at 40µm thick and collected as free-floating sections. Sections were washed three times with PBS, blocked for one hour in PBS with 0.25% triton-X100 (PBST) and 5% donkey serum, then incubated overnight at 4°C with primary antibodies in PBST plus 5% donkey serum. The next day, sections were washed three times, incubated with Alexa Fluor conjugated secondary antibodies (Life Technologies, Grand Island, NY; Jackson ImmunoResearch, West Grove, PA) for 2.5 hours at room temperature, counterstained with DAPI (1µg/ml) (Sigma-Aldrich, St. Louis, MO) and washed three final times in PBS. Antibodies used for fluorescent immunohistochemistry were: rabbit anti-α-actin (1:1000) (Clontech Laboratories Inc., Mountain View, CA, catalog #632496, RRID:AB_10013483), monoclonal G-A-5 anti-GFAP (1:200) (Sigma-Aldrich, catalog #G3893, RRID:AB_2314539), guinea pig anti-vGlut1 (1:1000) (EMO Millipore, catalog #AB5905, RRID:AB_2301751), and rabbit anti-GFAP (1:750) (Dako, Carpinteria, CA, catalog #Z0334, RRID:AB_10013382).

[0219] *COS-7 Cell Transfection:* COS-7 cells were transfected with pcDNA4-Ryk using FuGene6 (Roche) to express full length Ryk. Cells were either fixed for 30min with ice-cold 4% wt/vol paraformaldehyde in PBS for immunocytochemistry, or lysed with lysis buffer for Western blot.

[0220] *Image acquisition and analysis:* Images were acquired on an inverted Zeiss LSM510 confocal microscope with LSM acquisition software (Carl Zeiss Microscopy, LLC, Thornwood, NY). Image density quantification was done on thresholded images using ImageJ (NIH, Bethesda, MD). An investigator blinded to the experimental group performed all analyses. Axon index is the total thresholded pixels at every 0.411 µm in 8 total serial sagittal spinal cord cryosections spaced 140µm apart for mice, or 0.741 µm in 6 total serial sagittal spinal cord cryosections spaced 280µm apart for rats, divided by

thresholded pixels in transverse sections of the pyramid at the level of the obex. Lesion volume was calculated using the Cavalieri estimator tool in StereoInvestigator (MBF Bioscience, Williston, VT) on every seventh 40 μ m sagittal section. For tdTomato and vGlut1 colocalization, all axons within the gray matter were quantified over a region 210 μ m wide at a distance of 600 μ m rostral to the CS lesion in the 8 total serial sagittal cryosections used for tdTomato quantification. The location of 600 μ m was chosen as it was observed that the highest density of axon collaterals in this region in both groups of animals (Figure 8G) and it was located between the original CS and secondary C3 lesions.

[0221] *Behavioral testing:* All animals were trained on skilled forelimb reach over a period of two weeks prior to bilateral spinal cord injury. Animals were food restricted during training and then for 24 hours prior to weekly training after injury. Animals reached through a vertical slot in the front of an acrylic chamber and over a small gap to retrieve a reward pellet. Mice performed 25 reaches per session for 20mg sucrose reward tablets (TestDiet, St. Louis, MO). Rats performed 50 reaches per session for 45mg sugar pellets (Bio-Serv, Flemington, NJ). Successful retrieval rate was calculated as the number of pellets that were retrieved and eaten divided by the number contacted by the forepaw. Forelimb reach was trained twice weekly by two independent investigators blind to genotype or experimental treatment; the two independent scores were averaged. Mice in the group without weekly training during recovery were only tested twice, at 8 weeks after injury. In addition to forelimb reach, rats were tested once weekly on a grid crossing task, where forelimb footfalls were calculated as a percentage of total forelimb steps in 3 passages over a 60 inch span of 1 inch equidistant wire grid.

[0222] *Statistics:* Statistical tests indicated in main text were performed using JMP 9 software (SAS Institute, Cary, NC). It was previously demonstrated that inhibition of repulsive Wnt signaling results in sprouting and plasticity of descending corticospinal and ascending dorsal column sensory axons after spinal cord injury. In addressing the hypothesis that *Ryk* cKO or *Ryk* monoclonal antibody enhanced axon sprouting, the increases were tested by one-tailed t-test (Figures 8E, 10B, 12I, and 18). In order to test longitudinal behavioral studies with multiple, equally spaced measurements, repeated measures ANOVA were utilized (Figures 8F, 12B, and 21). In testing multiple groups with continuous, parametric data, ANOVA with post-hoc Bonferroni correction was utilized on

appropriate post-hoc comparisons (Figures 11C and 15C). Bivariate correlation was performed to determine the relationship between forelimb function and cortical maps (Figure 15D). Continuous data was tested with parametric tests and data was assumed to be normally distributed, but this was not formally tested.

EXAMPLE 9

Ryk cKO Enhances Recovery of Fine Motor Control After SCI

[0223] Mice underwent two weeks of training for the reaching and grasping task, followed by a CS dorsal column spinal cord lesion: a partial spinal cord injury model leaving the dorsal gray matter, lateral white matter and the entire ventral spinal cord intact (Figures 8A and 8D). Immediately after dorsal column lesion, forelimb reaching and grasping function is lost (Figure 8F). With continued training, the success rate of sugar pellet retrieval recovers due to reconfiguration of neural circuits. It has been shown that the CST undergoes robust collateral sprouting after injury and some of the new sprouts are thought to be responsible for new functional circuits. However, axon sprouting is inhibited by molecular cues that limit axon plasticity.

[0224] Members of the Wnt glycoprotein family are phylogenetically conserved axon guidance molecules that direct the growth along the rostro-caudal axis of both ascending sensory axons and descending CST axons during development. The repulsive Wnt receptor, Ryk, which mediates Wnt repulsion of the developing CST neurons is either not expressed in normal adult motor cortex and the CST neurons or expressed at extremely low levels to be detected by in situ hybridization or immunohistochemistry. Spinal cord injury re-induces expression of Ryk mRNA and protein in the injured CST. By injecting function-blocking antibodies to Ryk and diffusible Wnt inhibitors, it was found that inhibiting Wnt-Ryk signaling enhanced the plasticity of both sensory and motor axons following injury. However, Ryk antibodies or Wnt inhibitors may exert the effects by impacting on the environment, such as the glial cells around the lesion, rather than CST axons *per se*.

[0225] To specifically test the role of Ryk in neurons, a Ryk conditional allele (cKO) was created and these mice were crossed with Ai14 86.Cg mice containing a loxP-flanked stop cassette preventing tdTomato expression, in order to specifically label recombined corticospinal axons after viral transduction (Figures 8B and 8C). Ryk cKO::tdTomato Ai14

mice were injected with an adeno-associated virus (AAV) that expresses Cre recombinase under the control of the cytomegalovirus (CMV) into the primary motor cortex and the enhancement of corticospinal circuit remodeling was assessed (Figure 8A). AAV-Cre was injected to adult motor cortex an average of 2.3 weeks prior to CS dorsal column lesion in order to ensure sufficient time for Cre expression, so that injury would not lead to *Ryk* expression. It was found that *Ryk* deletion in the CST significantly enhanced recovery of skilled forelimb function as assessed by forelimb reach over a period of 12 weeks (Repeated measures ANOVA $P < 0.005$, Figures 8F and 16). The effects of *Ryk* deletion were observed early on, with a trend towards better performance at early testing sessions. This was consistently observed and may be due to reduced retraction of axons and collaterals as previously demonstrated at 5 weeks post-injury in animals infused with *Ryk* antibodies. Following *Ryk* conditional deletion, mice recovered to $81 \pm 7\%$ of peak pre-injury success rates, compared to only $60 \pm 5\%$ in control mice.

EXAMPLE 10

Ryk cKO Enhances CST Collateral Sprouting After SCI

[0226] To begin to address the mechanisms underlying improved functional recovery in *Ryk* cKO mice, CST collaterals and synapse density were analyzed along collateral sprouts in the cervical spinal cord. It was found that conditional *Ryk* deletion did not significantly reduce axonal die-back of the injured CST (one-tailed t-test $P = 0.12$), but did lead to significantly increased numbers of CST collaterals within the spinal gray matter, both rostral and caudal to the site of CS injury, at 12 weeks post-injury (one-tailed t-test $P < 0.05$, Figure 9E). In addition to a greater number of axon collaterals, mice with *Ryk* conditionally deleted from cortical pyramidal neurons exhibited pre-synaptic vesicular glutamate transporter 1 (vGlut1)-labeled puncta on identified corticospinal axon collaterals at 600 μm rostral to the injury site, suggesting enhanced functional connectivity following *Ryk* conditional deletion (Figures 10C and 10D).

[0227] The majority of CST axons reside within the dorsal columns and are lesioned by the CS dorsal column injury. A sparse, minor, component of CST axons descend down the spinal cord within the lateral columns (lateral CST), which remained intact in the CS lesion paradigm and may contribute to functional recovery (Figure 8D). To test this, the

distribution of axon collaterals were first characterized in the spinal cord both rostral and caudal to the CS lesion. It was observed that an increase in axon collateral density in *Ryk* conditional deleted mice throughout the gray matter, with the highest density more medial, in close proximity to the principal dorsal column corticospinal tract (Figures 10A and 10B), suggesting that increased branches may come from the dorsal column CST following *Ryk* deletion. No axons were present within the dorsal column CST caudal to CS lesion in any of the mice studied.

[0228] To further assess the contribution of the dorsal column CST to behavioral recovery, a second dorsal column lesion was performed in these animals 12 weeks after CS injury at the C3 level, 1.15 ± 0.07 mm rostral to the original CS injury (Figures 11A and 11B). The lateral CST is again spared in this lesion. After a one-week delay to allow the mice to recover from the immediate hyporeflexic stage of spinal shock, behavioral testing began. The secondary injury at C3 ablated the enhanced functional recovery that was observed in the *Ryk* conditional deletion mice, leaving only the modest levels of partial recovery achieved by control mice (Figure 11C). This suggests that the axon sprouts from the dorsal column CST are indeed responsible for the enhanced functional recovery in the *Ryk* conditional knockout. This also suggests that the spared lateral CST axons may provide only a minor contribution to a basal level functional recovery independent of the dorsal column corticospinal tract. Quantification of axon distribution at 2 weeks after C3 lesion confirmed that the secondary C3 injury eliminated a majority of axon collaterals between the two injury sites, thereby disrupting the remodeled corticospinal circuit (Figures 11D-11F).

EXAMPLE 11

Monoclonal *Ryk* Antibody Promotes Functional Recovery

[0229] To test whether inhibition of the Wnt-*Ryk* signaling axis in the injured spinal cord after spinal cord injury is sufficient to increase CST remodeling and enhance behavioral recovery, a new monoclonal *Ryk* antibody was generated using half of the Wnt binding domain (amino acid range 90-183) as the antigen and infused into adult rats immediately following spinal cord injury (Figures 12A, 17A and 17B). Function-blocking polyclonal antibodies were previously generated using the same region. Following a CS

dorsal column wire-knife lesion, Ryk antibody infusion via osmotic minipump for 4 weeks promoted recovery of skilled forelimb function in the forelimb reach task with all rats recovering to peak pre-injury levels, as compared to only half of rats infused with IgG control (Figure 12B). Ryk antibody did not enhance recovery in the grid crossing locomotor task since rats exhibited similar levels of forelimb stepping impairment irrespective of treatment group (Figure 12C). Corticospinal axons were labeled with biotinylated dextran amine (BDA) injections into motor cortex. Consistent with the conditional Ryk deletion prior to injury, it was found that the Ryk monoclonal antibody infusion at the time of injury resulted in an increase in corticospinal axon collaterals both rostral and caudal to the level of injury (one-tailed t-test $P < 0.05$, Figures 12E-12I and 18). The extent of increase of collateral sprouts after Ryk antibody infusion in rats was similar to that observed in CST axons lacking Ryk expression in mice (Figures 9E and 12I). These results also suggest that Ryk signaling is a feasible therapeutic target, since functional recovery can be promoted by blocking its function after spinal cord injury.

EXAMPLE 12

Cortical Map Re-organization during Recovery

[0230] In order to address the circuit mechanisms with which the primary motor cortex regains control over the remodeled spinal cord, an optogenetics approach was used to monitor cortical output. Cortical motor maps have been studied using intracortical electrical stimulation in rodents and primates, as well as transcranial magnetic stimulation in humans. Recent advances in optogenetic tools allow for the stimulation of specific neural populations in a minimally invasive manner. Specifically, the expression of the light-activated, non-selective, cation channel channelrhodopsin-2 (ChR2) under control of the Thy1 promoter (*Thy1-ChR2*) allows for selective activation of layer V projection neurons within the motor cortex. Unilateral craniotomies were performed on *Thy1-ChR2* mice contralateral to the dominant forelimb in order to investigate motor map changes through repeated optogenetic mapping of evoked motor output after injury (Figure 19). Following craniotomy, AAV-Cre was injected unilaterally into the motor cortex contralateral to the dominant forelimb as contralateral cortex exhibits motor plasticity in response to forelimb training; and CST collateral plasticity, which supports functional recovery, was specifically

induced in CST axons with *Ryk* deletion. Motor map output was assessed by observing evoked, contralateral motor outputs in sedated mice.

[0231] Massive remapping of cortical motor output was observed immediately after spinal cord injury in the mouse. Acutely (3 days) after CS injury, the total area of motor representations for limb muscles at or below the level of lesion was reduced or eliminated (Figure 12C). Conversely, motor maps expanded for muscle groups with motor neurons above the injury site, most notably elbow flexion mediated by biceps brachii and brachialis (Figures 13B, 14E, and 20C). Over the next two months following spinal cord lesion, cortical maps underwent gradual changes with continued forelimb reaching and grasping training (Figure 20). Behavioral recovery after CS spinal cord injury plateaued between 4 and 8 weeks post-injury (Figures 8F and 21), with a median time to reach 90% of peak, post-injury, performance of either 6 weeks in control mice or 5 weeks in *Ryk* conditional deletion. Therefore, cortical motor maps were examined before and after peak recovery at 4 weeks and 8 weeks, respectively, post-injury. At 4 weeks post-injury, significant differences were observed in the proportion of motor cortex allocated to forelimb extensor (biceps) or forelimb flexor (triceps) activation, with *Ryk* deleted mice exhibiting larger flexor motor maps at the expense of reduced extensor maps ($P < 0.05$ one-tailed t-test, Figures 14E and 14F). Expansion of elbow extensor areas at 4 weeks into regions originally occupied by the flexor was inversely correlated with behavioral recovery ($n = 21$ mice: 10 (control), 11 (*Ryk* cKO), Spearman's $\rho = -0.5766$, $P = 0.0062$). By 8 weeks post-injury, *Ryk* deleted mice exhibited a similar pattern of extensor and flexor motor maps as controls, however the total area occupied by all elbow movements (flexor and extensor) was significantly larger in *Ryk* deleted mice (one-tailed t-test, $P = 0.0480$ $t(14) = 1.79$). Additionally, at 8 weeks post-injury, wrist flexor representations returned to (or exceeded) maximal pre-injury size in 64% of *Ryk* deleted mice compared to 10% of control mice (Wilcoxon rank sum $P = 0.0136$ $\chi^2 = 6.086$, Figure 20C). Recovery of wrist flexor control correlated with improvement of forelimb reach performance at 8 weeks post-injury (Spearman's $\rho = 0.4555$, $P = 0.0380$). Over the course of the experiment, there was a strong correlation of wrist movement and skilled forelimb reach performance, regardless of injury or genotype (Pearson's $\rho = 0.665$, $P < 0.0001$, Figure 15D).

[0232] In order to further characterize the remapped cortical output, mice were subjected to a second dorsal column lesion at C3, rostral to the level of extensor motor units, at 8 weeks after CS injury (Figure 11B). It was found that the C3 injury significantly reduced flexor motor maps in all mice but, surprisingly, had little effect on the recovered extensor motor maps, suggesting the flexor control is routed from connections rostral to C3 or from the lateral corticospinal tract (Figure 13D). Importantly, a subsequent unilateral pyramidotomy abolished unilateral forelimb responses to cortical stimulation and also the ability of mice to perform the forelimb reach task (Figures 13D and 21). Although plasticity of other supraspinal pathways, such as the rubrospinal tract or reticulospinal tract may also contribute to functional recovery, the effects of unilateral pyramidotomy suggest that a direct connection between the primary motor cortex with the cervical spinal cord is essential for the recovery of voluntary skilled forelimb movement.

EXAMPLE 13

Cortical Re-organization Requires Rehabilitative Training

[0233] The repeated testing of skilled forelimb reach over the course of the experiment essentially constitutes a rehabilitative training paradigm that can promote motor recovery from spinal cord injury and cortical reorganization. In order to determine if the induced axonal plasticity mediated by Ryk deletion alone was required to promote functional recovery, the recovery of skilled forelimb reach was tested at 8 weeks after CS injury in another cohort of mice that did not undergo weekly behavioral testing after injury (Figure 15A). Mice that did not undergo weekly behavioral testing displayed only limited skilled forelimb recovery with performance similar to that of mice tested at one week after injury (Figures 15C and 8F). In the absence of weekly testing, refinement of cortical motor maps was also impaired, irrespective of Ryk conditional deletion (Figures 15B and 22).

[0234] References

[0235] 1. Saxena, et al. (2007) Mechanisms of axon degeneration: from development to disease. (Translated from eng) Progress in neurobiology 83(3):174-191 (in eng).

- [0236] 2. Wang, et al. (2012) Axon degeneration: molecular mechanisms of a self-destruction pathway. (Translated from eng) *The Journal of cell biology* 196(1):7-18 (in eng).
- [0237] 3. Yan, et al. (2010) Axon degeneration: Mechanisms and implications of a distinct program from cell death. (Translated from eng) *Neurochemistry international* 56(4):529-534 (in eng).
- [0238] 4. Schmidt, et al. (2009) Axon guidance proteins: novel therapeutic targets for ALS? (Translated from eng) *Prog Neurobiol* 88(4):286-301 (in eng).
- [0239] 5. Van Hoecke, et al. (EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. (Translated from eng) *Nat Med* 18(9):1418-1422 (in eng).
- [0240] 6. Tury, et al. (Altered expression of atypical PKC and Ryk in the spinal cord of a mouse model of amyotrophic lateral sclerosis. (Translated from Eng) *Dev Neurobiol* (in Eng).
- [0241] 7. Shi, et al. (2003) Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. (Translated from eng) *Cell* 112(1):63-75 (in eng).
- [0242] 8. Nishimura, et al. (2004) Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. (Translated from eng) *Nature cell biology* 6(4):328-334 (in eng).
- [0243] 9. Chen, et al. (2006) Microtubule affinity-regulating kinase 2 functions downstream of the PAR-3/PAR-6/atypical PKC complex in regulating hippocampal neuronal polarity. (Translated from eng) *Proceedings of the National Academy of Sciences of the United States of America* 103(22):8534-8539 (in eng).
- [0244] 10. Parker, et al. (2013) Competing molecular interactions of aPKC isoforms regulate neuronal polarity. (Translated from eng) *Proceedings of the National Academy of Sciences of the United States of America* 110(35):14450-14455 (in eng).

- [0245] 11. Zhang, et al. (2007) Dishevelled promotes axon differentiation by regulating atypical protein kinase C. (Translated from eng) Nature cell biology 9(7):743-754 (in eng).
- [0246] 12. Mori, et al. (2009) An essential role of the aPKC-Aurora A-NDEL1 pathway in neurite elongation by modulation of microtubule dynamics. (Translated from eng) Nature cell biology 11(9):1057-1068 (in eng).
- [0247] 13. Parker, et al. (Competing molecular interactions of aPKC isoforms regulate neuronal polarity. (Translated from eng) Proc Natl Acad Sci U S A 110(35):14450-14455 (in eng).
- [0248] 14. Wolf, et al. (2008) Phosphatidylinositol-3-kinase-atypical protein kinase C signaling is required for Wnt attraction and anterior-posterior axon guidance. (Translated from eng) The Journal of neuroscience : the official journal of the Society for Neuroscience 28(13):3456-3467 (in eng).
- [0249] 15. Onishi, et al. (Antagonistic Functions of Dishevelleds Regulate Frizzled3 Endocytosis via Filopodia Tips in Wnt-Mediated Growth Cone Guidance. (Translated from eng) J Neurosci 33(49):19071-19085 (in eng).
- [0250] 16. Wang, et al. (1999) Atypical PKC zeta is activated by ceramide, resulting in coactivation of NF-kappaB/JNK kinase and cell survival. (Translated from eng) Journal of neuroscience research 55(3):293-302 (in eng).
- [0251] 17. Wooten, et al. (1999) Overexpression of atypical PKC in PC12 cells enhances NGF-responsiveness and survival through an NFkappaB dependent pathway. (Translated from eng) Cell death and differentiation 6(8):753-764 (in eng).
- [0252] 18. Xie, et al. (2000) Protein kinase C iota protects neural cells against apoptosis induced by amyloid beta-peptide. (Translated from eng) Brain research. Molecular brain research 82(1-2):107-113 (in eng).
- [0253] 19. Huang, et al. (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in

PC12 cells. (Translated from eng) The Journal of biological chemistry 276(17):13838-13846 (in eng).

[0254] 20. Kim, et al. (2007) Polarity proteins PAR6 and aPKC regulate cell death through GSK-3beta in 3D epithelial morphogenesis. (Translated from eng) Journal of cell science 120(Pt 14):2309-2317 (in eng).

[0255] 21. Joung, et al. (2005) p62 modulates Akt activity via association with PKCzeta in neuronal survival and differentiation. (Translated from eng) Biochemical and biophysical research communications 334(2):654-660 (in eng).

[0256] 22. Xin, et al. (2007) Protein kinase Czeta abrogates the proapoptotic function of Bax through phosphorylation. (Translated from eng) The Journal of biological chemistry 282(29):21268-21277 (in eng).

[0257] 23. Reyland ME (2009) Protein kinase C isoforms: Multi-functional regulators of cell life and death. (Translated from eng) Front Biosci (Landmark Ed) 14:2386-2399 (in eng).

[0258] 24. Liu, et al. (2005) Ryk-mediated Wnt repulsion regulates posterior-directed growth of corticospinal tract. (Translated from eng) Nature neuroscience 8(9):1151-1159 (in eng).

[0259] 25. Schmitt, et al. (2006) Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. (Translated from eng) Nature 439(7072):31-37 (in eng).

[0260] 26. Keeble, et al. (2006) Ryk: a novel Wnt receptor regulating axon pathfinding. (Translated from eng) The international journal of biochemistry & cell biology 38(12):2011-2017 (in eng).

[0261] 27. Keeble, et al. (2006) The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. (Translated from eng) The Journal of neuroscience : the official journal of the Society for Neuroscience 26(21):5840-5848 (in eng).

- [0262] 28. Gonzalez, et al. (2013) The ryk receptor is expressed in glial and fibronectin-expressing cells after spinal cord injury. (Translated from eng) Journal of neurotrauma 30(10):806-817 (in eng).
- [0263] 29. Hollis ER, 2nd & Zou Y (2012) Reinduced Wnt signaling limits regenerative potential of sensory axons in the spinal cord following conditioning lesion. (Translated from eng) Proceedings of the National Academy of Sciences of the United States of America 109(36):14663-14668 (in eng).
- [0264] 30. Hollis ER, 2nd & Zou Y (2012) Expression of the Wnt signaling system in central nervous system axon guidance and regeneration. (Translated from eng) Frontiers in molecular neuroscience 5:5 (in eng).
- [0265] 31. Fradkin, et al. (2010) Ryks: new partners for Wnts in the developing and regenerating nervous system. (Translated from eng) Trends in neurosciences 33(2):84-92 (in eng).
- [0266] 32. Liu, et al. (2008) Repulsive Wnt signaling inhibits axon regeneration after CNS injury. (Translated from eng) The Journal of neuroscience : the official journal of the Society for Neuroscience 28(33):8376-8382 (in eng).
- [0267] 33. Miyashita, et al. (2009) Wnt-Ryk signaling mediates axon growth inhibition and limits functional recovery after spinal cord injury. (Translated from eng) Journal of neurotrauma 26(7):955-964 (in eng).
- [0268] 34. Guenther, et al. (2012) Increased atypical PKC expression and activity in the phrenic motor nucleus following cervical spinal injury. (Translated from eng) Experimental neurology 234(2):513-520 (in eng).
- [0269] 35. Luo L & O'Leary DD (2005) Axon retraction and degeneration in development and disease. (Translated from eng) Annual review of neuroscience 28:127-156 (in eng).

- [0270] 36. Watts, et al. (2003) Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. (Translated from eng) *Neuron* 38(6):871-885 (in eng).
- [0271] 37. Zhai, et al. (2003) Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. (Translated from eng) *Neuron* 39(2):217-225 (in eng).
- [0272] 38. Xiao, et al. (2006) Insights into the mechanism of microtubule stabilization by Taxol. (Translated from eng) *Proceedings of the National Academy of Sciences of the United States of America* 103(27):10166-10173 (in eng).
- [0273] 39. Drewes, et al. (1997) MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. (Translated from eng) *Cell* 89(2):297-308 (in eng).
- [0274] 40. Hurov, et al. (2004) Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity. (Translated from eng) *Current biology : CB* 14(8):736-741 (in eng).
- [0275] 41. Matenia D & Mandelkow EM (2009) The tau of MARK: a polarized view of the cytoskeleton. (Translated from eng) *Trends in biochemical sciences* 34(7):332-342 (in eng).
- [0276] 42. Shen HM & Liu ZG (2006) JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. (Translated from eng) *Free radical biology & medicine* 40(6):928-939 (in eng).
- [0277] 43. Manning AM & Davis RJ (2003) Targeting JNK for therapeutic benefit: from junk to gold? (Translated from eng) *Nature reviews. Drug discovery* 2(7):554-565 (in eng).
- [0278] 44. Yoshimura K, et al. (2011) c-Jun N-terminal kinase induces axonal degeneration and limits motor recovery after spinal cord injury in mice. (Translated from eng) *Neuroscience research* 71(3):266-277 (in eng).

- [0279] 45. Li, et al. (2004) JNK-dependent phosphorylation of c-Jun on serine 63 mediates nitric oxide-induced apoptosis of neuroblastoma cells. (Translated from eng) The Journal of biological chemistry 279(6):4058-4065 (in eng).
- [0280] 46. Onishi K, et al. (2013) Antagonistic Functions of Dishevelleds Regulate Frizzled3 Endocytosis via Filopodia Tips in Wnt-Mediated Growth Cone Guidance. (Translated from eng) The Journal of neuroscience : the official journal of the Society for Neuroscience 33(49):19071-19085 (in eng).
- [0281] 47. Tury, et al. (2013) Altered expression of atypical PKC and Ryk in the spinal cord of a mouse model of amyotrophic lateral sclerosis. (Translated from Eng) Developmental neurobiology (in Eng).
- [0282] 48. Kamitori. Et al. (1999) Expression of receptor tyrosine kinase RYK in developing rat central nervous system. (Translated from eng) Brain research. Developmental brain research 114(1):149-160 (in eng).
- [0283] 49. Lyu, et al. (2008) Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. (Translated from eng) Developmental cell 15(5):773-780 (in eng).
- [0284] 50. Halford MM, et al. (2000) Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. (Translated from eng) Nature genetics 25(4):414-418 (in eng).
- [0285] 51. Wang, et al. (2002) Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. (Translated from eng) The Journal of neuroscience : the official journal of the Society for Neuroscience 22(19):8563-8573 (in eng).
- [0286] 52. Hua, et al. (Frizzled3 controls axonal development in distinct populations of cranial and spinal motor neurons. (Translated from eng) Elife 2(0):e01482 (in eng).
- [0287] 53. Shafer, et al. (Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance. (Translated from eng) Dev Cell 20(2):177-191 (in eng).

[0288] 54. Macheda ML, et al. (The Wnt Receptor Ryk Plays a Role in Mammalian Planar Cell Polarity Signaling. (Translated from Eng) J Biol Chem (in Eng).

[0289] 55. Andre P, et al. (The Wnt coreceptor Ryk regulates Wnt/planar cell polarity by modulating the degradation of the core planar cell polarity component Vangl2. (Translated from Eng) J Biol Chem (in Eng).

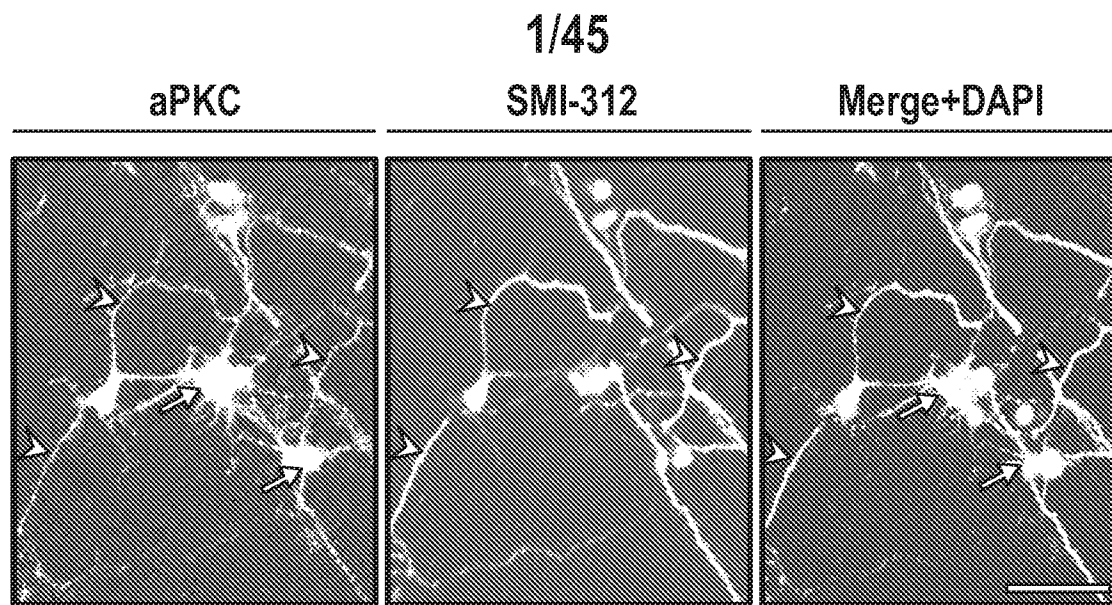
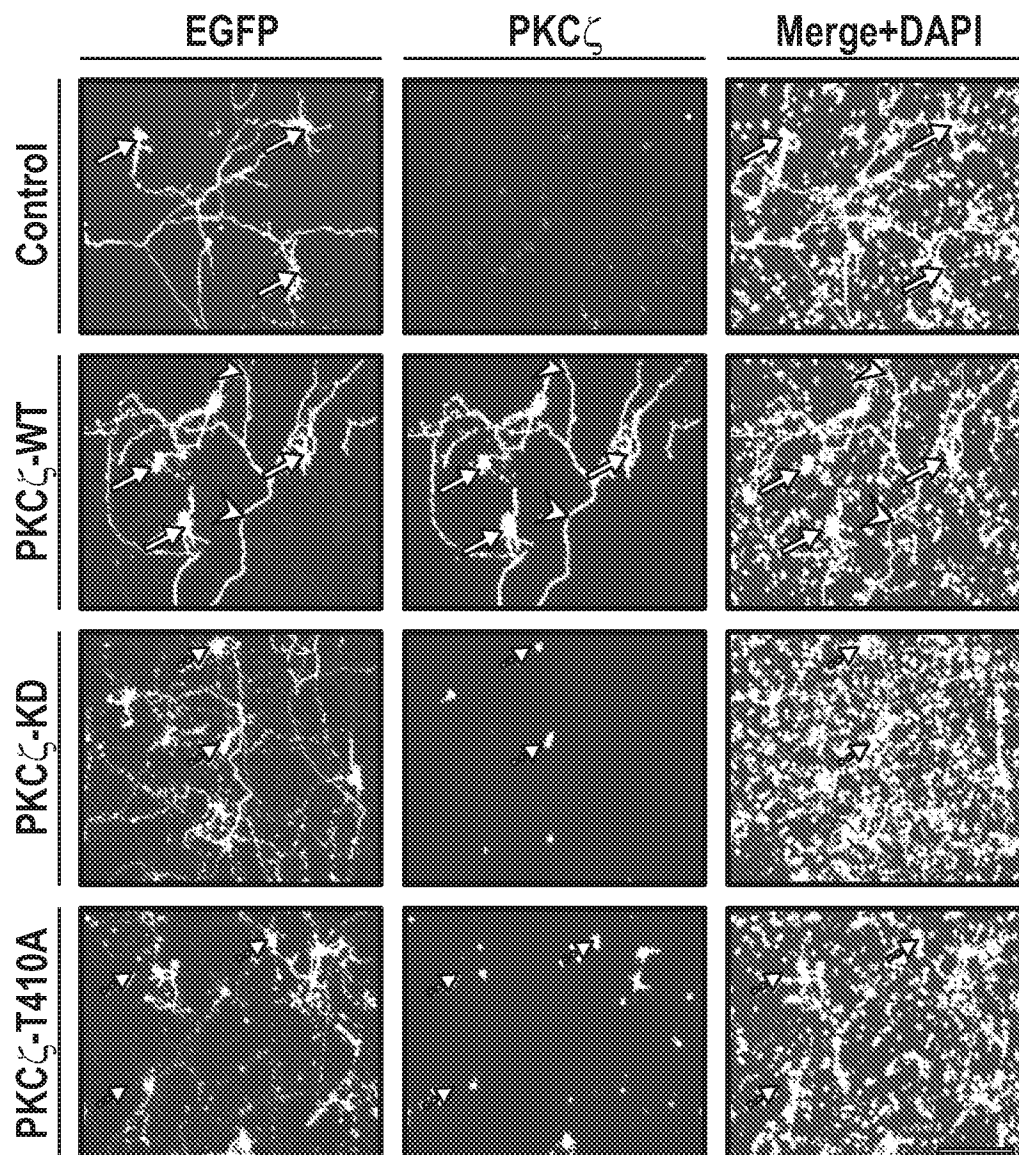
[0290] 56. Romanelli, et al. (1999) p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex. (Translated from eng) Molecular and cellular biology 19(4):2921-2928 (in eng).

[0291] 57. Mairet-Coello G, et al. (2013) The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation. (Translated from eng) Neuron 78(1):94-108 (in eng).

[0292] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. An isolated anti-Ryk antibody or antibody fragment that specifically binds to a WnT binding domain on Ryk or specifically binds to an epitope within a region of the ectodomain of Ryk, amino-acids 90-183 of Ryk, the antibody or antibody fragment comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 8 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 4.
2. The isolated anti-Ryk antibody or antibody fragment of claim 1, wherein the antibody or antibody fragment inhibits or reduces Ryk binding to Wnt.
3. The isolated anti-Ryk antibody or antibody fragment of claim 1, wherein the antibody or antibody fragment specifically binds to an epitope within amino acid residues 90-183 of Wnt.
4. A pharmaceutical composition comprising an effective amount of the antibody or antibody fragment according to claim 1, and a pharmaceutically acceptable carrier or excipient.
5. An immunoconjugate comprising the isolated anti-Ryk antibody or antibody fragment of claim 1, linked to a therapeutic agent.
6. The immunoconjugate of claim 5, wherein the therapeutic agent is a cytotoxin or a radioactive isotope.
7. A bispecific molecule comprising the isolated anti-Ryk antibody or antibody fragment of claim 1, linked to a second functional moiety having a different binding specificity than the isolated anti-Ryk antibody or antibody fragment of claim 1.

**FIG. 1A****FIG. 1B**

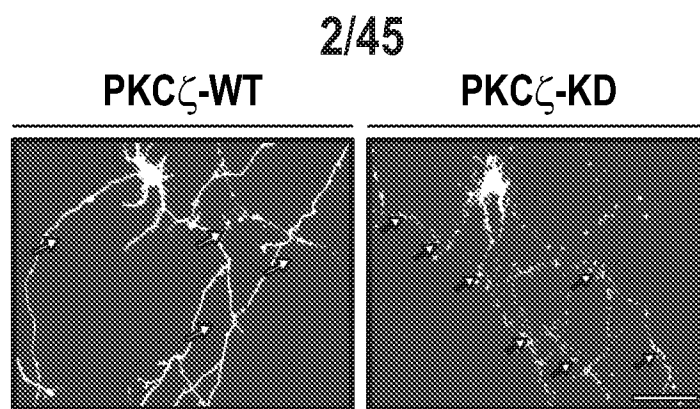


FIG. 1C

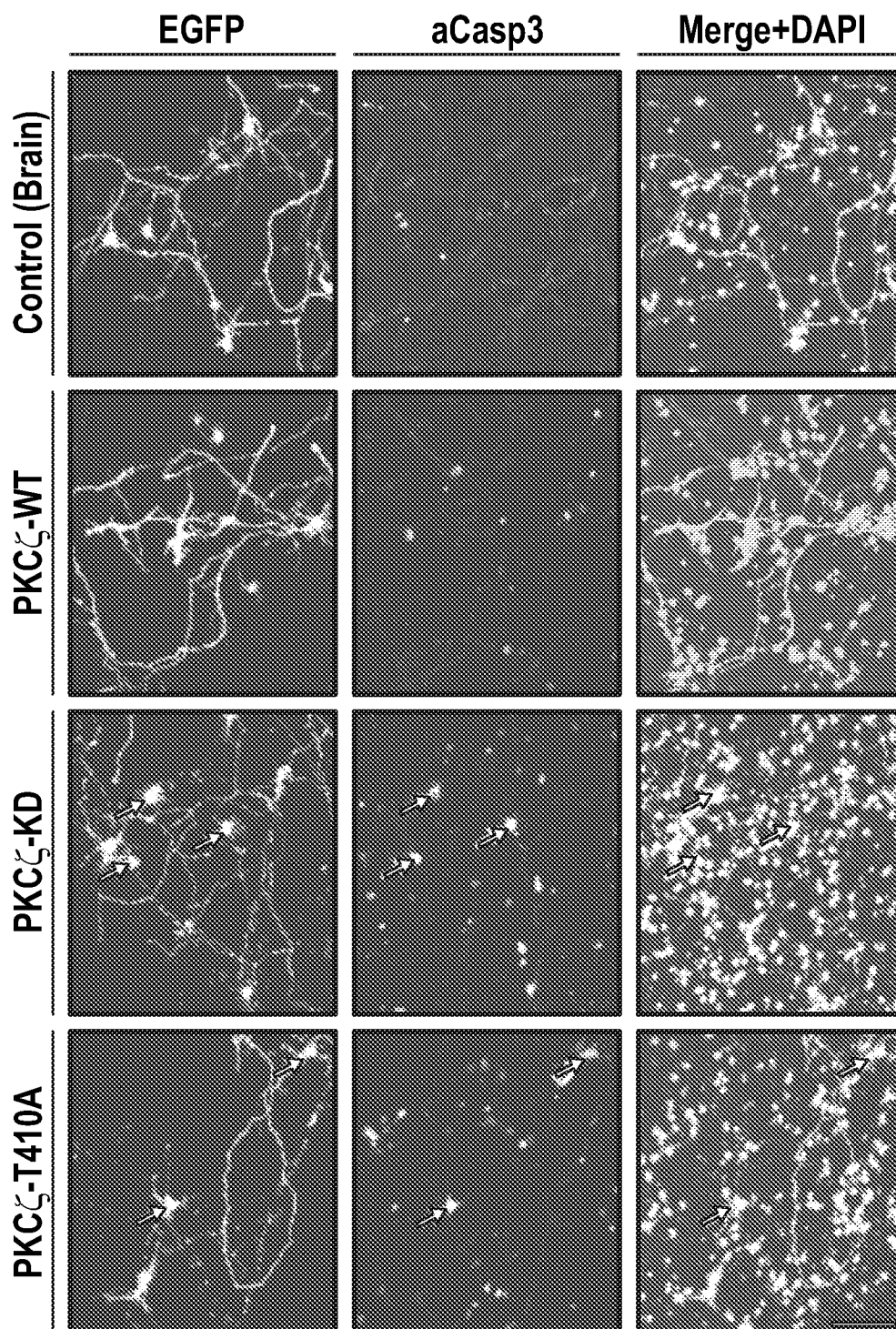


FIG. 1D

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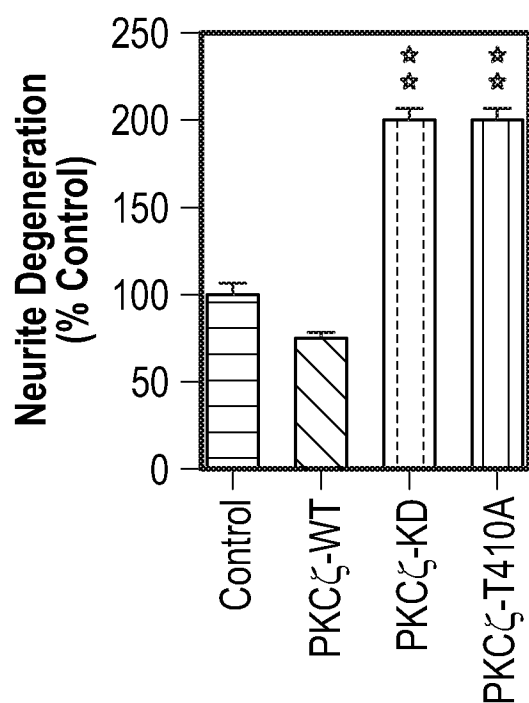


FIG. 1E

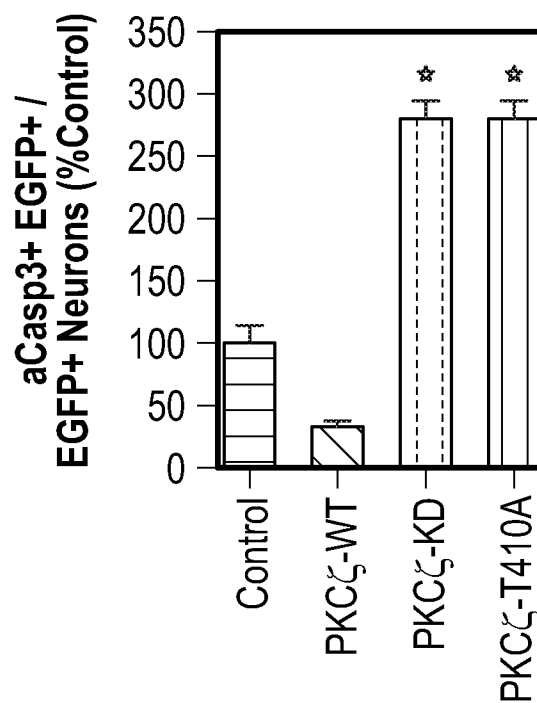


FIG. 1F

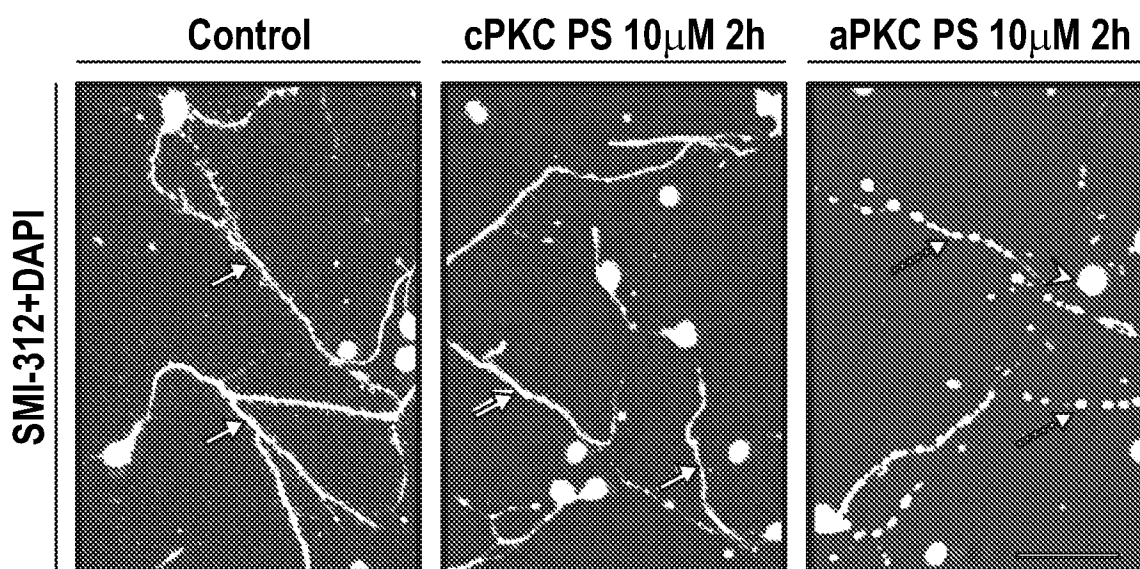


FIG. 2A

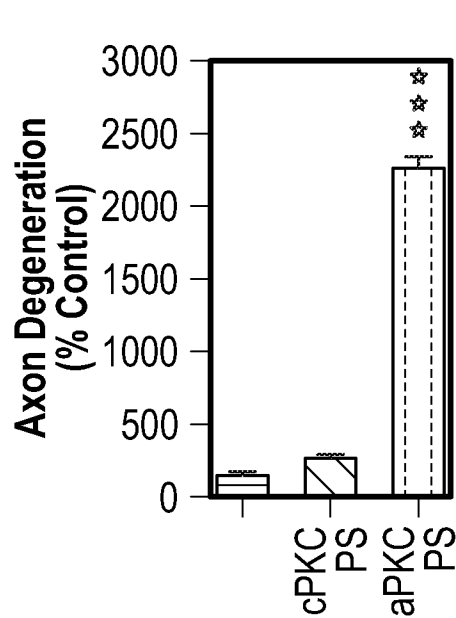


FIG. 2B

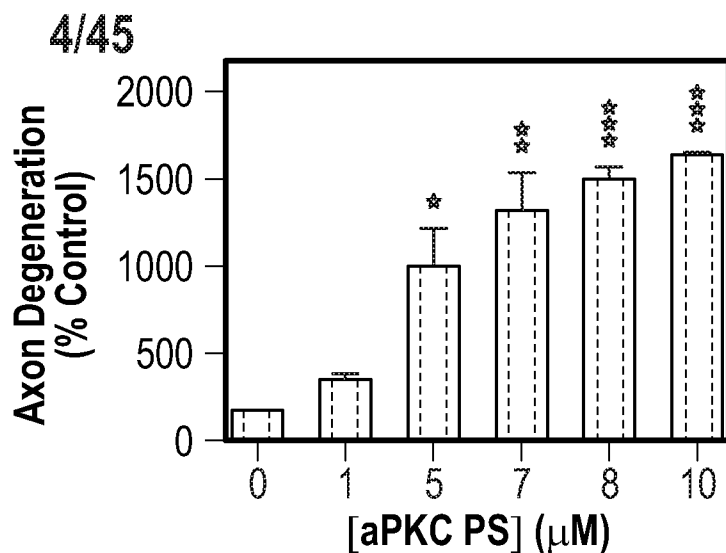


FIG. 2C

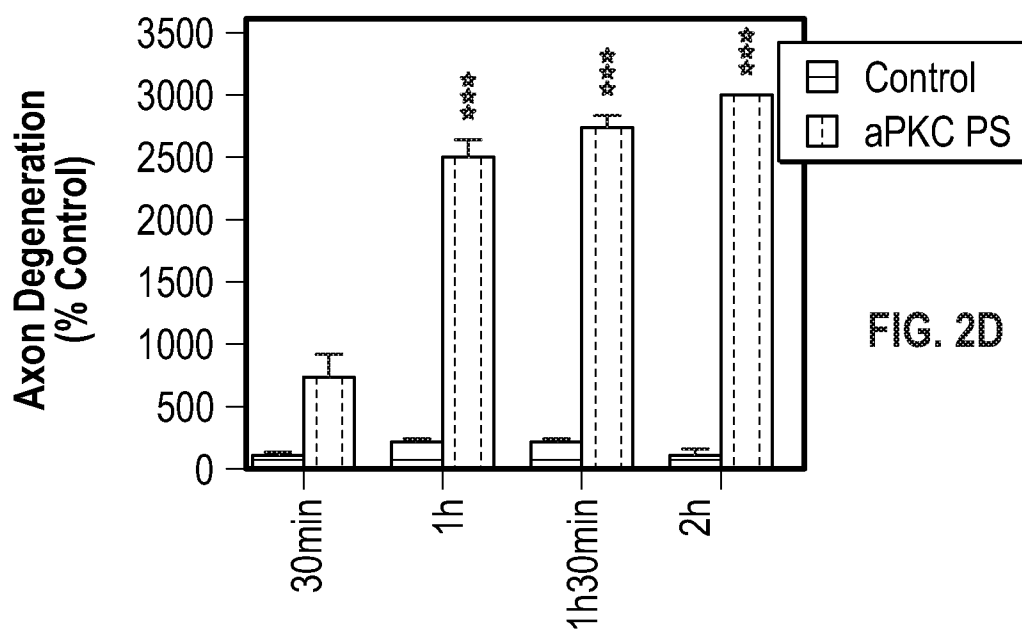


FIG. 2D

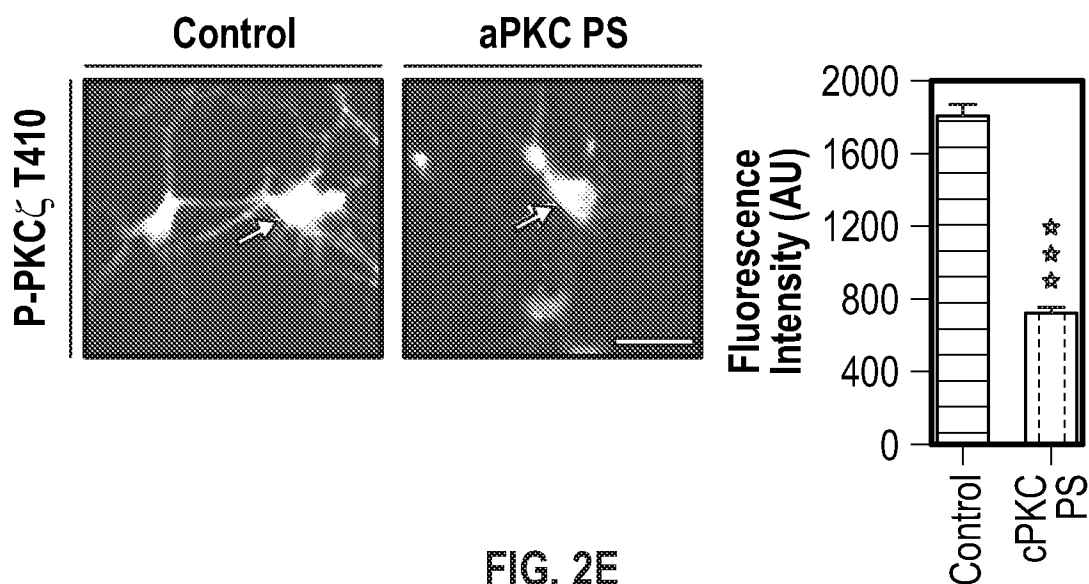
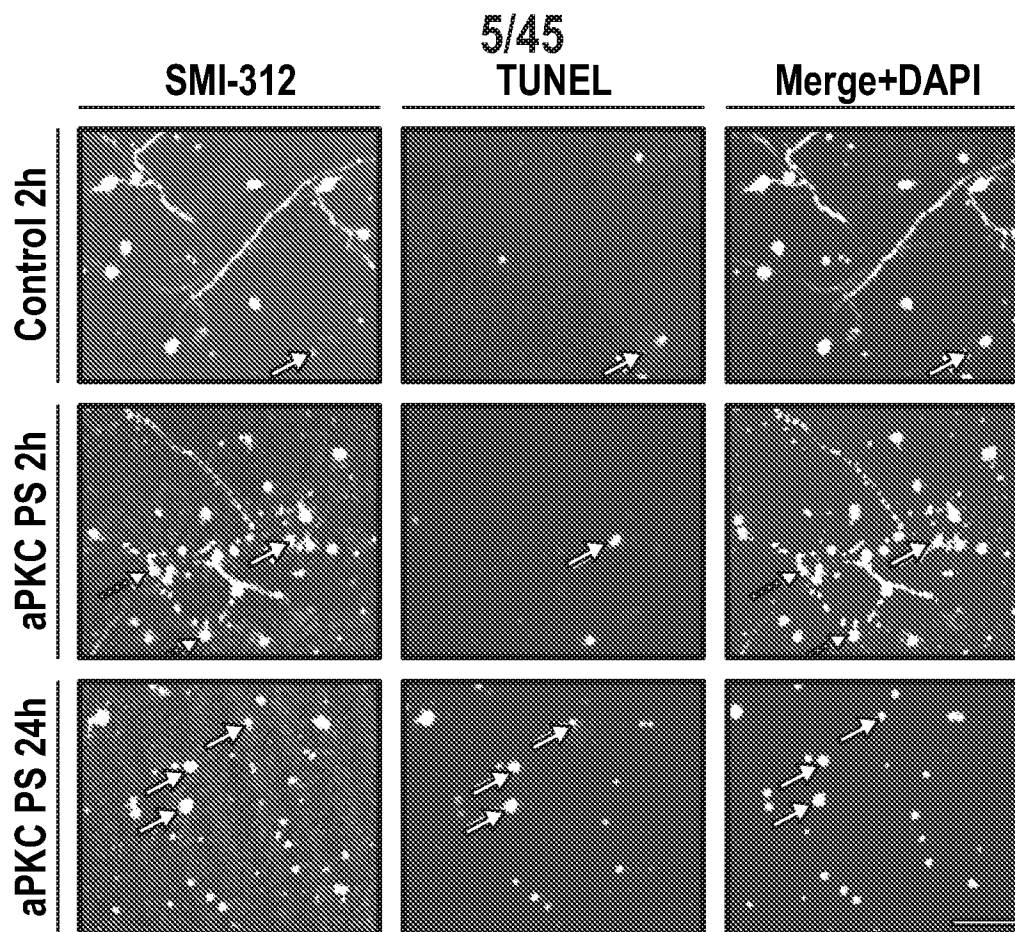
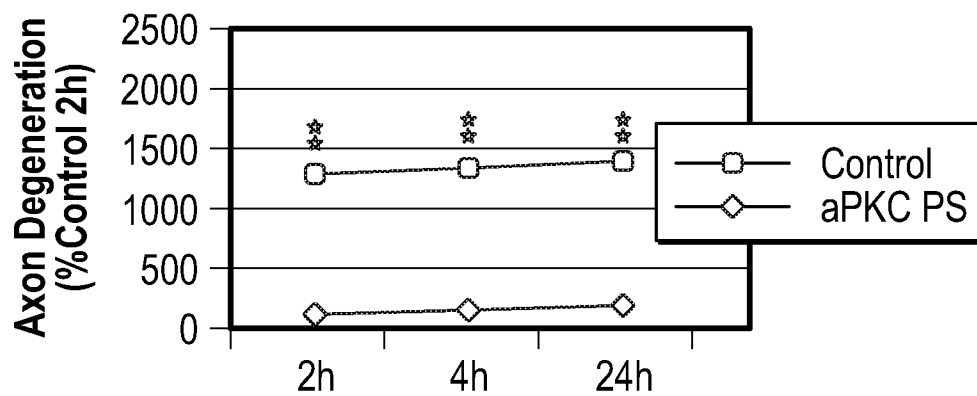
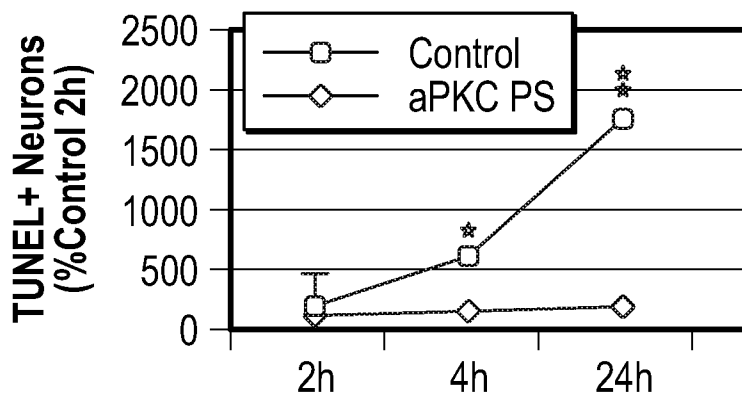


FIG. 2E

**FIG. 2F****FIG. 2G****FIG. 2H**

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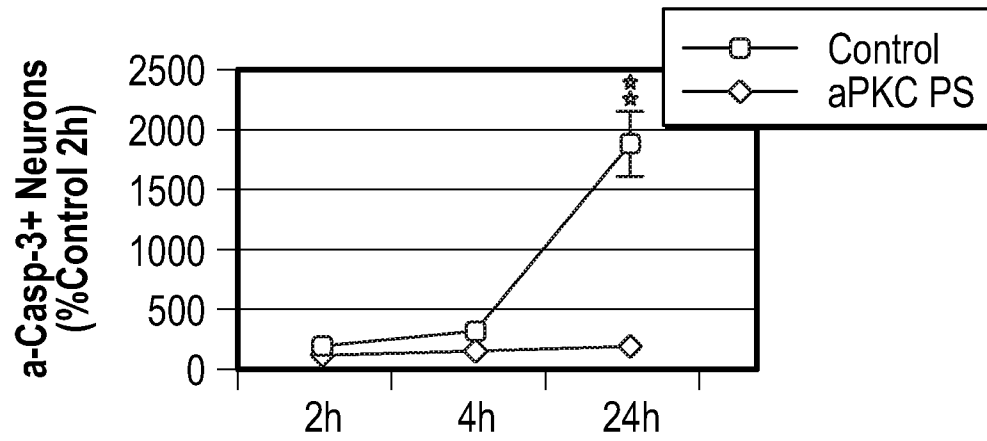


FIG. 2I

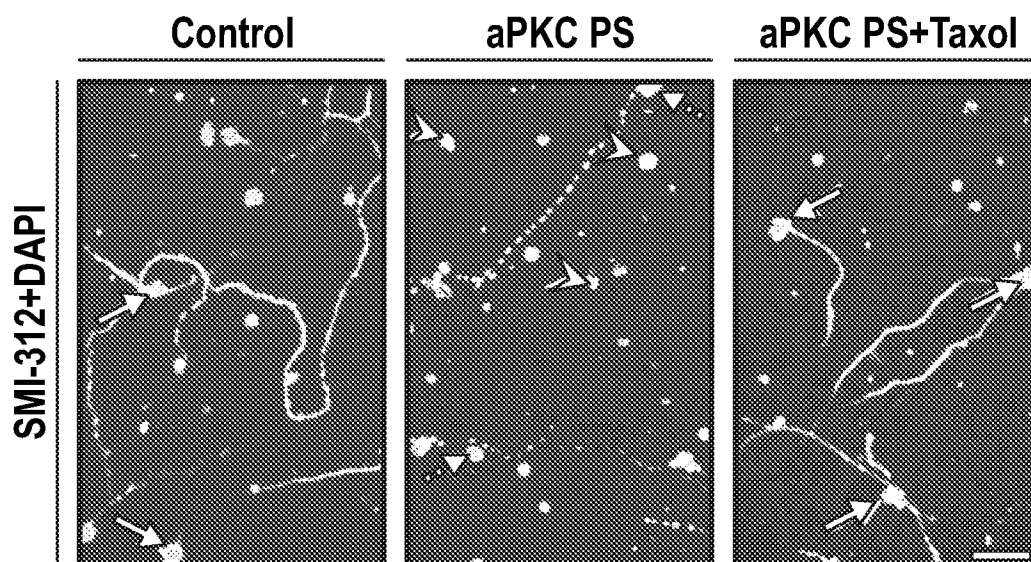


FIG. 3A

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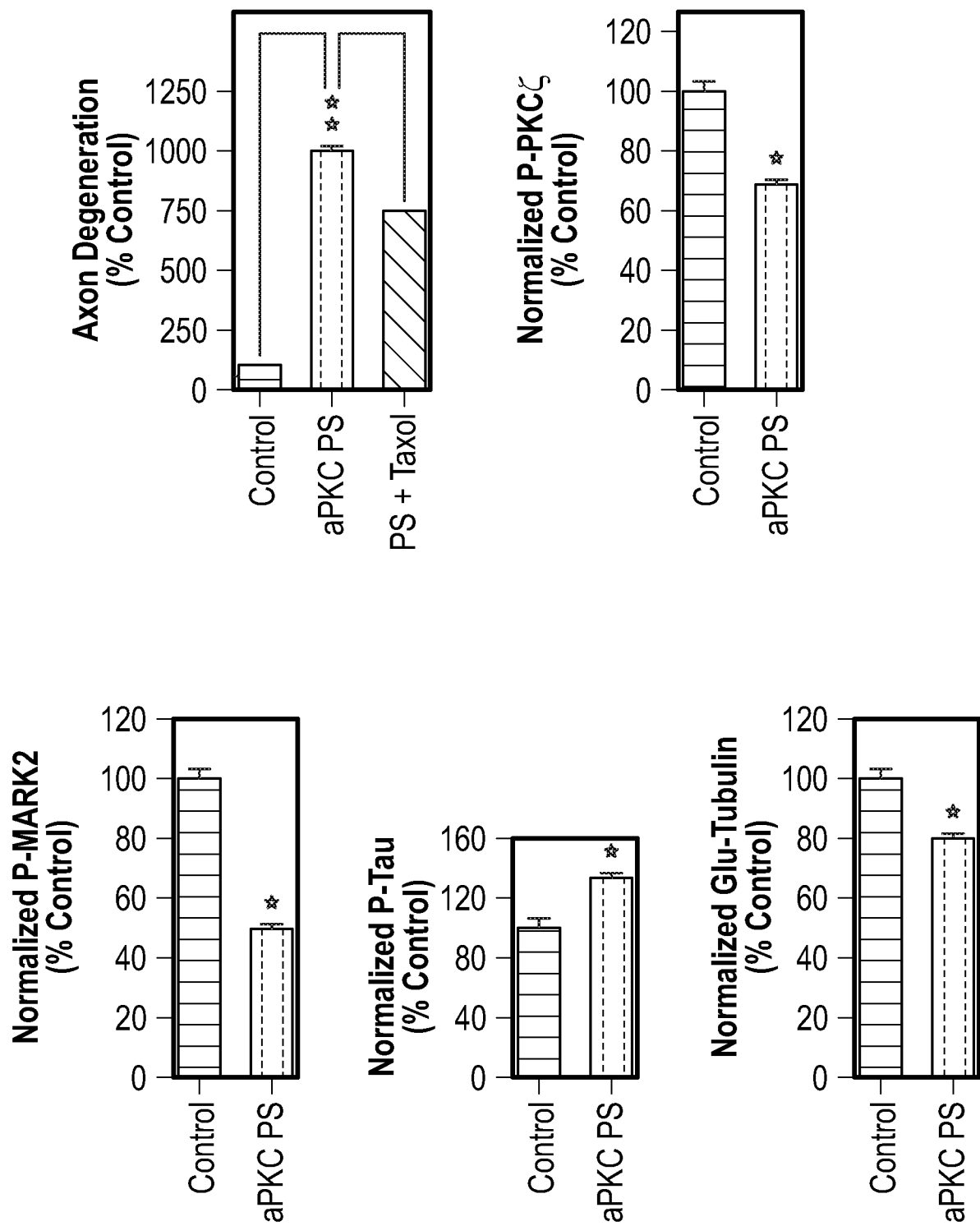


FIG. 3B

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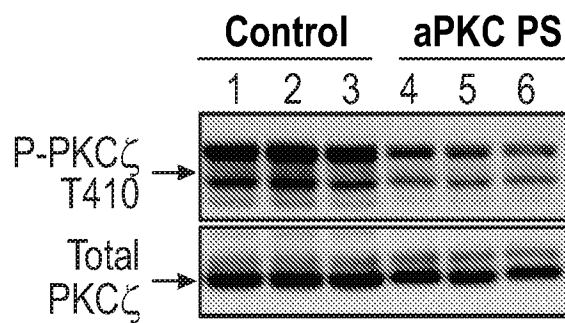


FIG. 3C

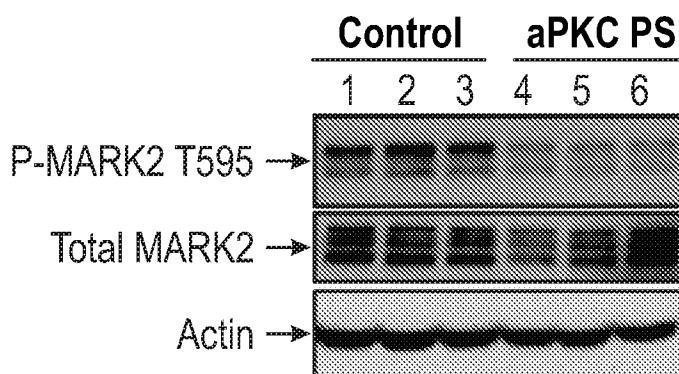


FIG. 3D

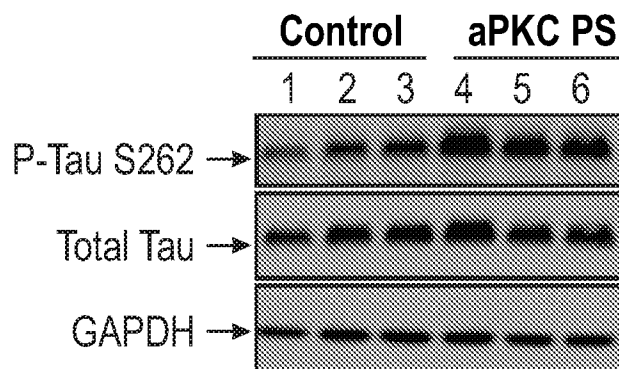


FIG. 3E

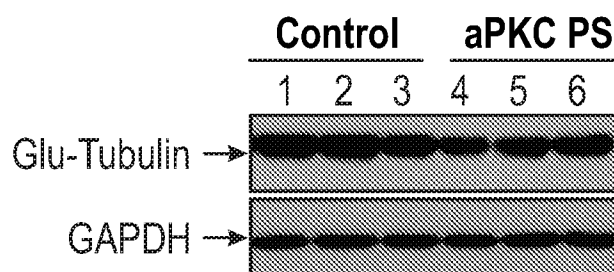


FIG. 3F

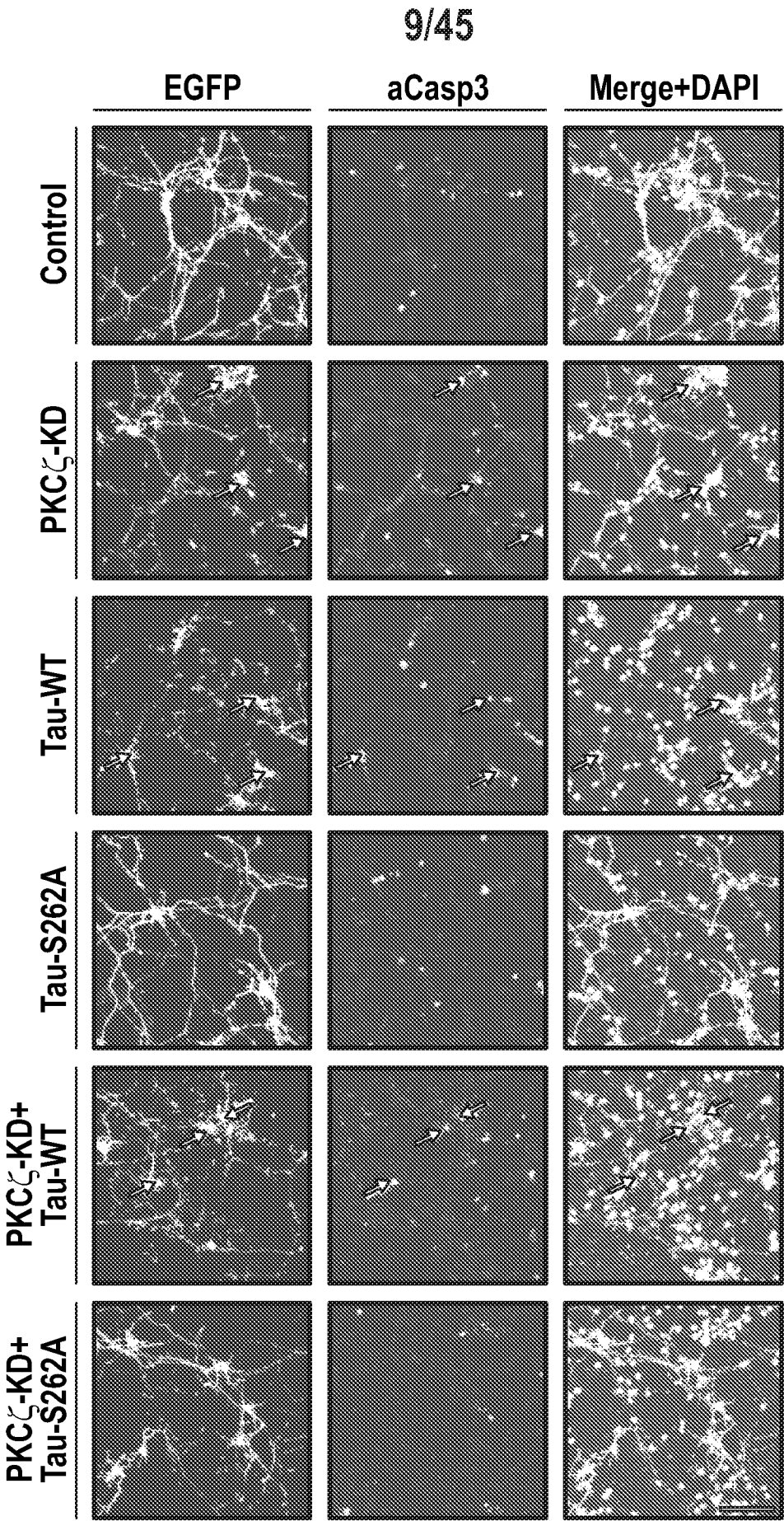


FIG. 3G

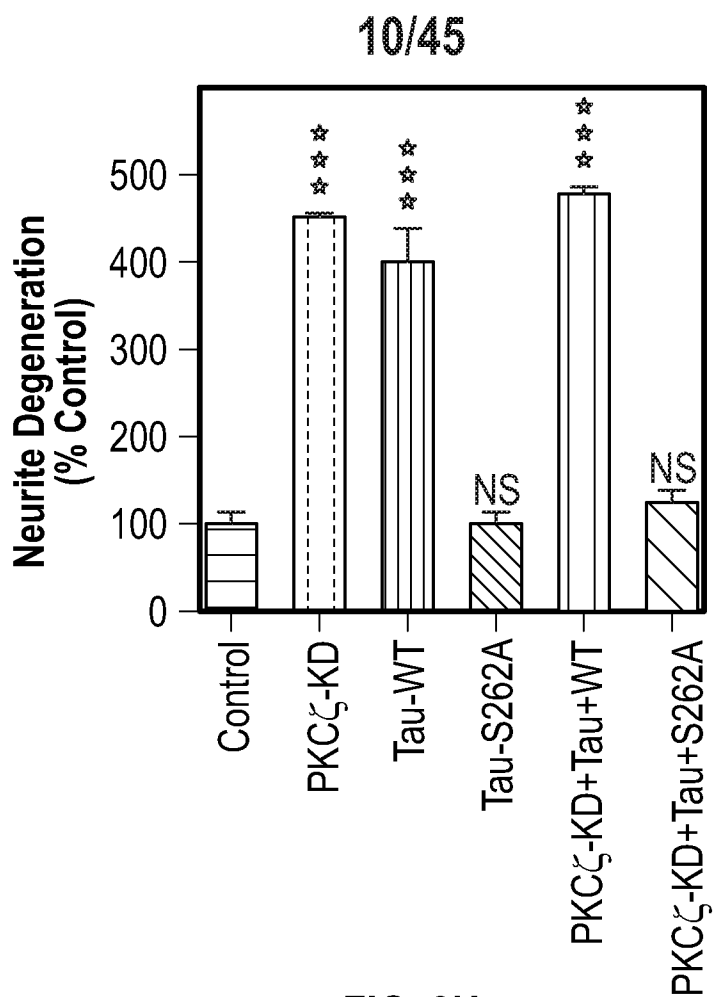


FIG. 3H

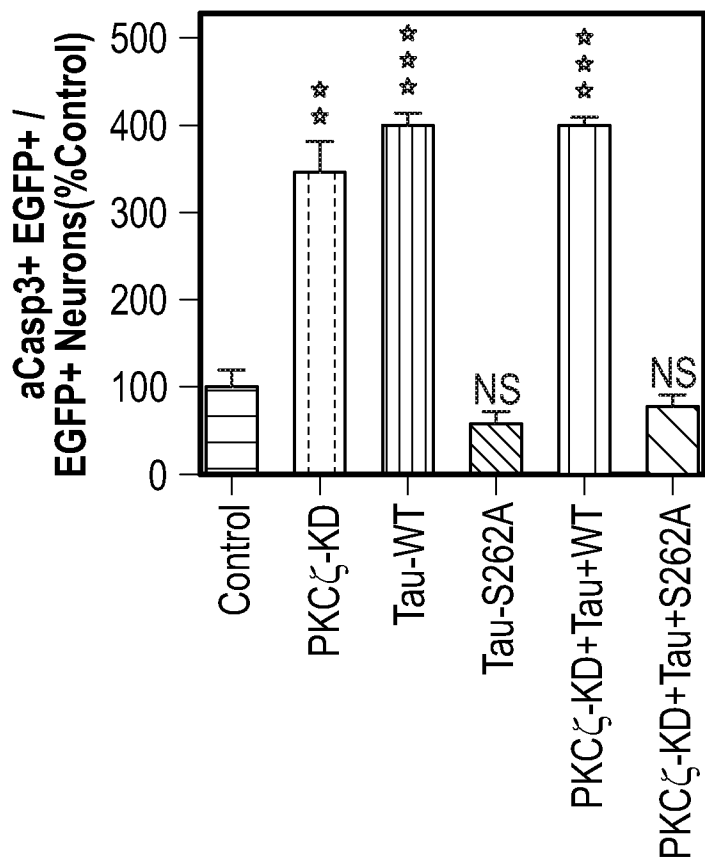


FIG. 3I

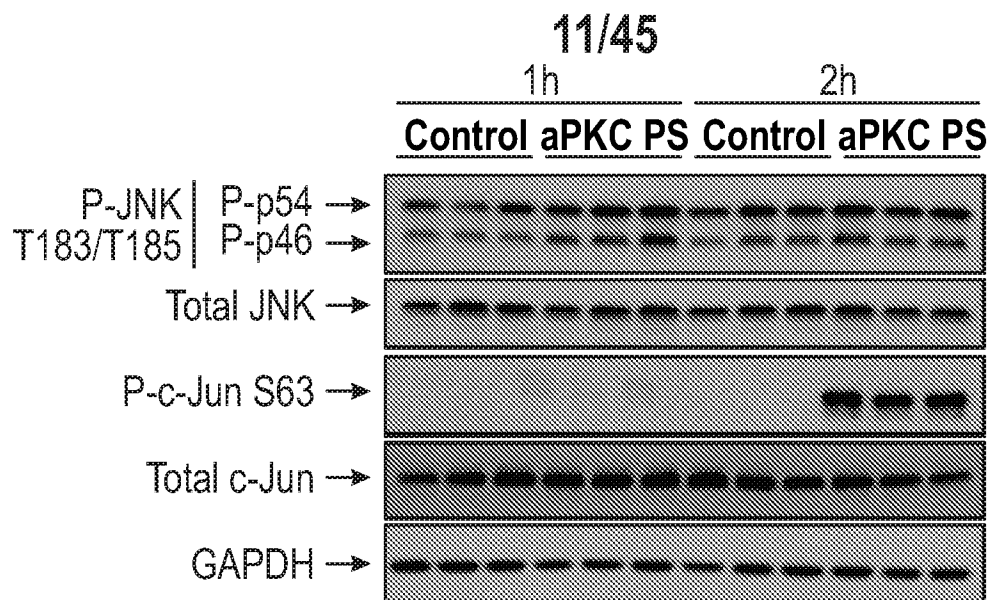


FIG. 4A

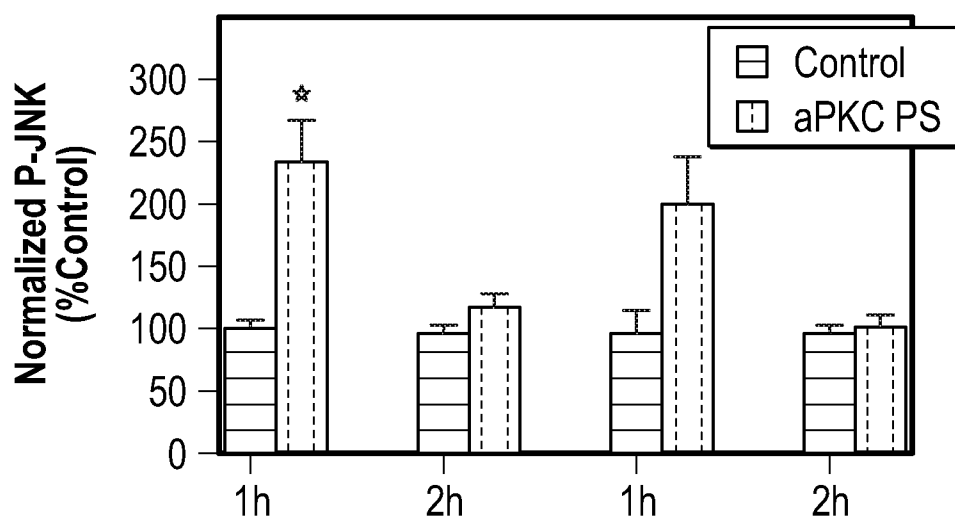


FIG. 4B

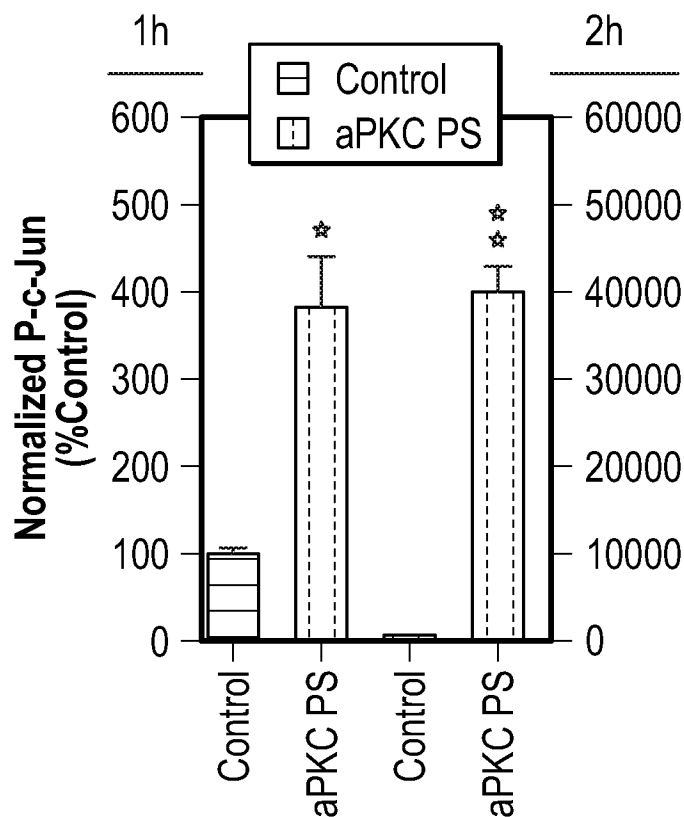


FIG. 4C

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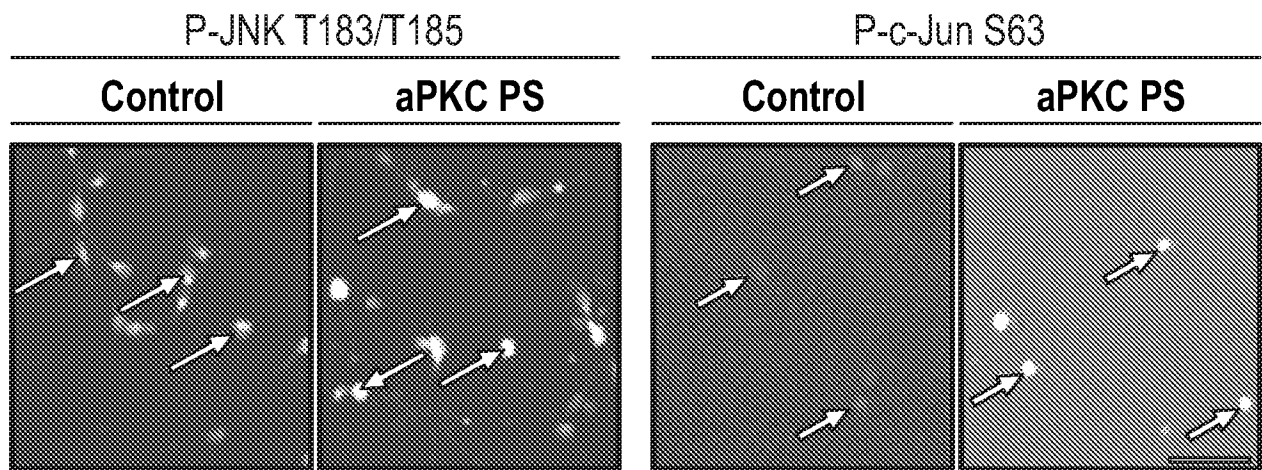


FIG. 4D

FIG. 4E

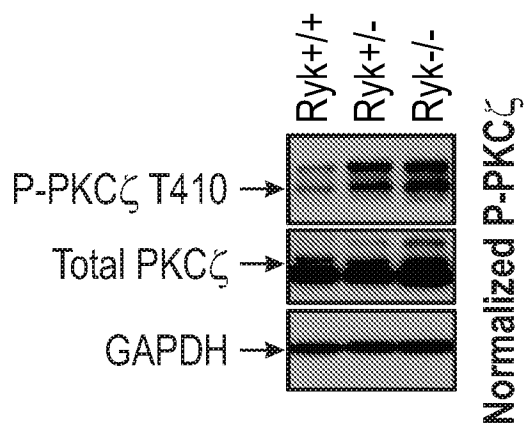


FIG. 5A

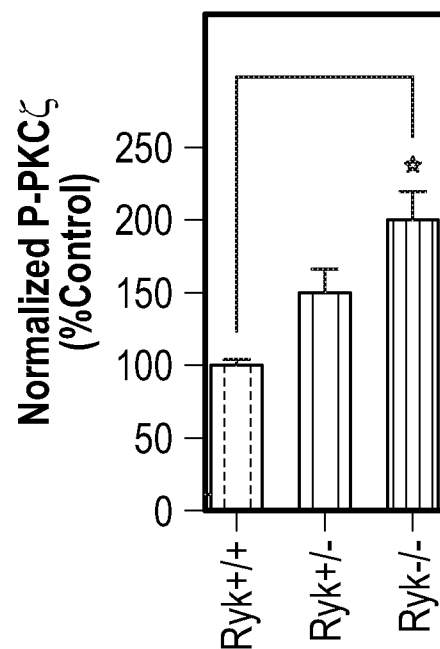


FIG. 5B

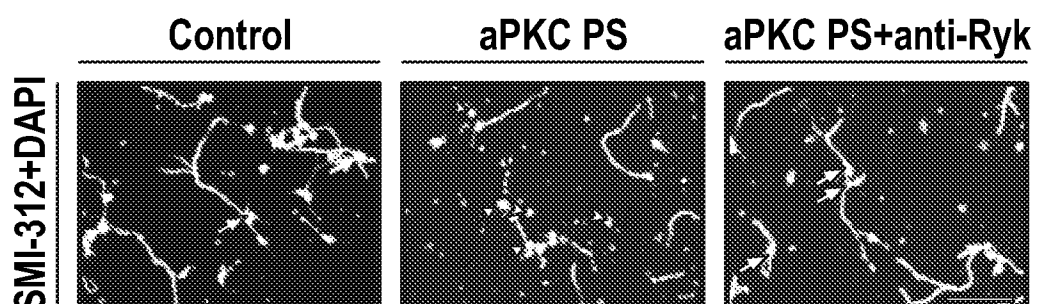


FIG. 5C

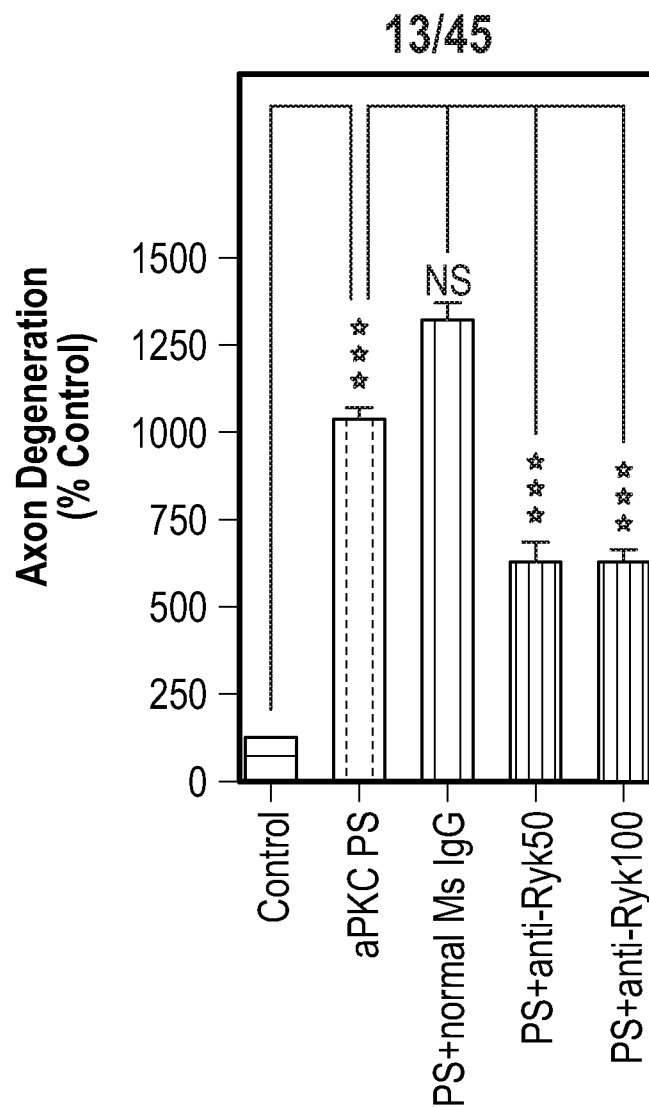


FIG. 5D

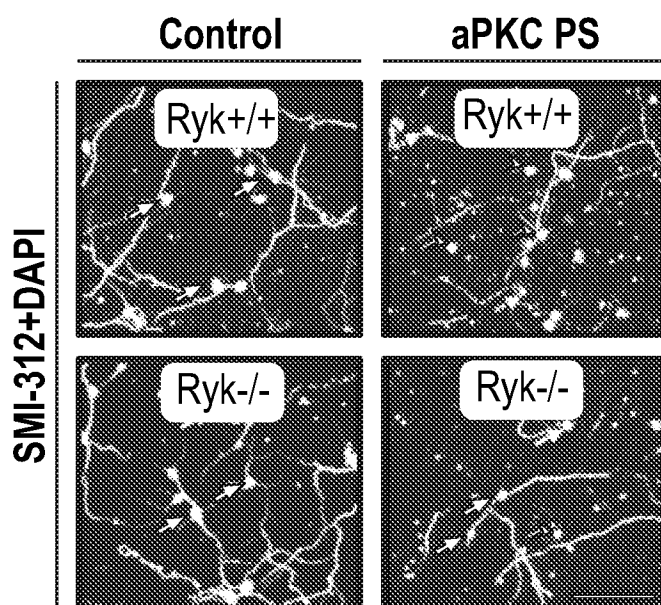


FIG. 5E

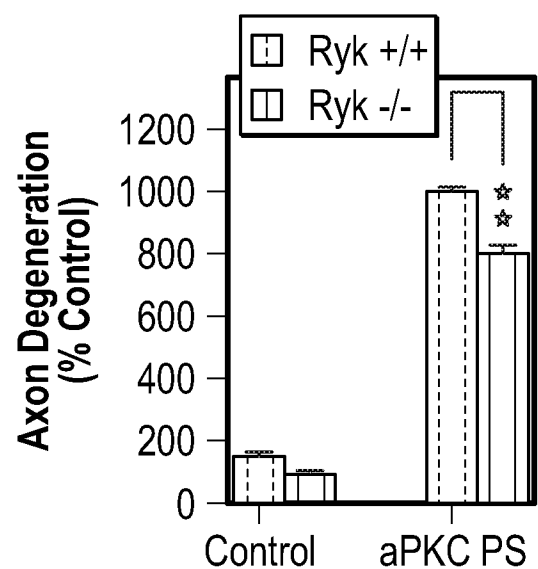


FIG. 5F

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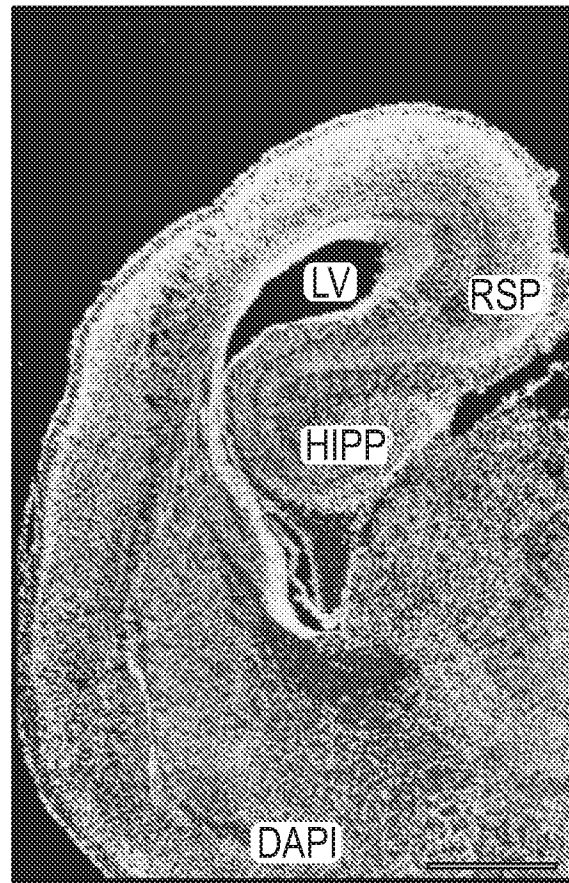


FIG. 6A

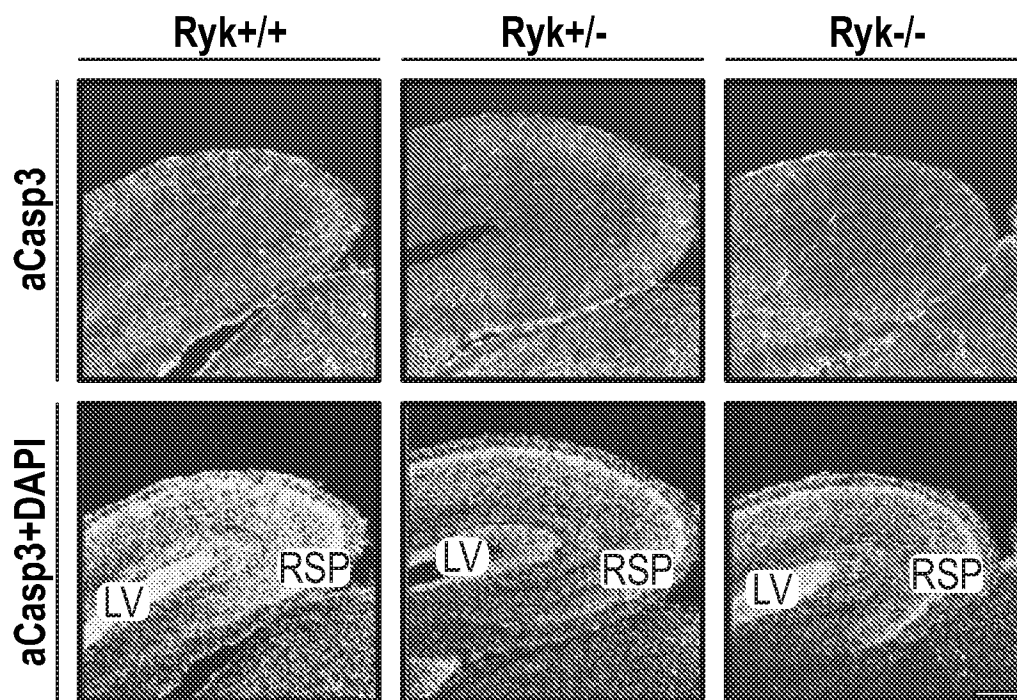


FIG. 6B

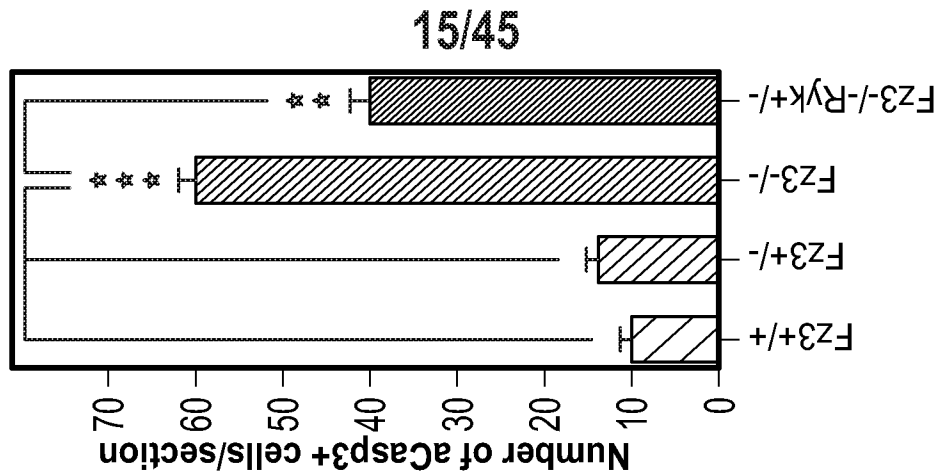


FIG. 6E

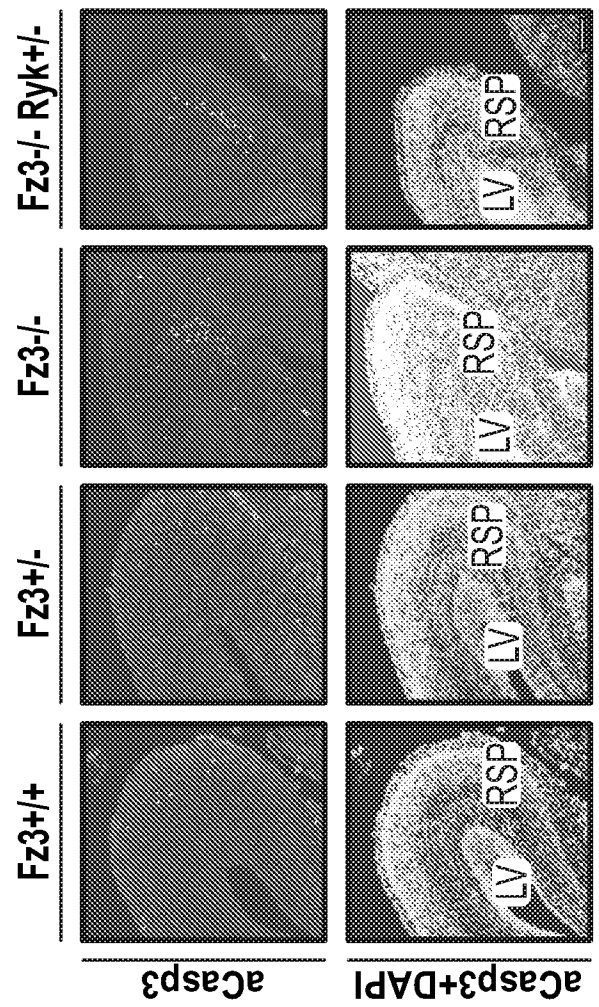


FIG. 6D

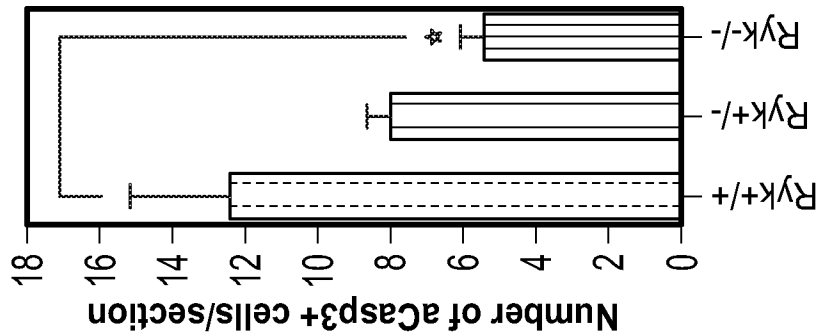


FIG. 6C

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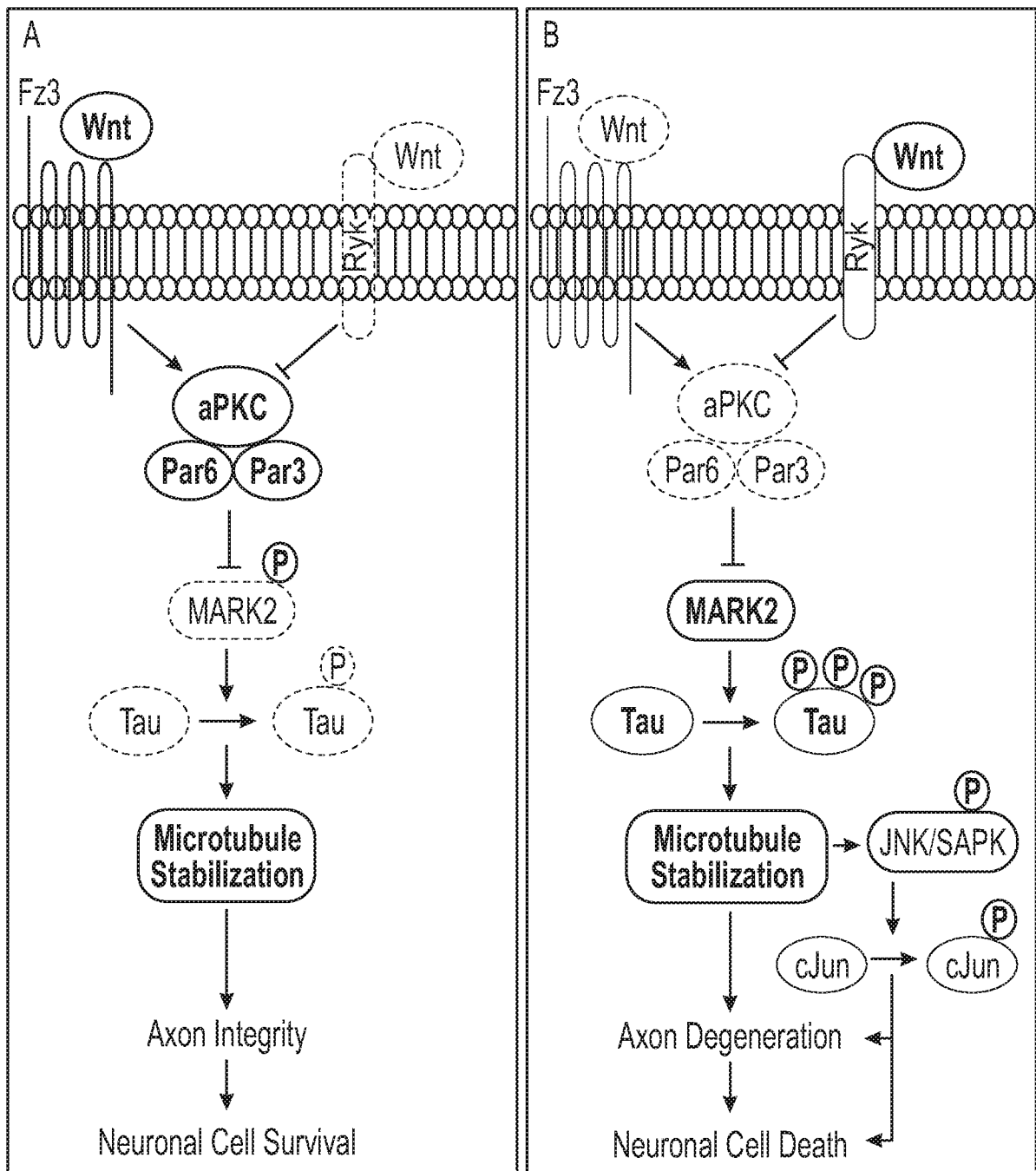


FIG. 7A

FIG. 7B

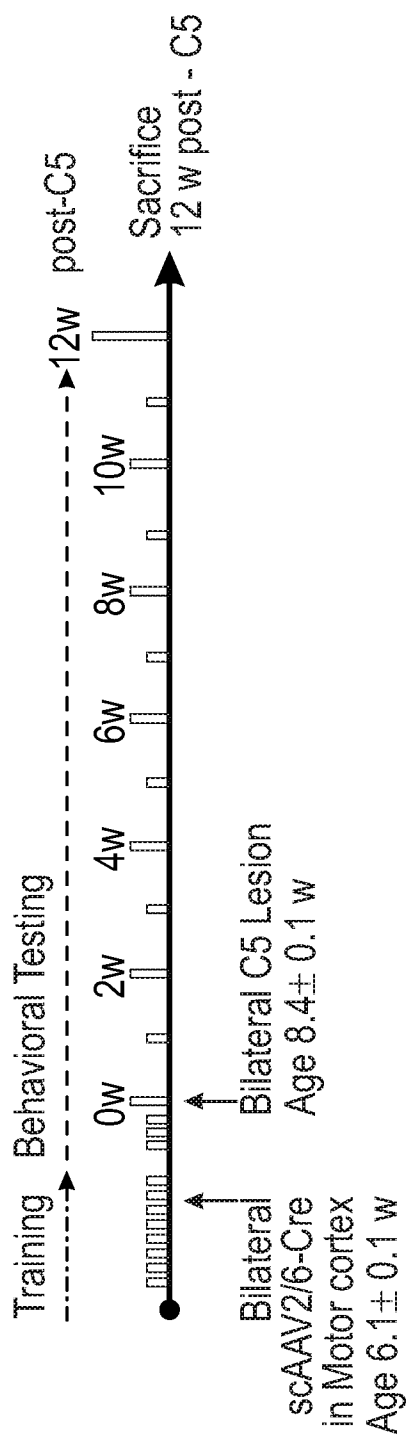


FIG. 8A

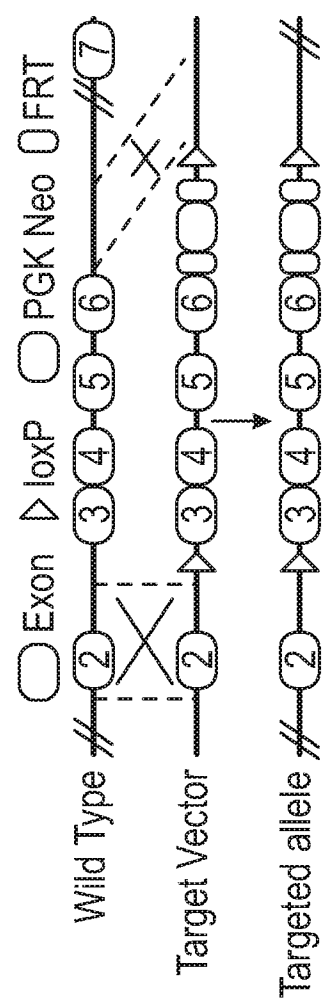


FIG. 8B

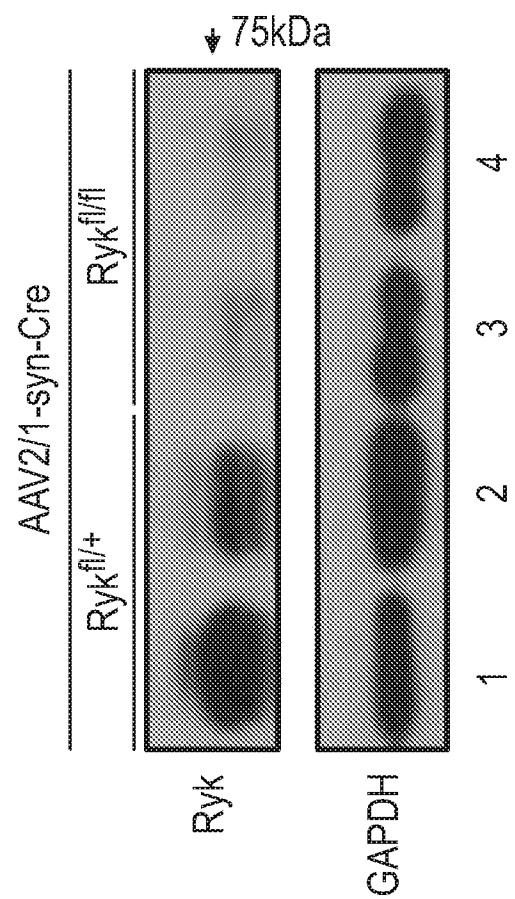


FIG. 8C

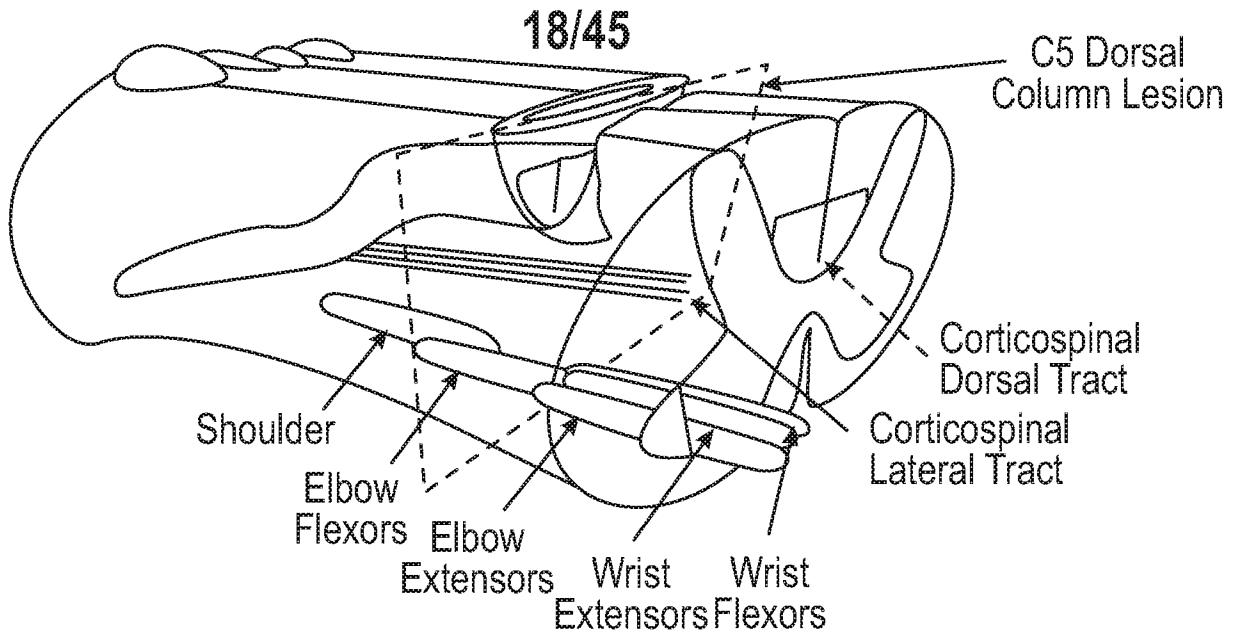


FIG. 8D

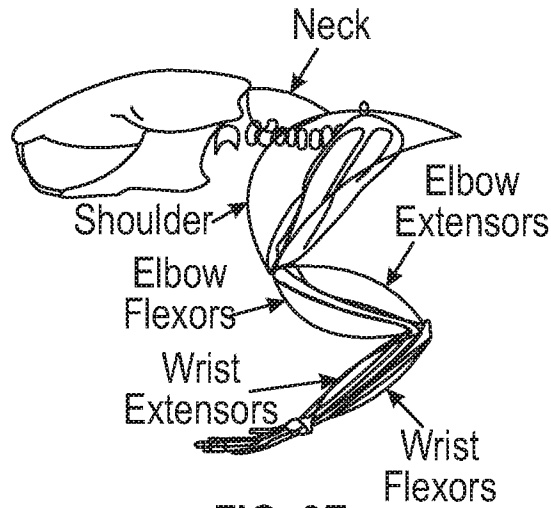


FIG. 8E

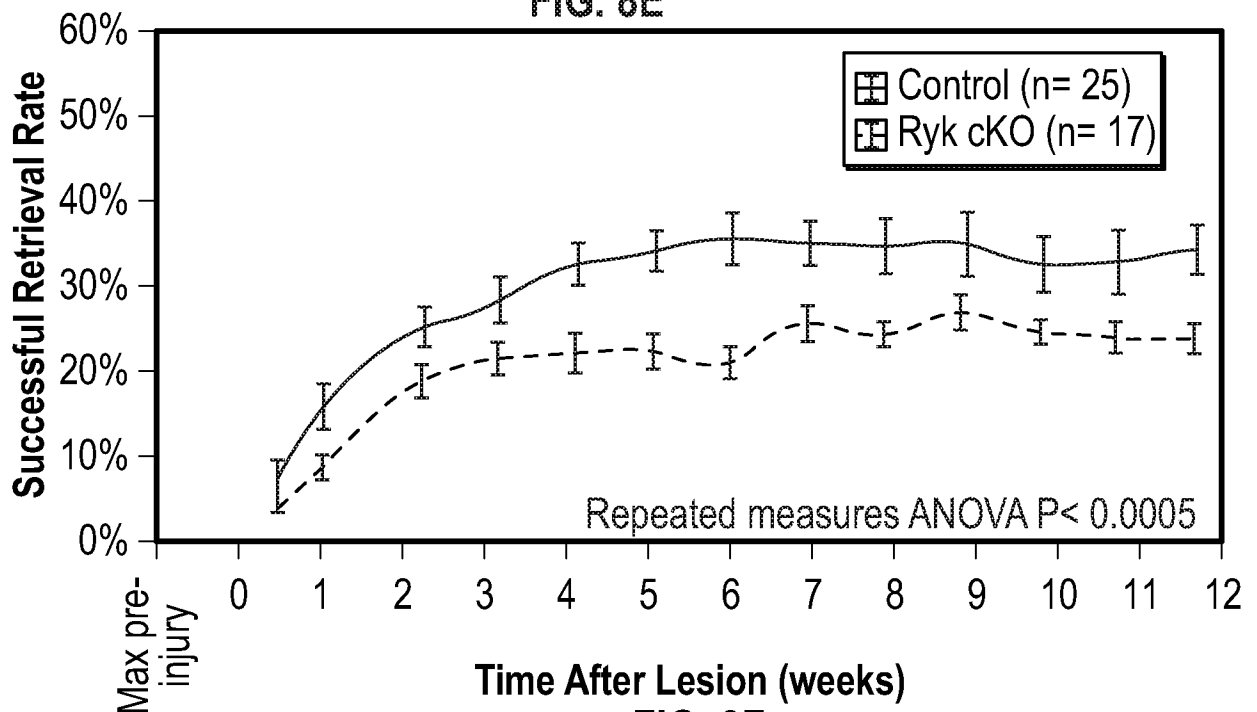


FIG. 8F

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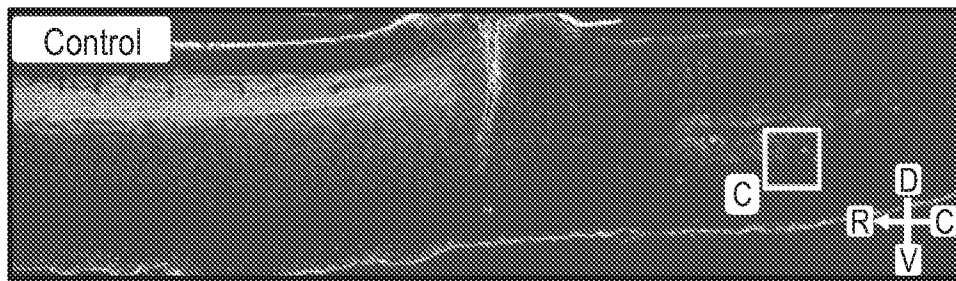


FIG. 9A

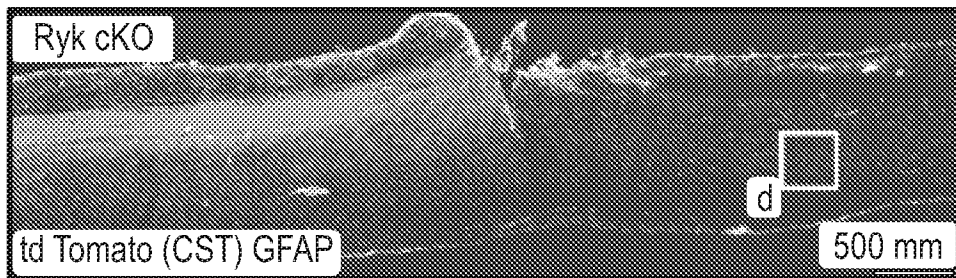


FIG. 9B

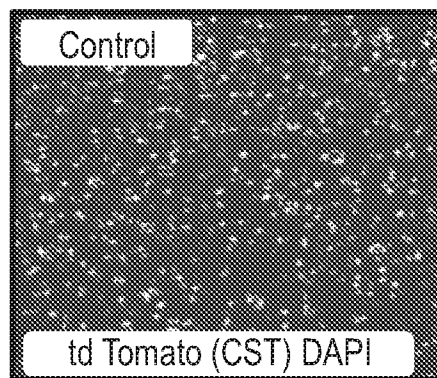


FIG. 9C

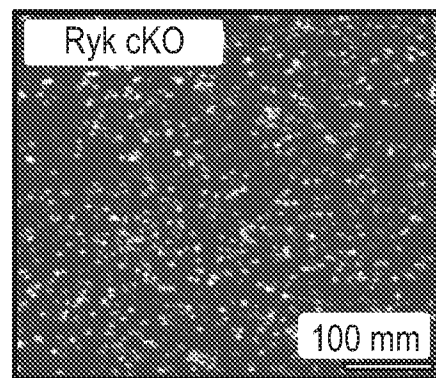


FIG. 9D

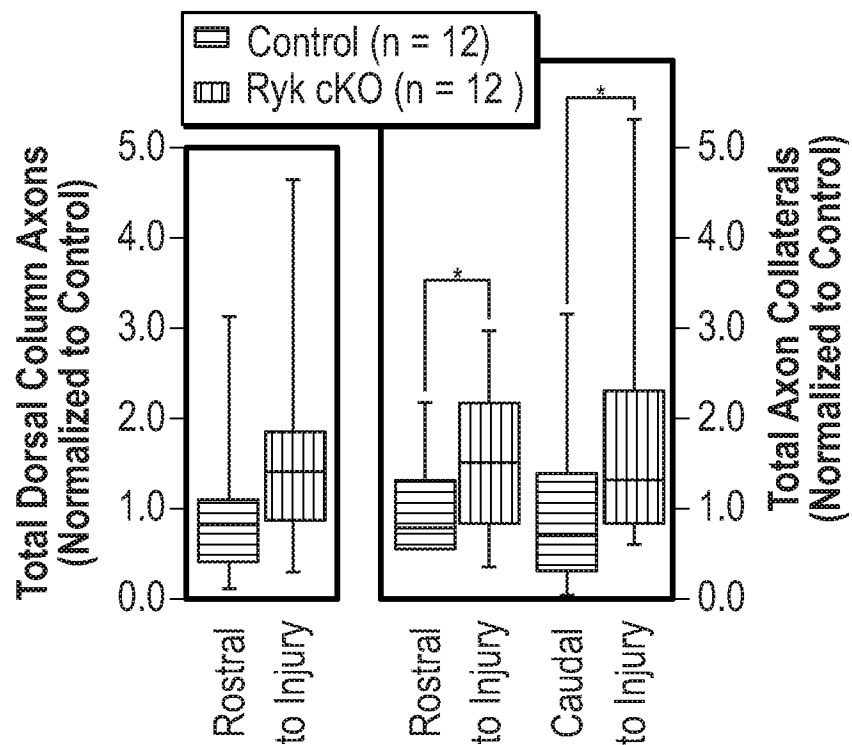


FIG. 9E

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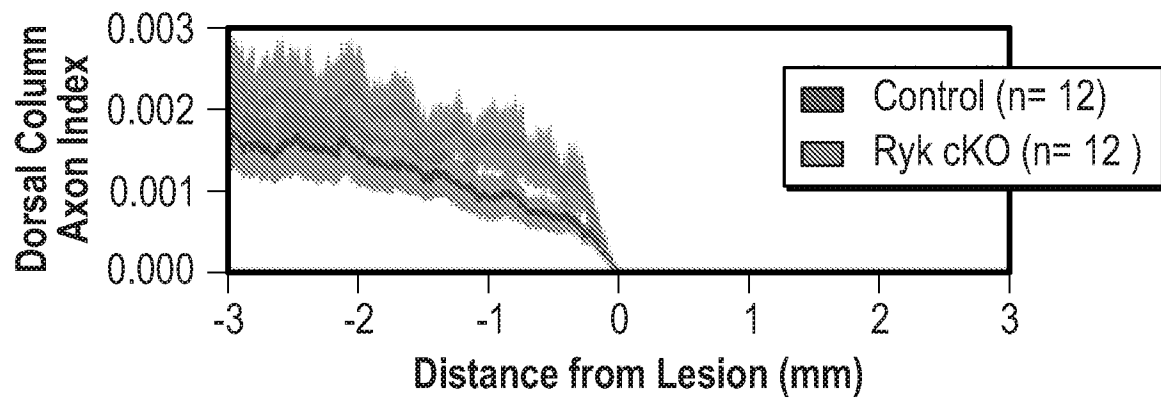


FIG. 9F

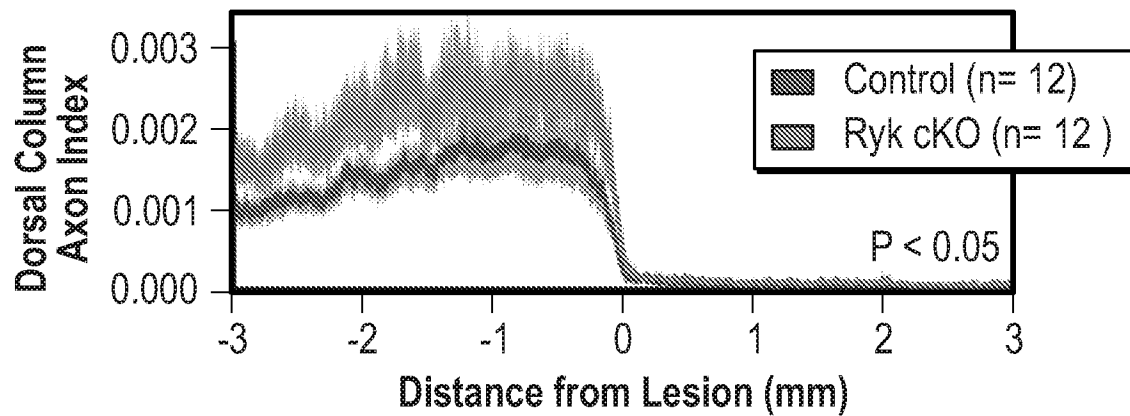


FIG. 9G

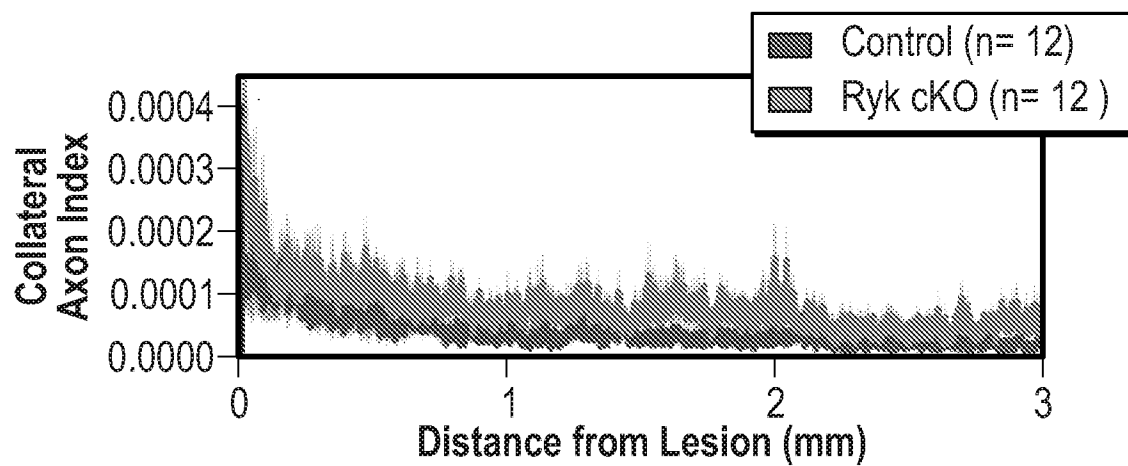


FIG. 9H

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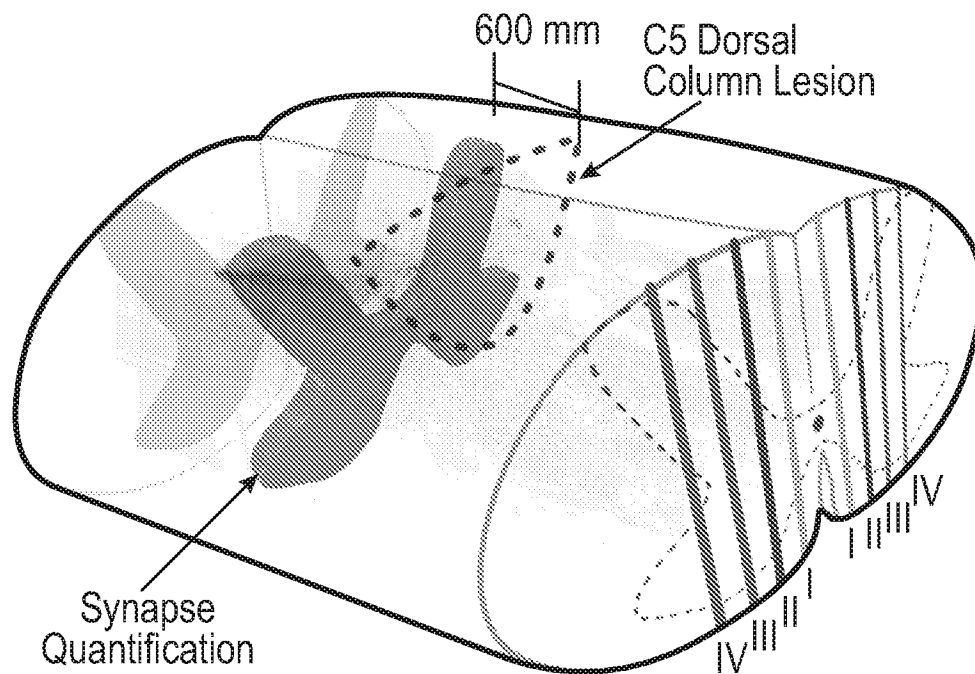


FIG. 10A

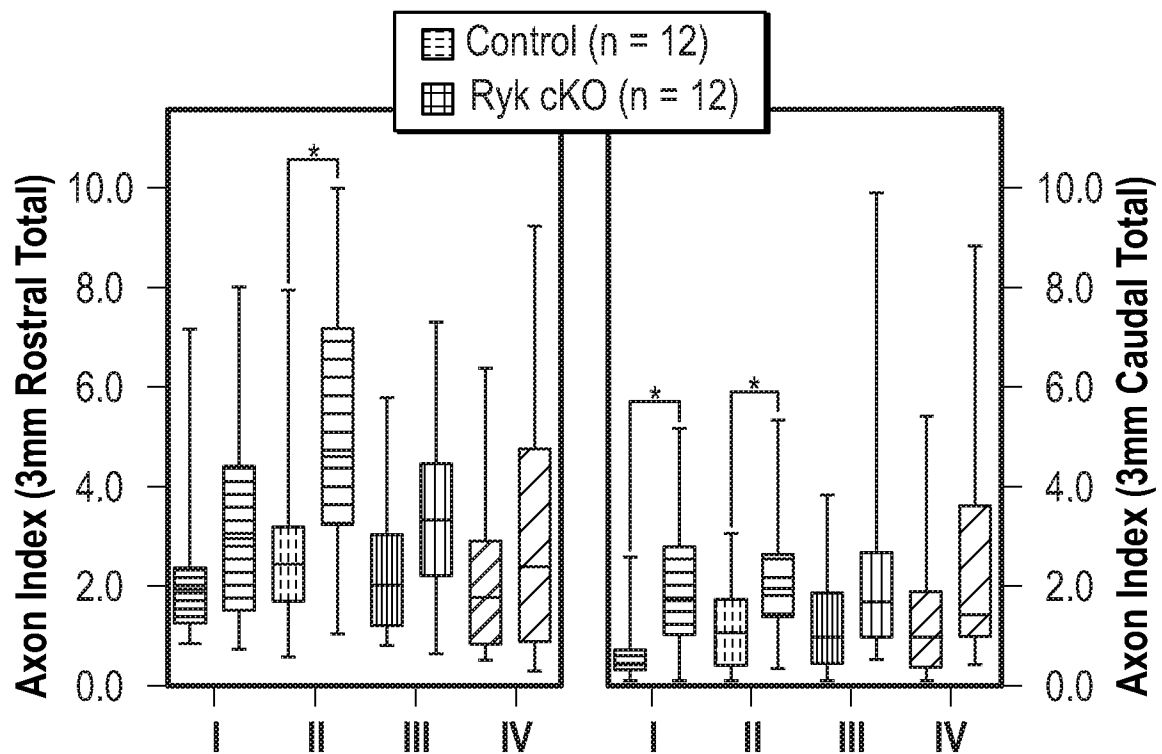


FIG. 10B

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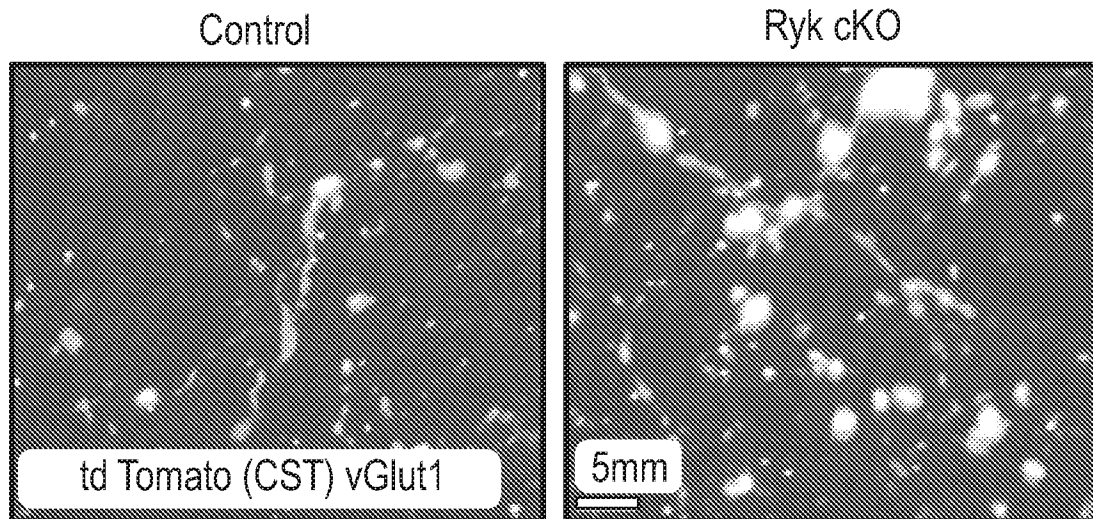


FIG. 10C

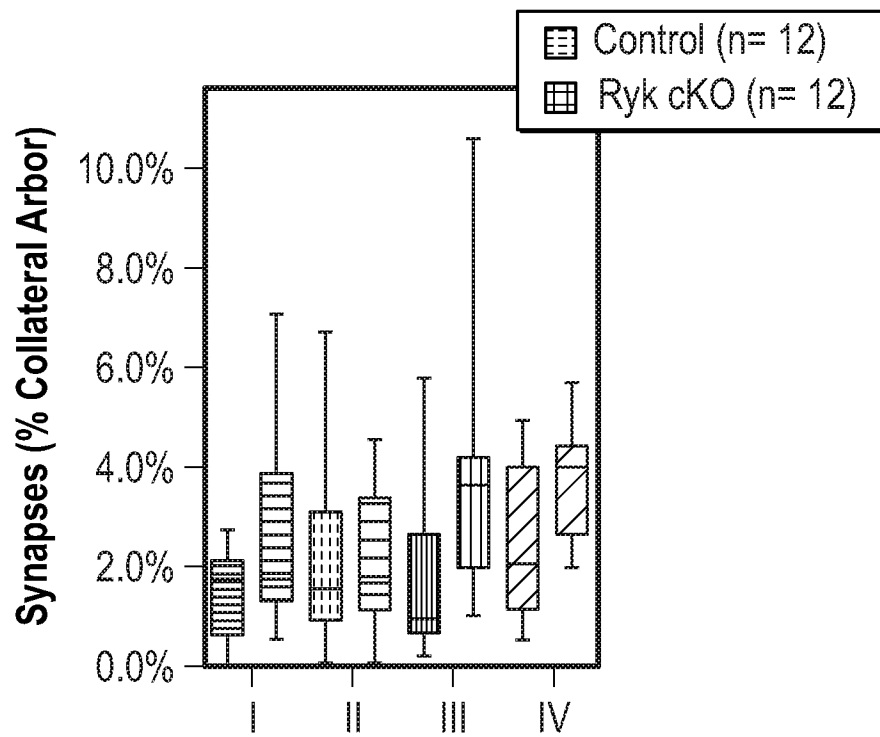


FIG. 10D

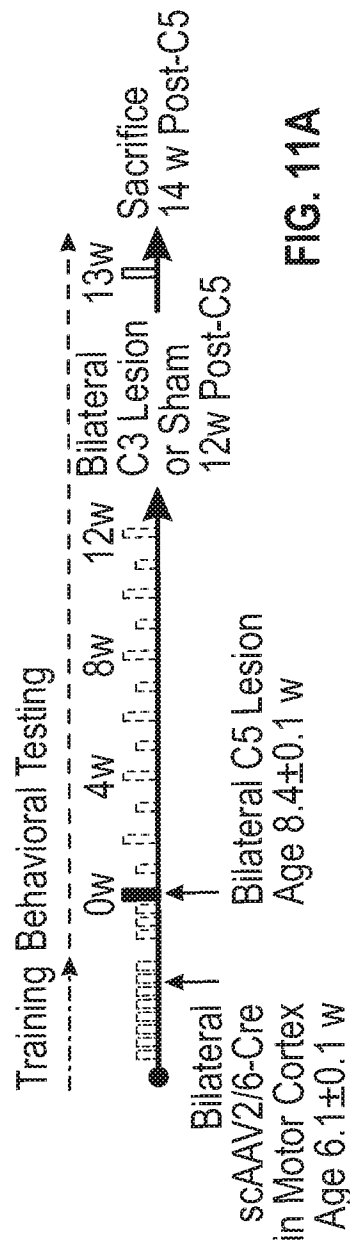


FIG. 11A

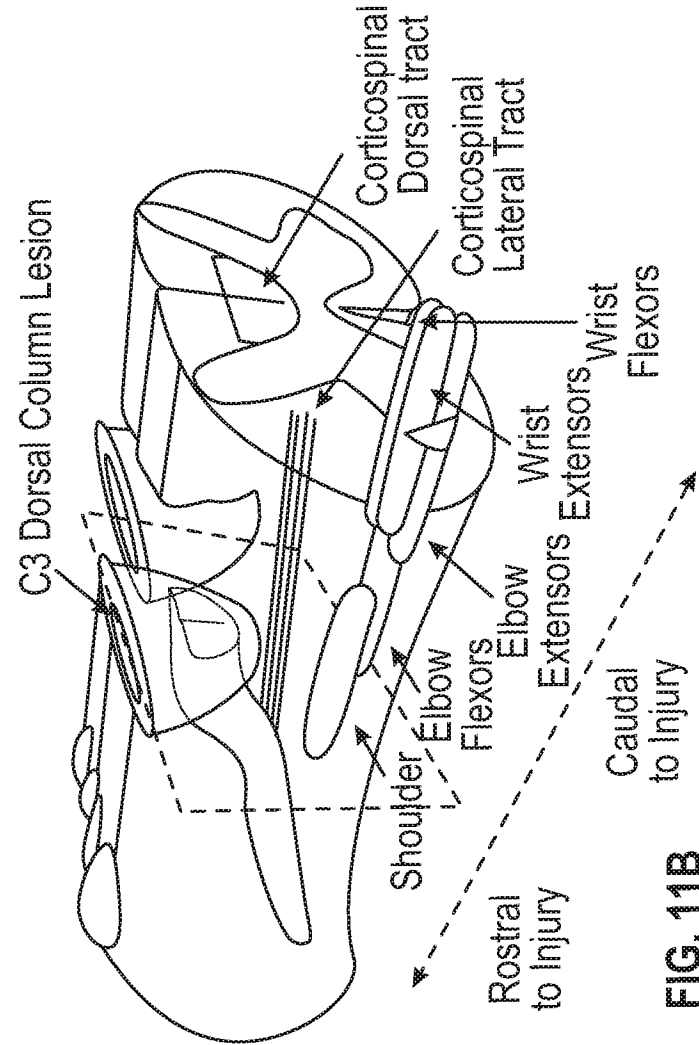


FIG. 11B

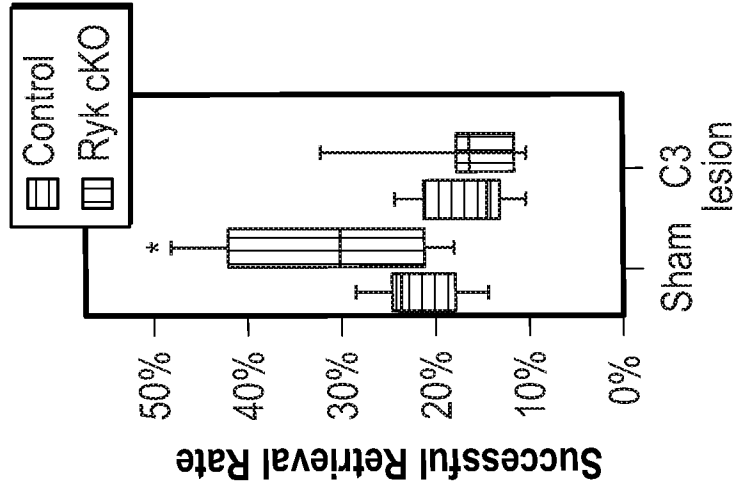
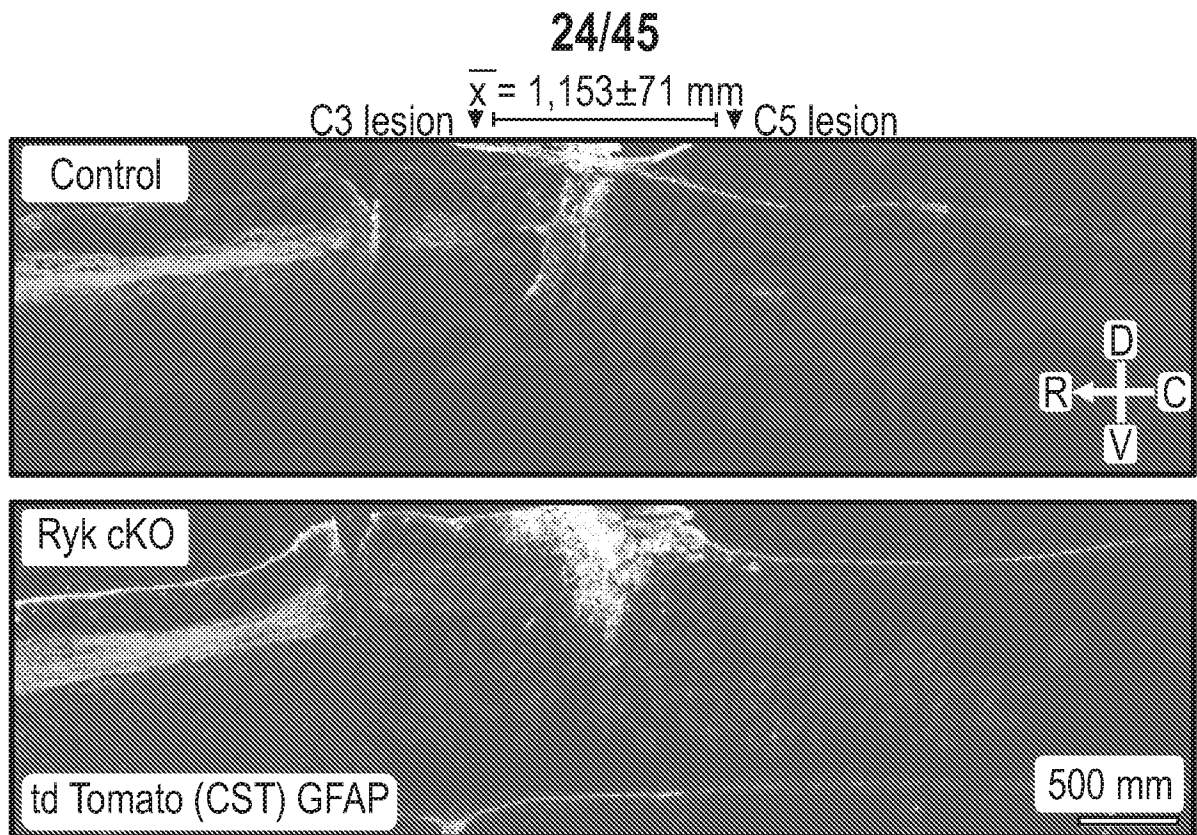
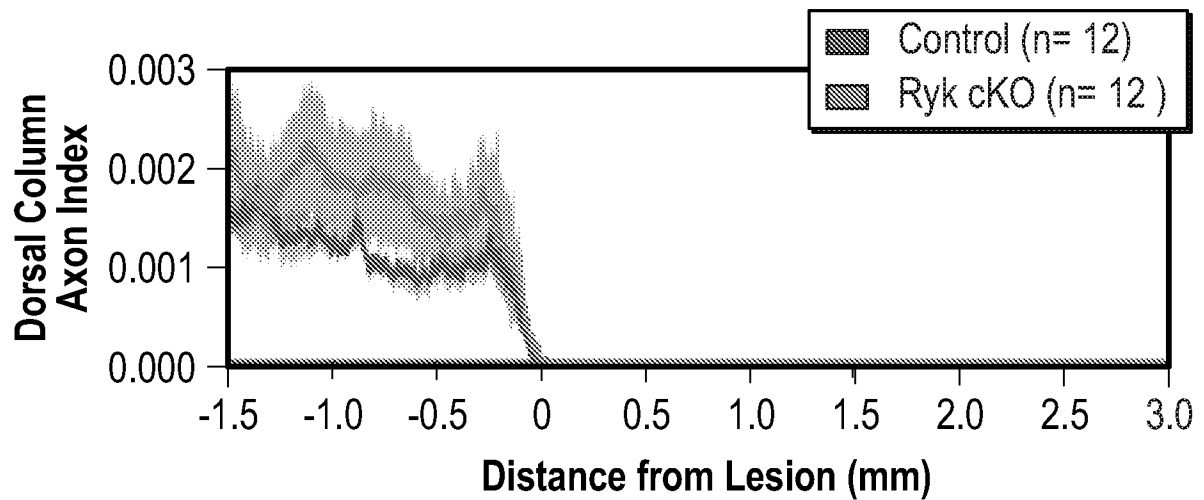
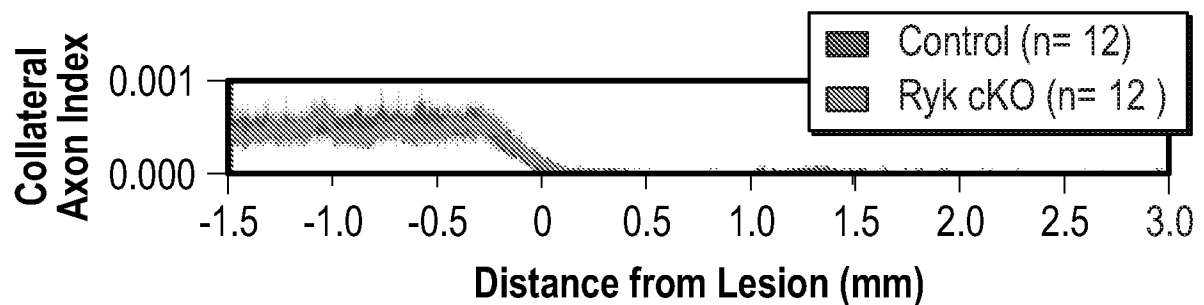


FIG. 11C

**FIG. 11D****FIG. 11E****FIG. 11F**

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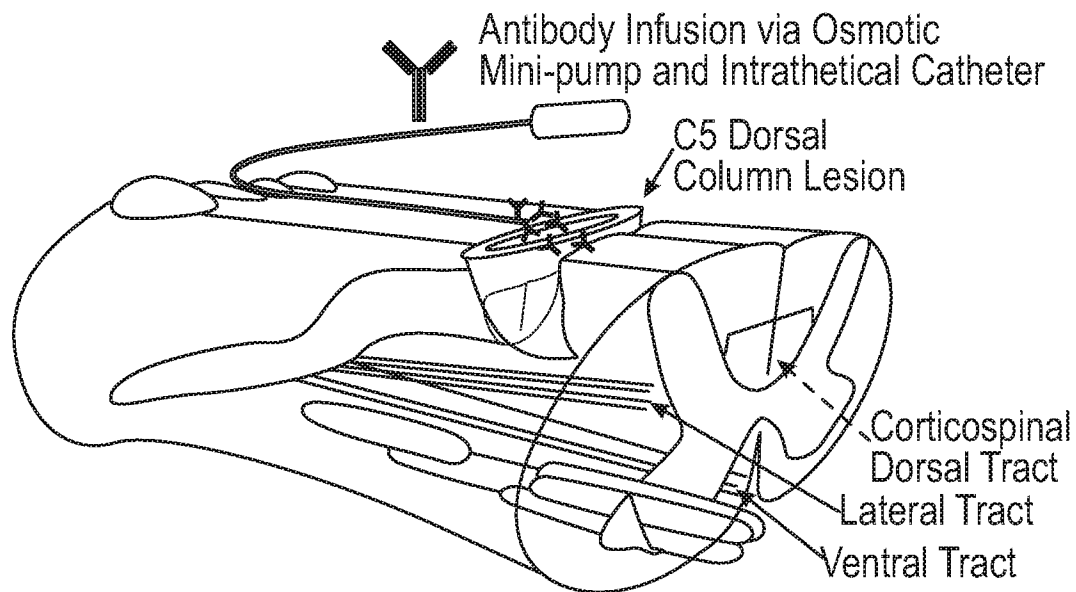


FIG. 12A

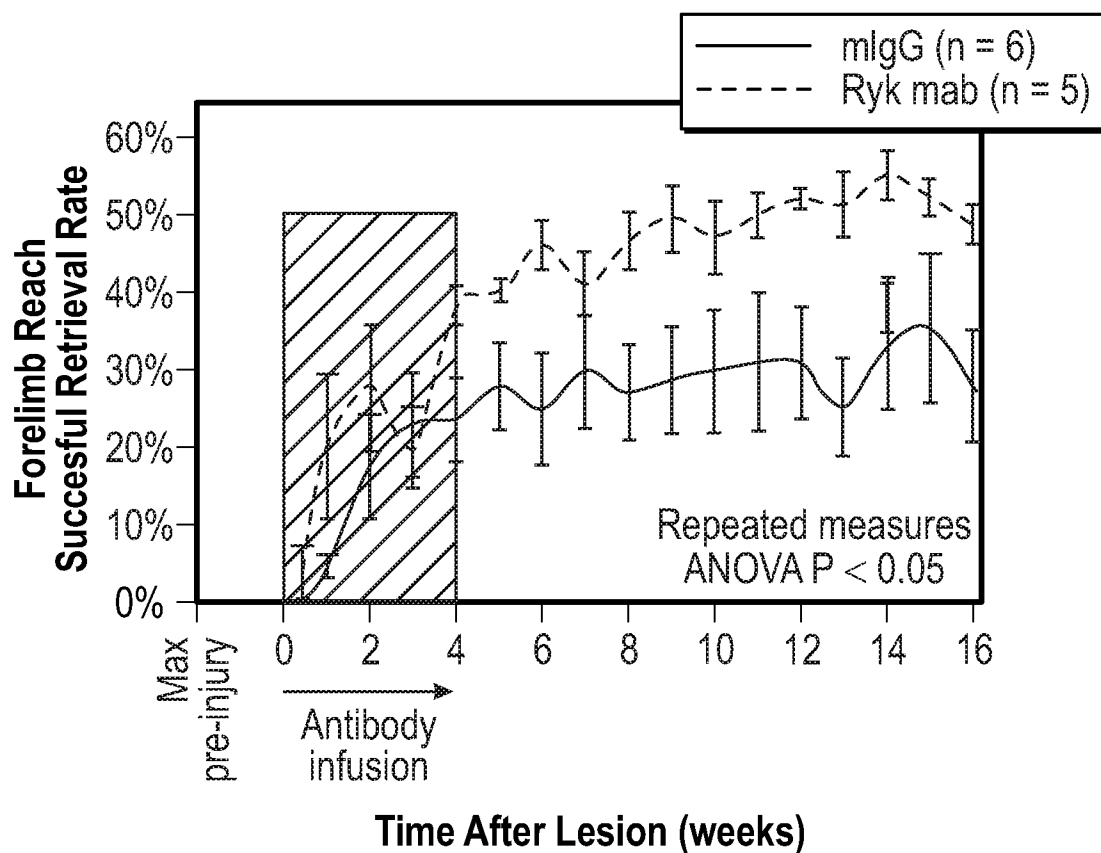


FIG. 12B

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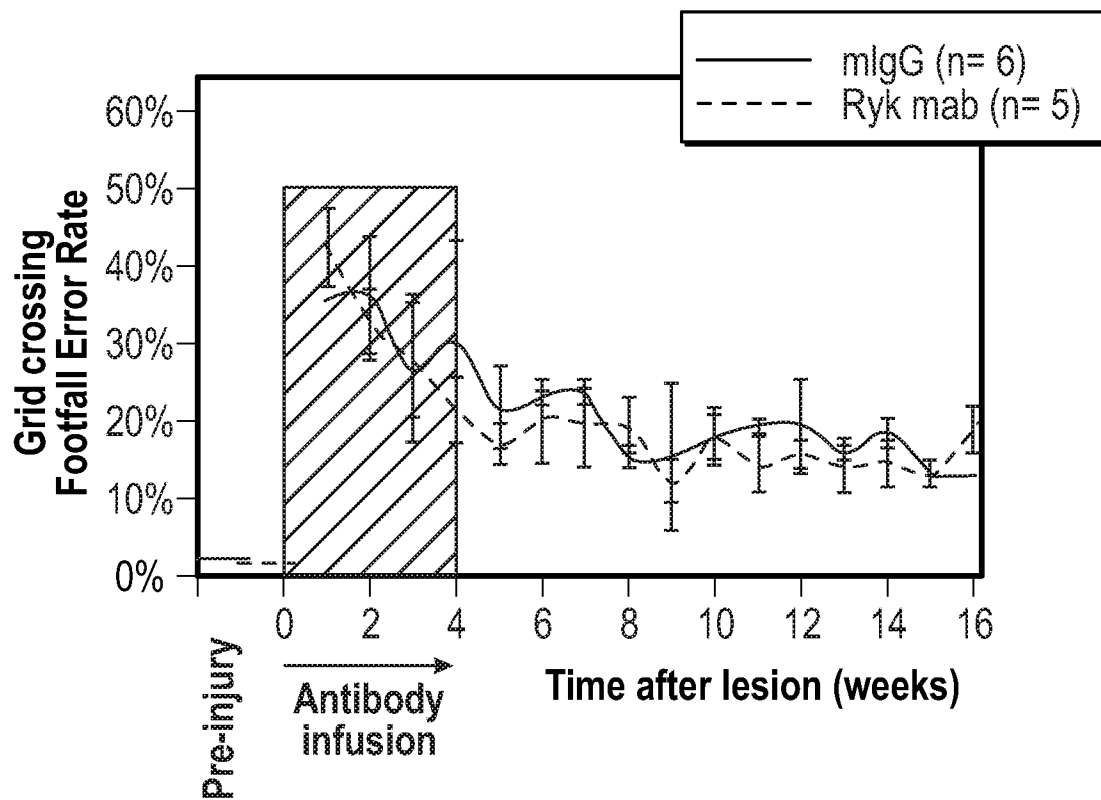


FIG. 12C

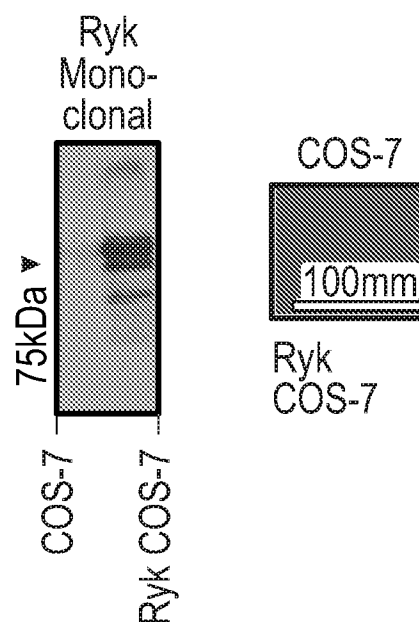


FIG. 12D

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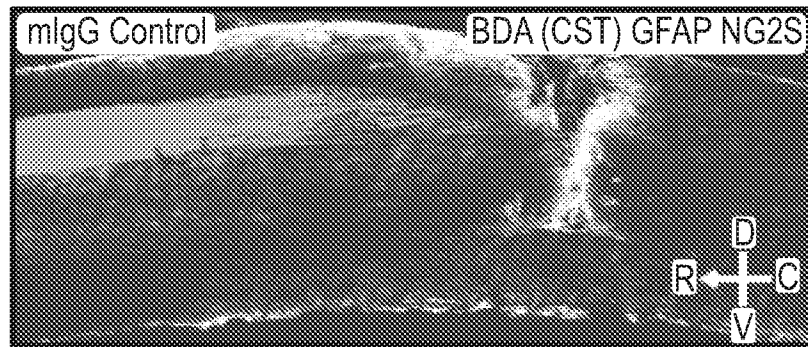


FIG. 12E

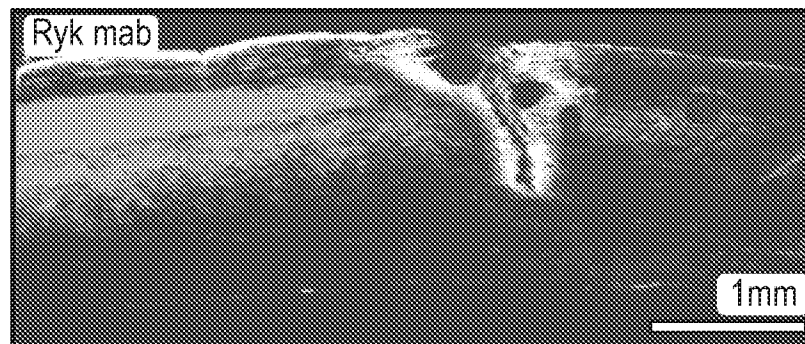


FIG. 12F

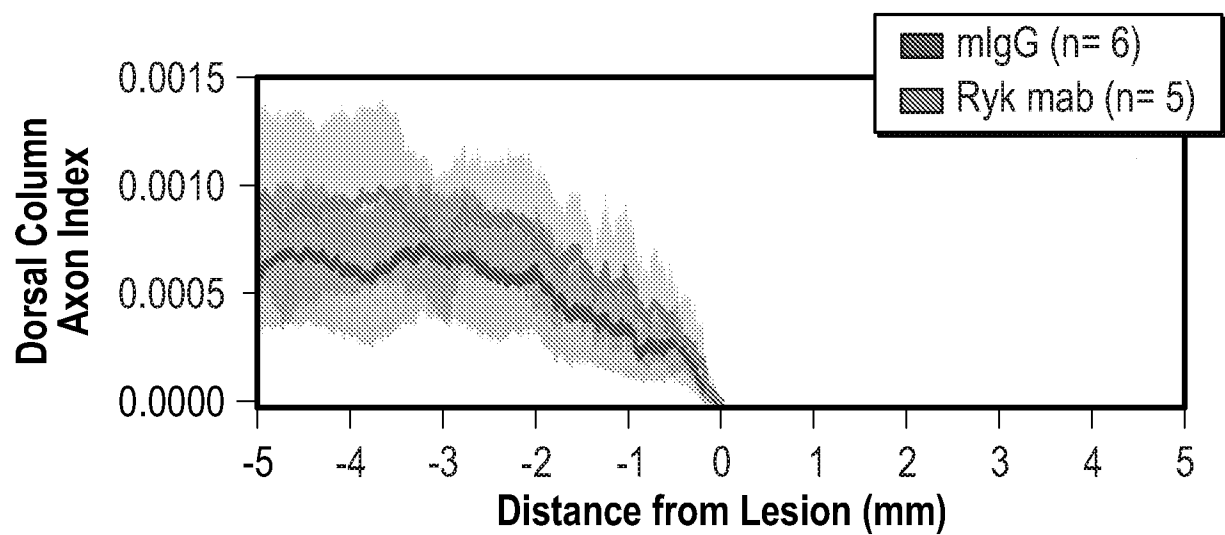
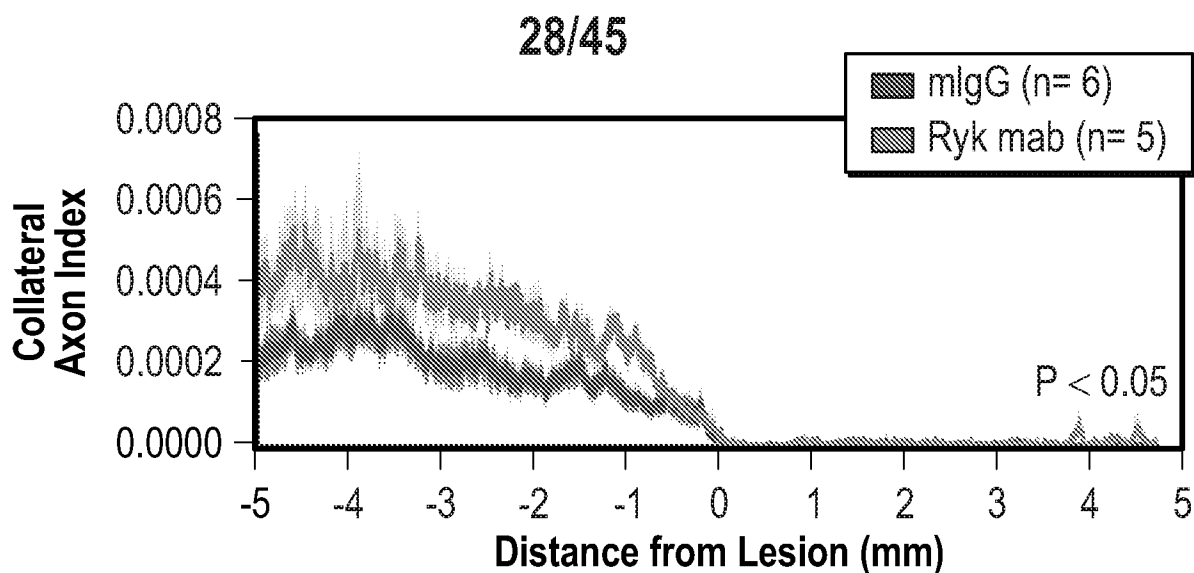
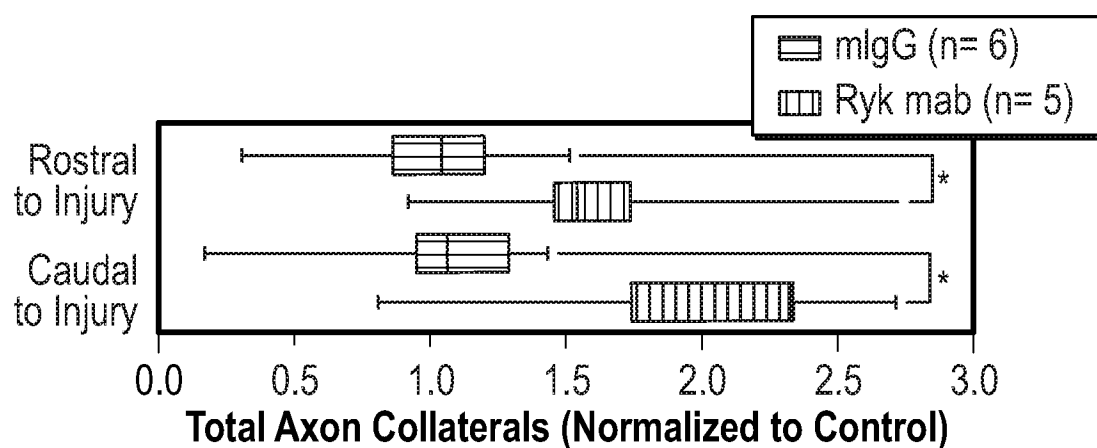
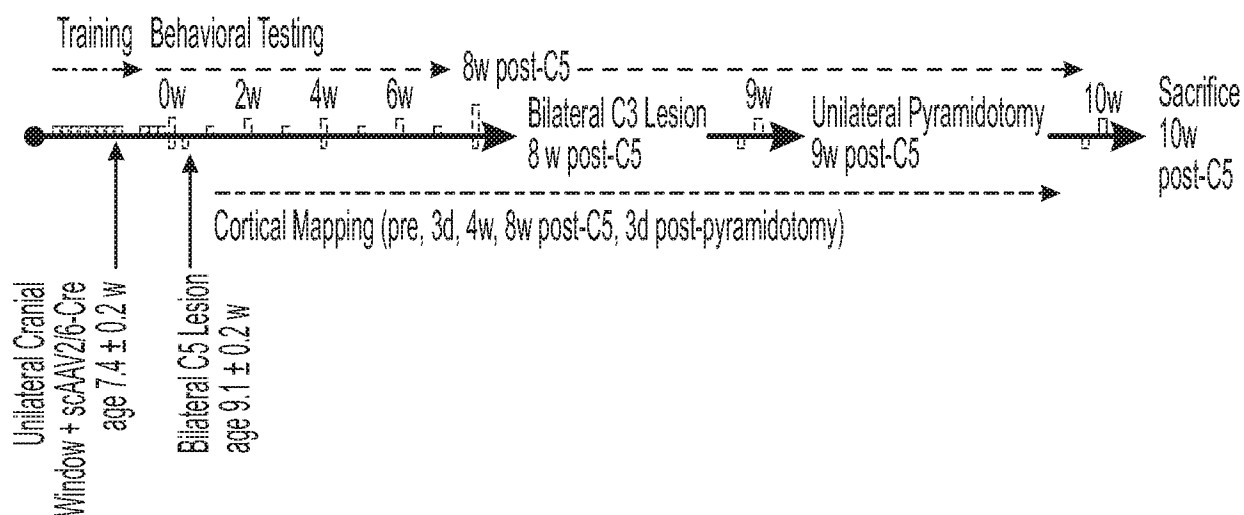


FIG. 12G

**FIG. 12H****FIG. 12I****FIG. 13A**

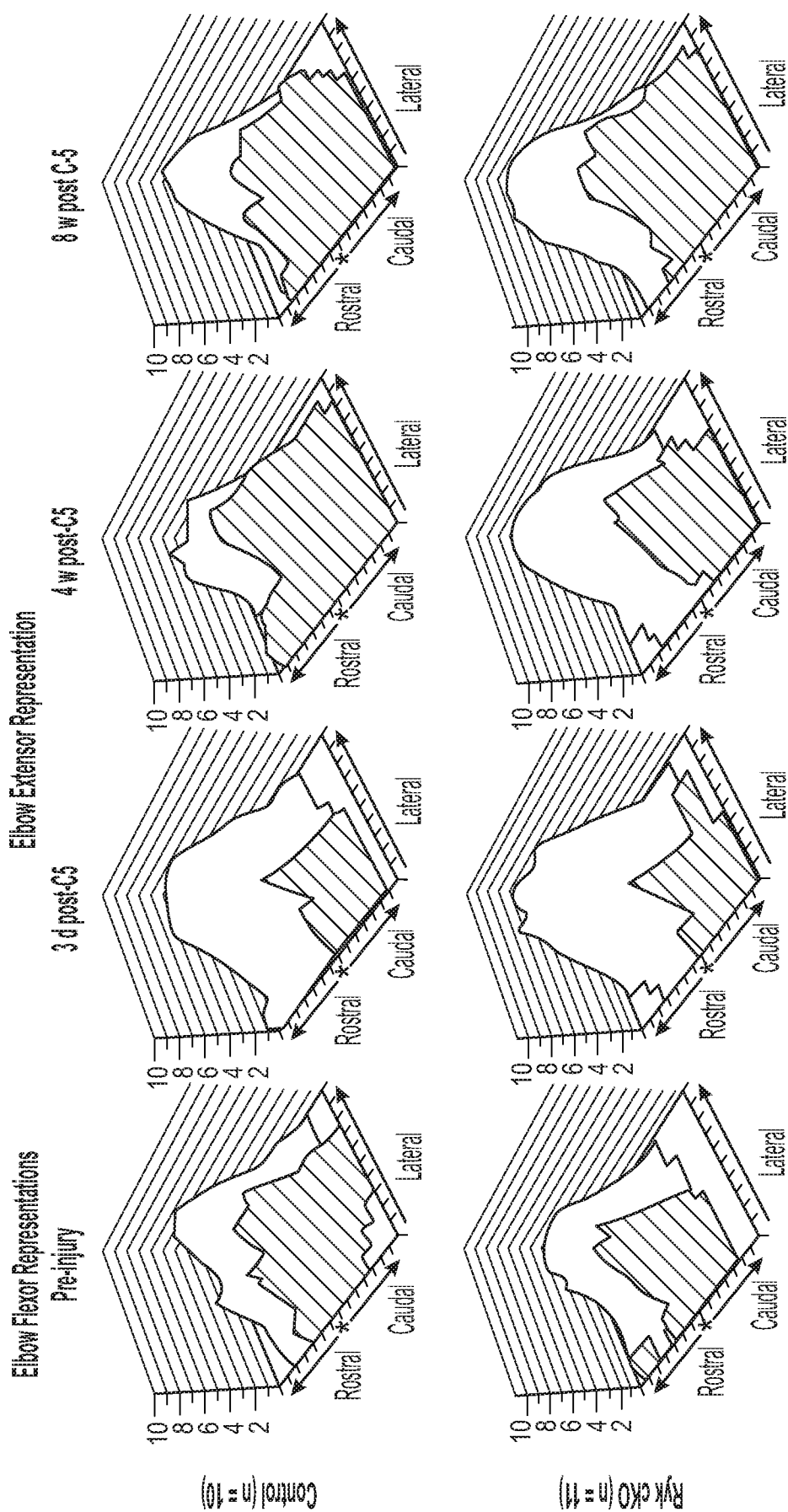


FIG. 13B

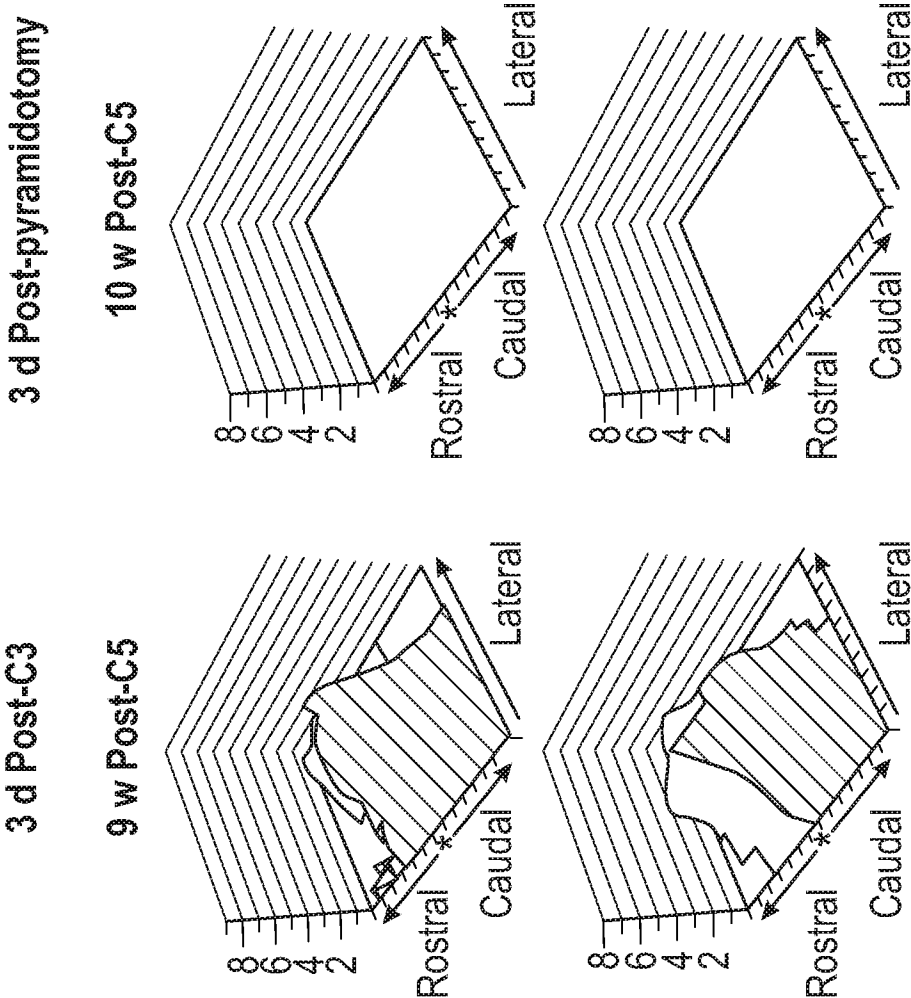
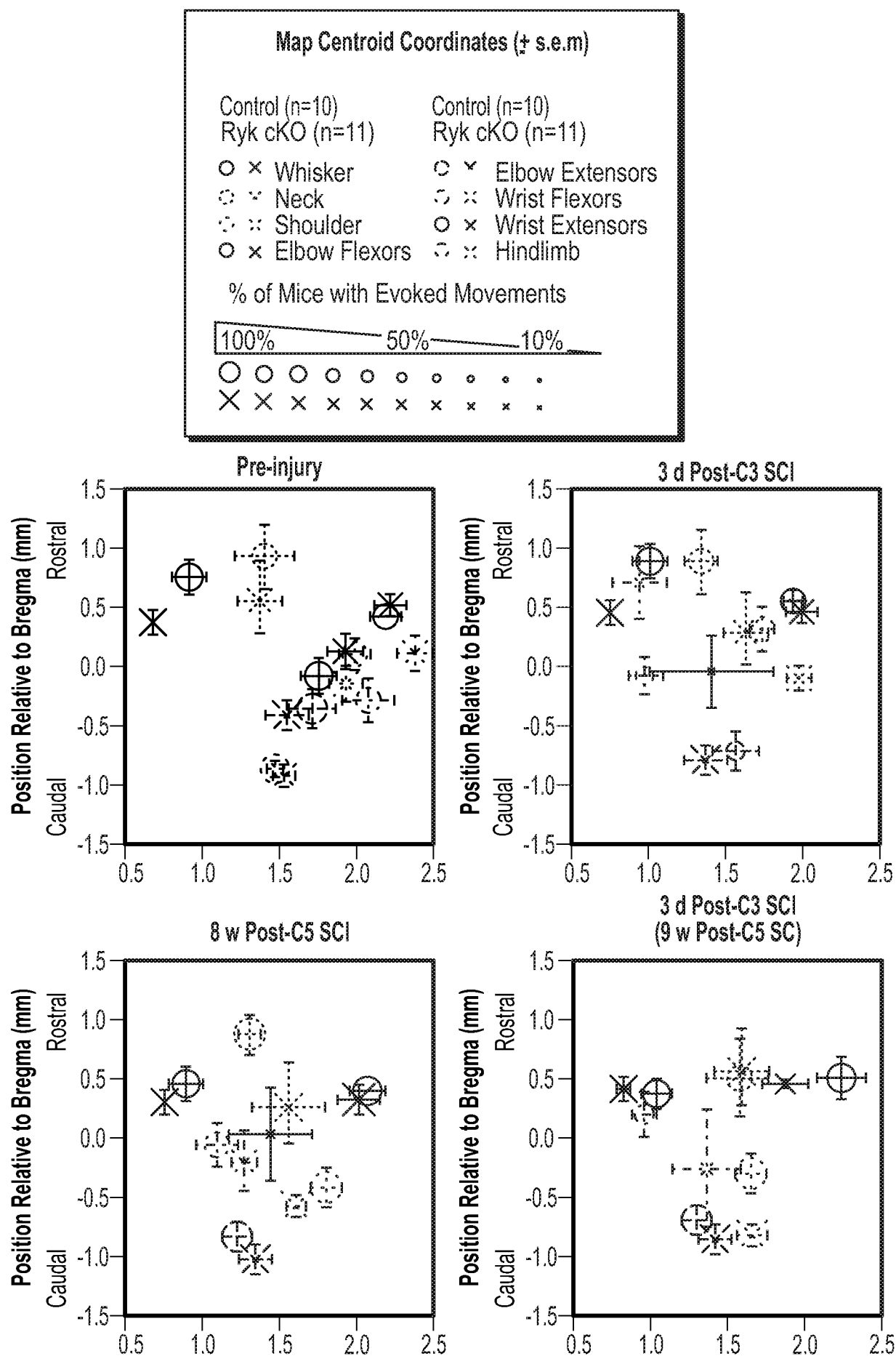


FIG. 13C

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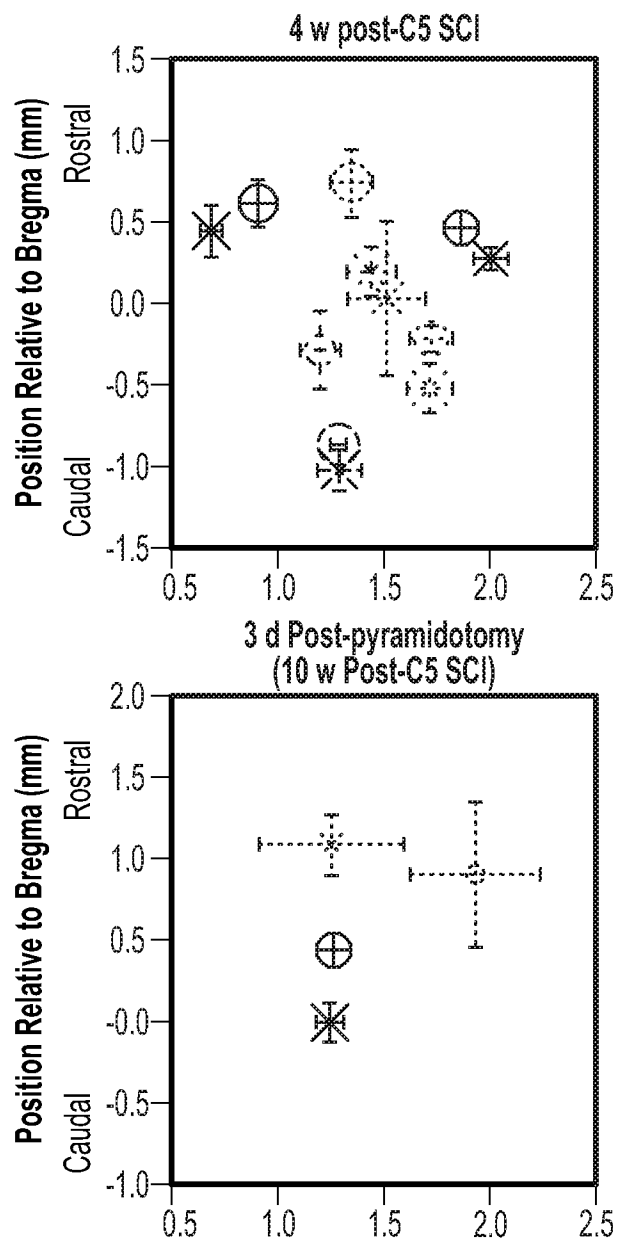
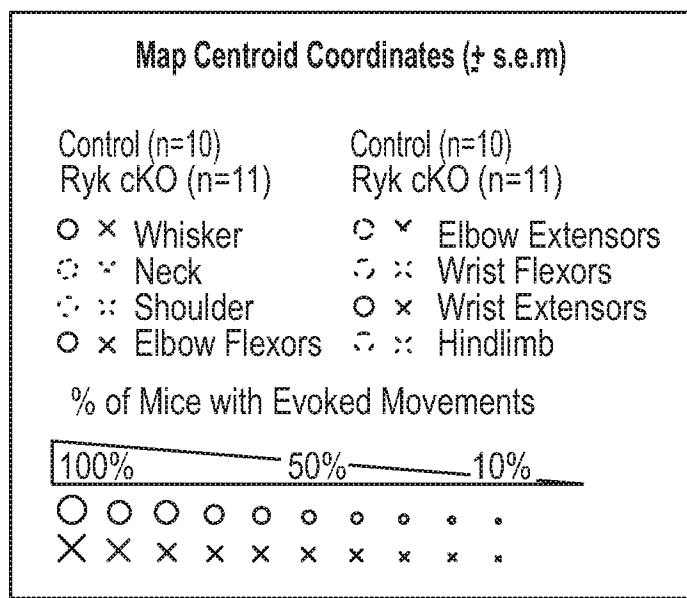


FIG. 14A

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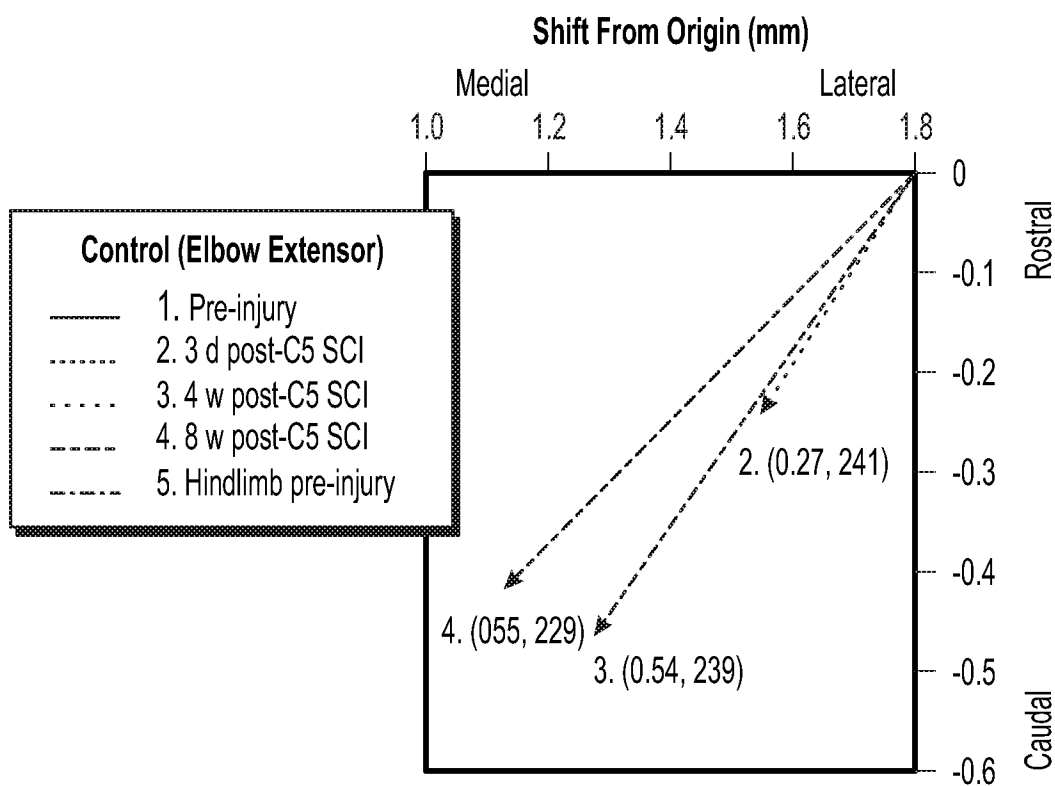
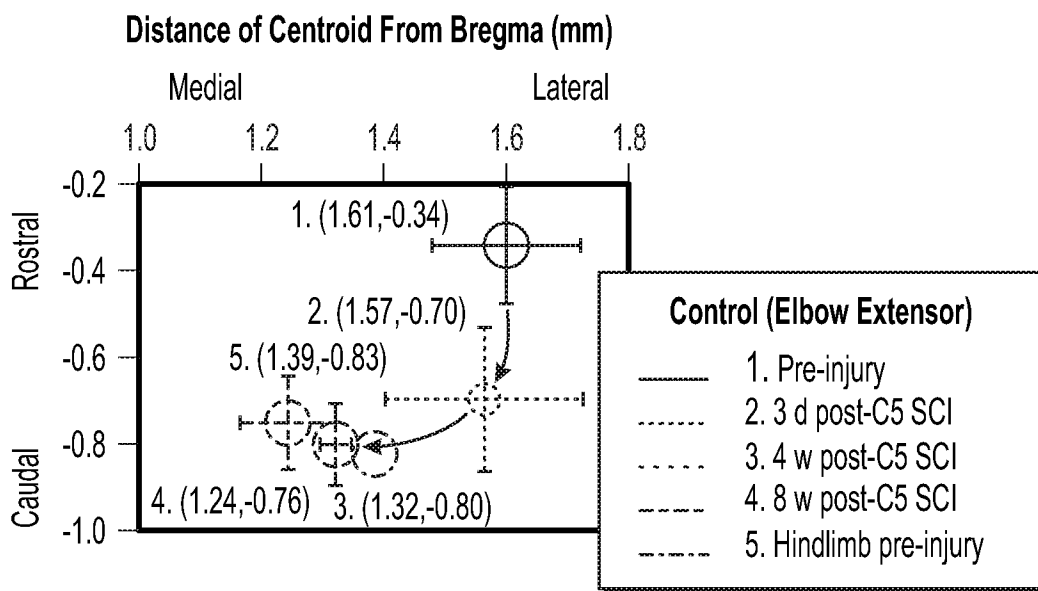


FIG. 14B

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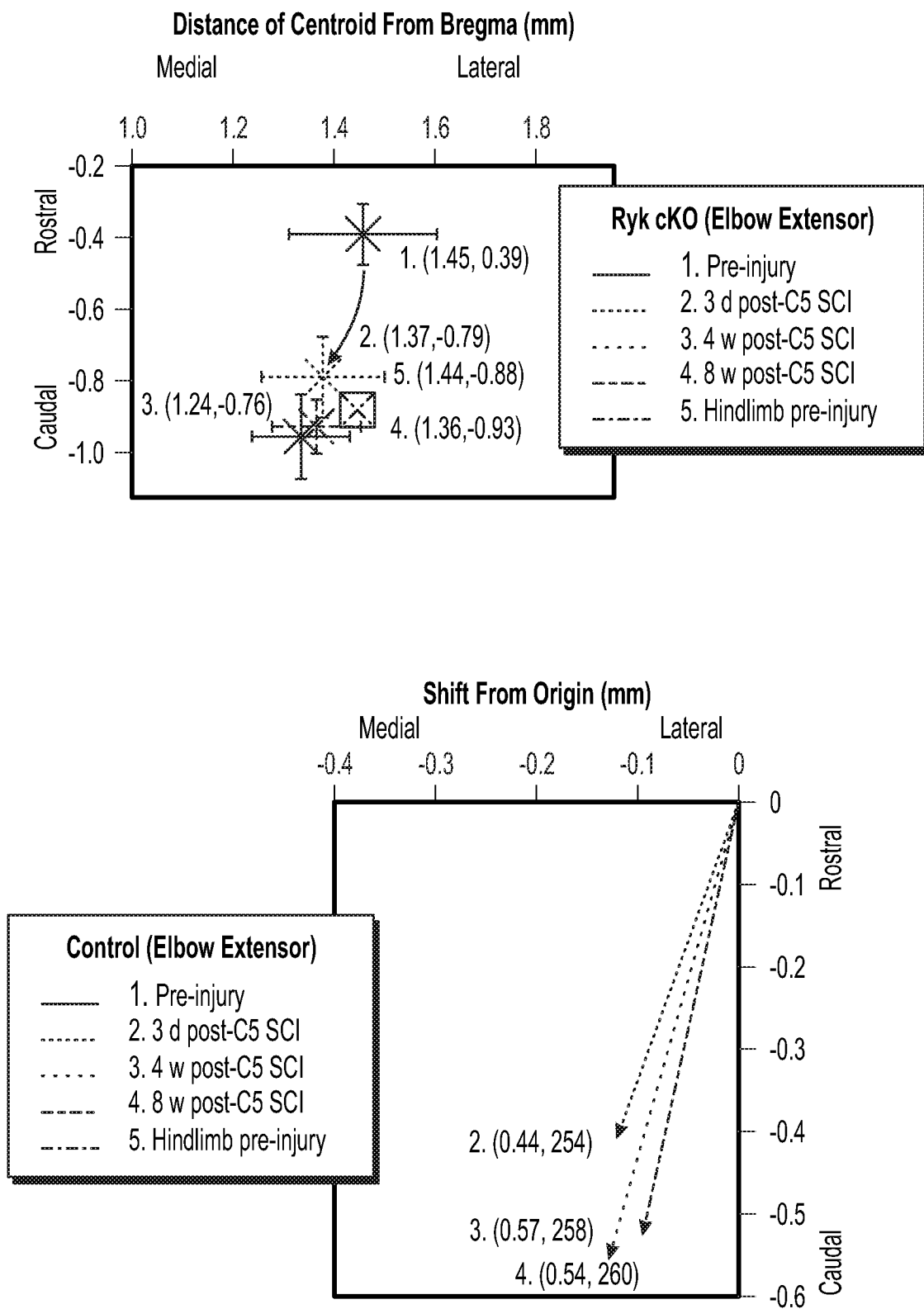


FIG. 14C

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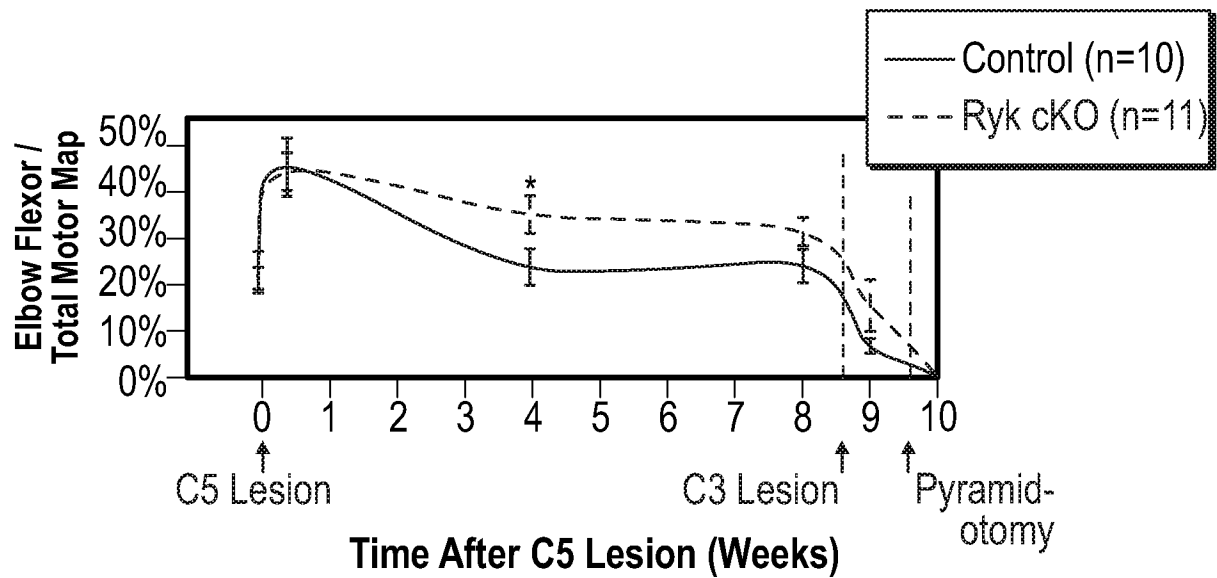


FIG. 14D

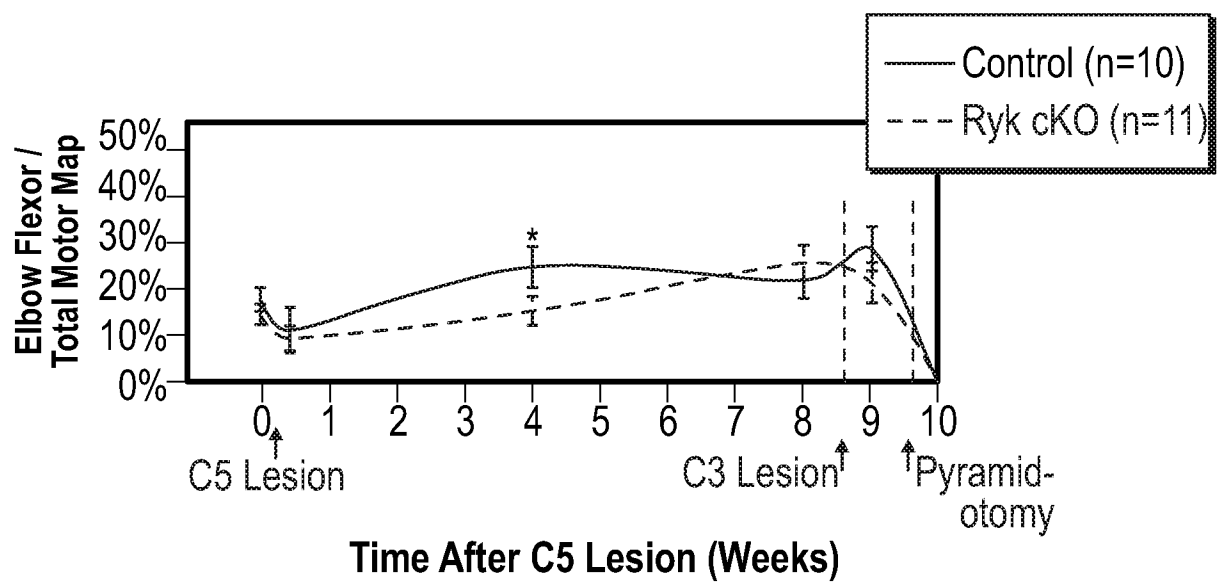


FIG. 14E

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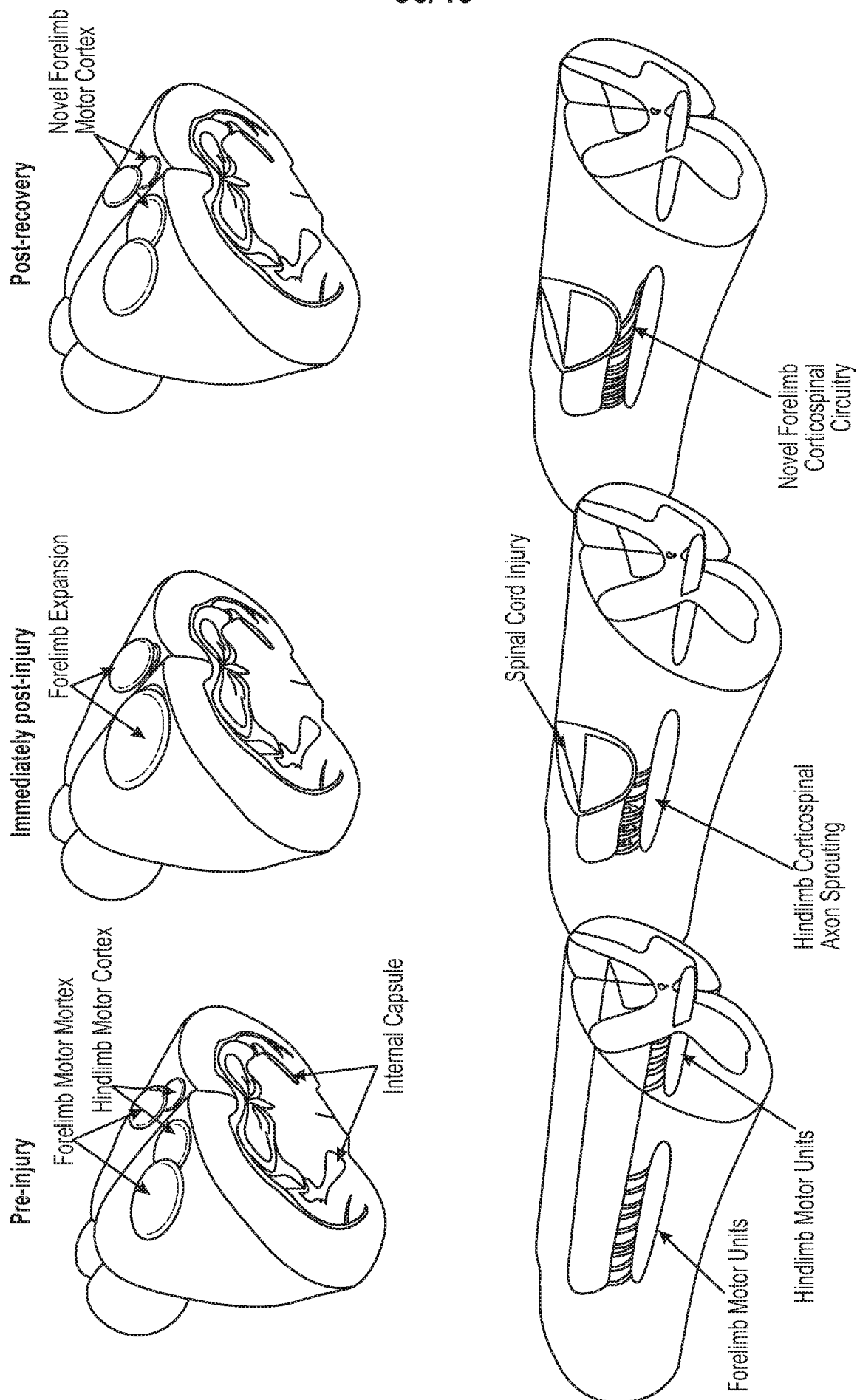


FIG. 14F

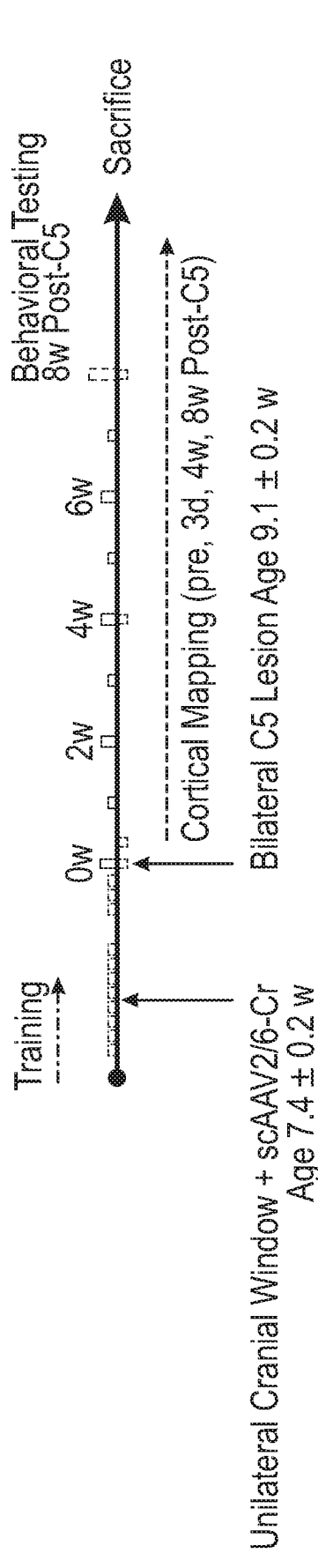


FIG. 15A

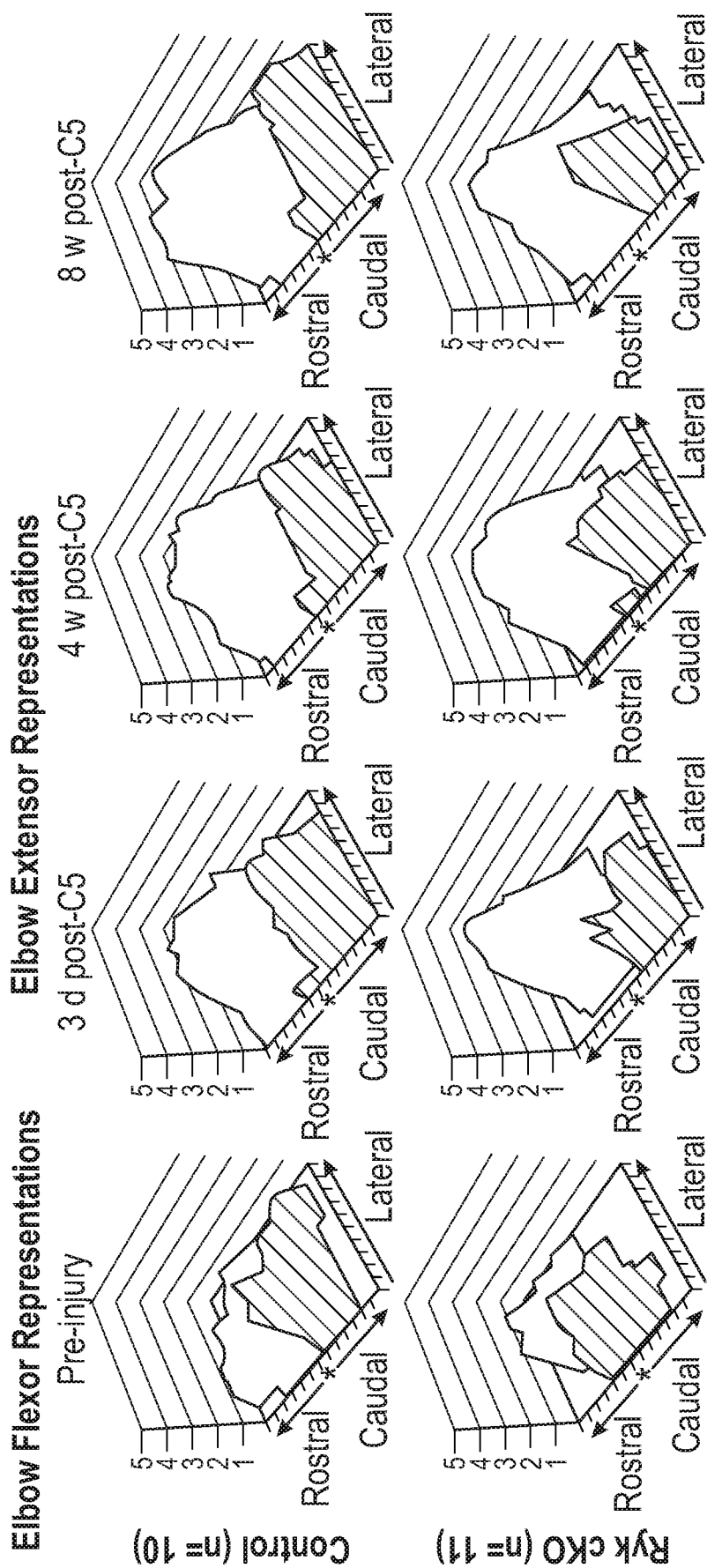


FIG. 15B

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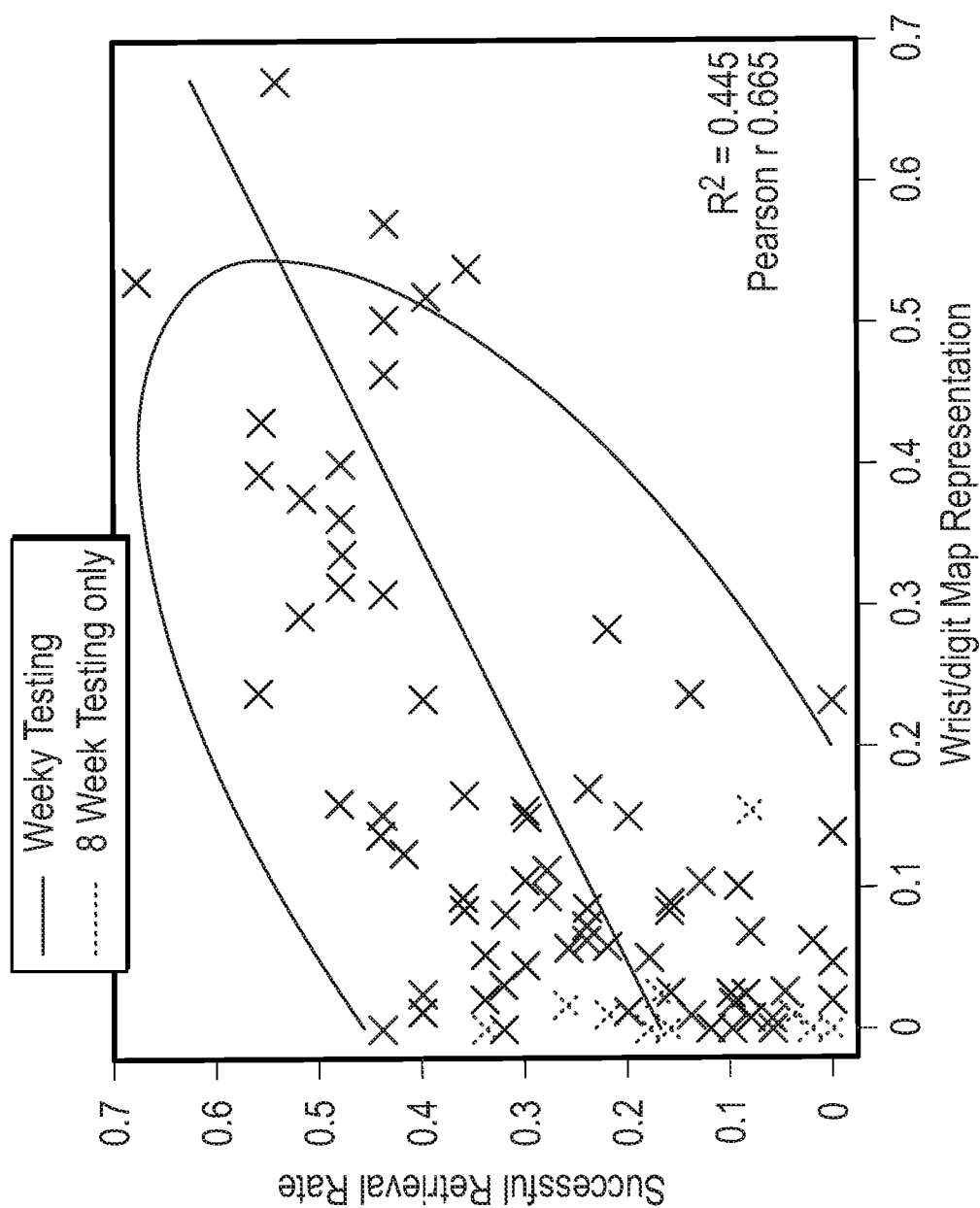


FIG. 15D

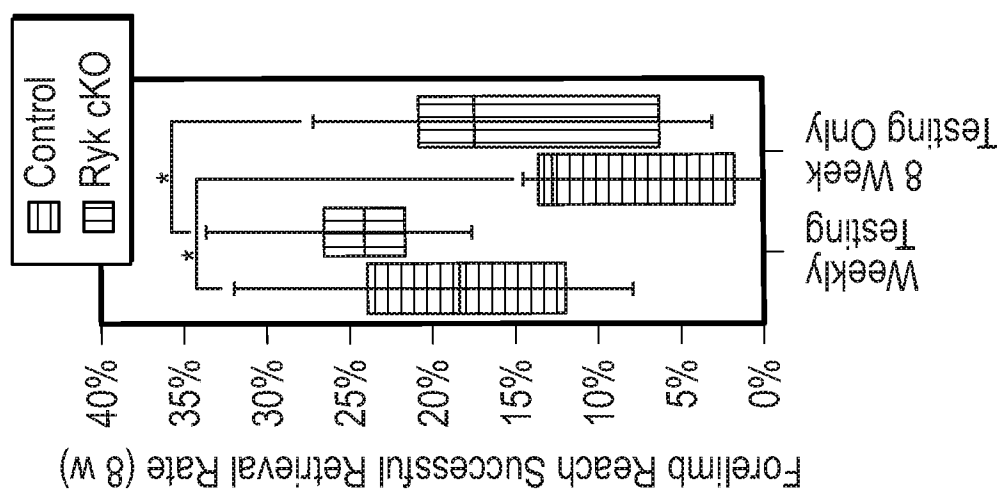


FIG. 15C

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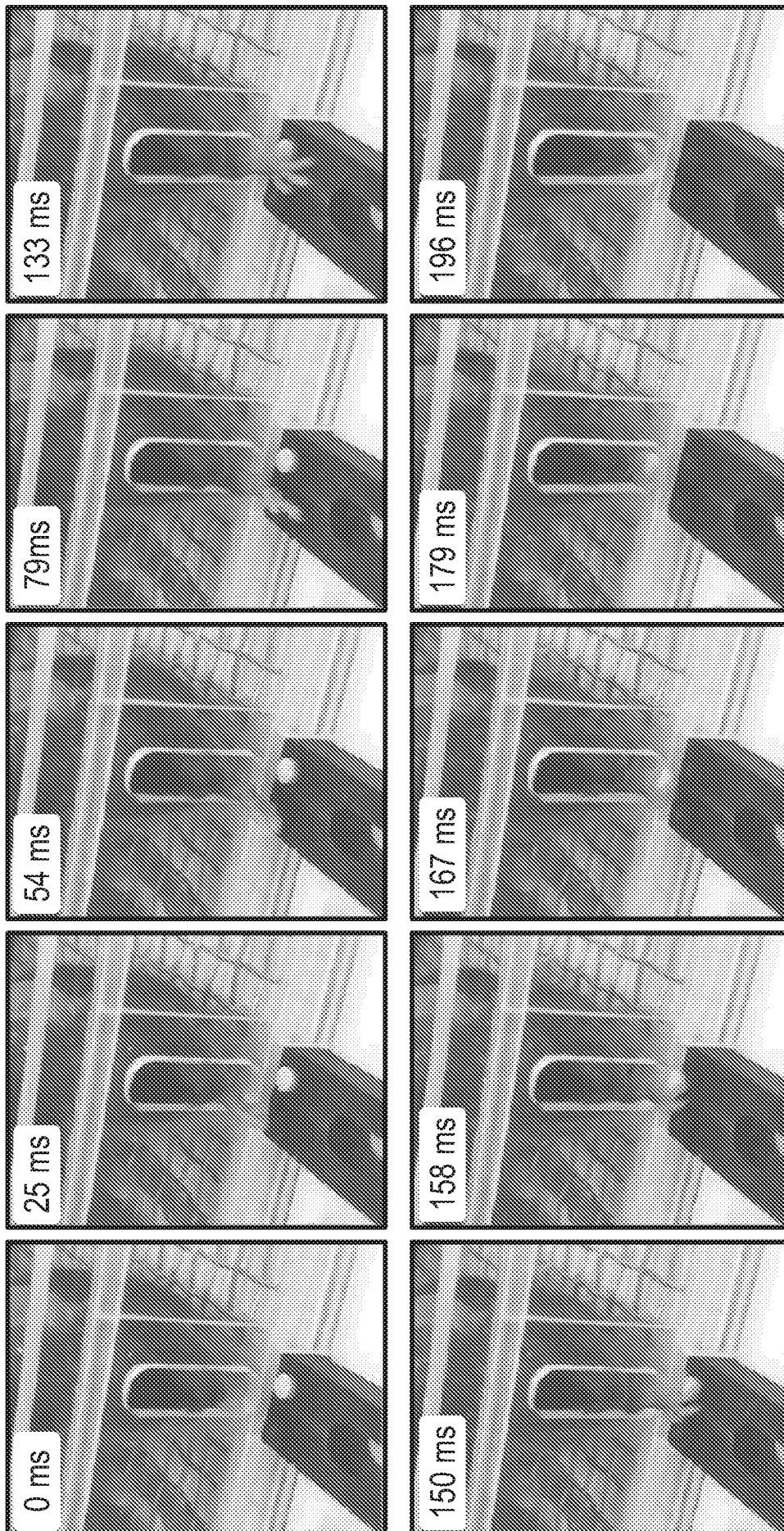


FIG. 16

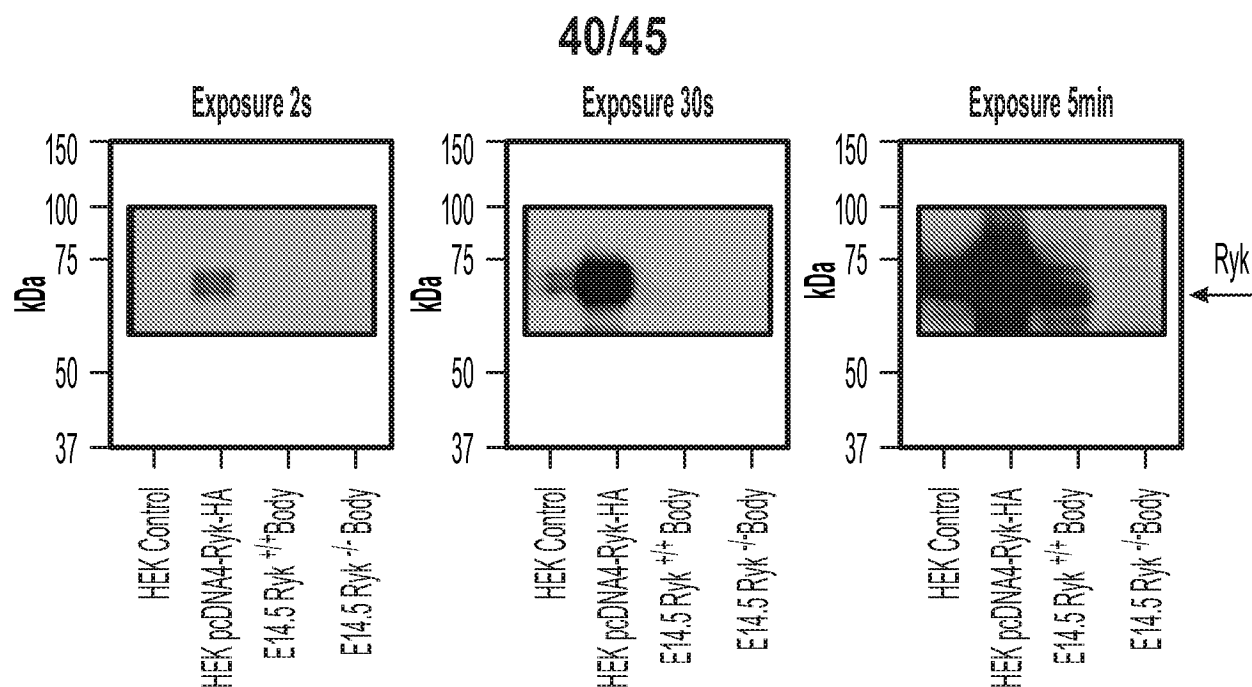


FIG. 17A

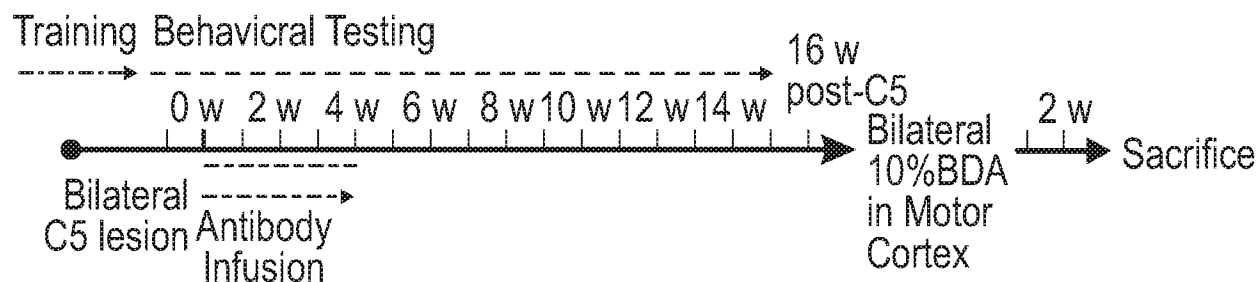


FIG. 17B

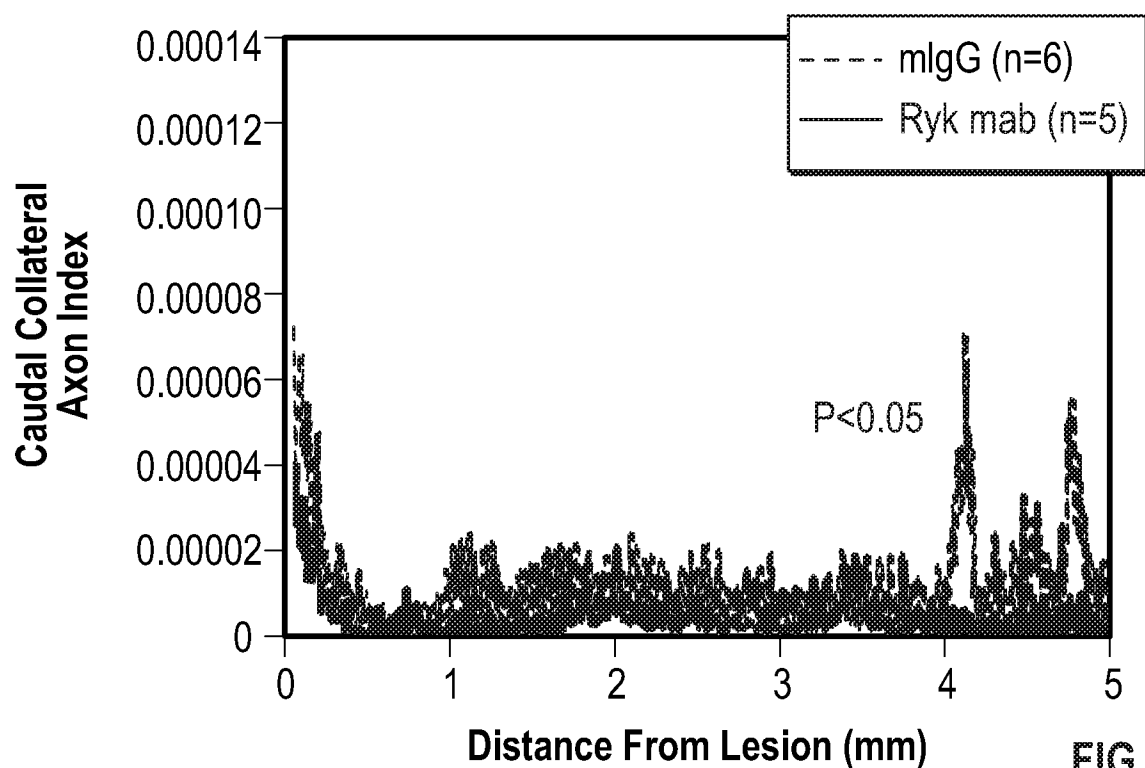
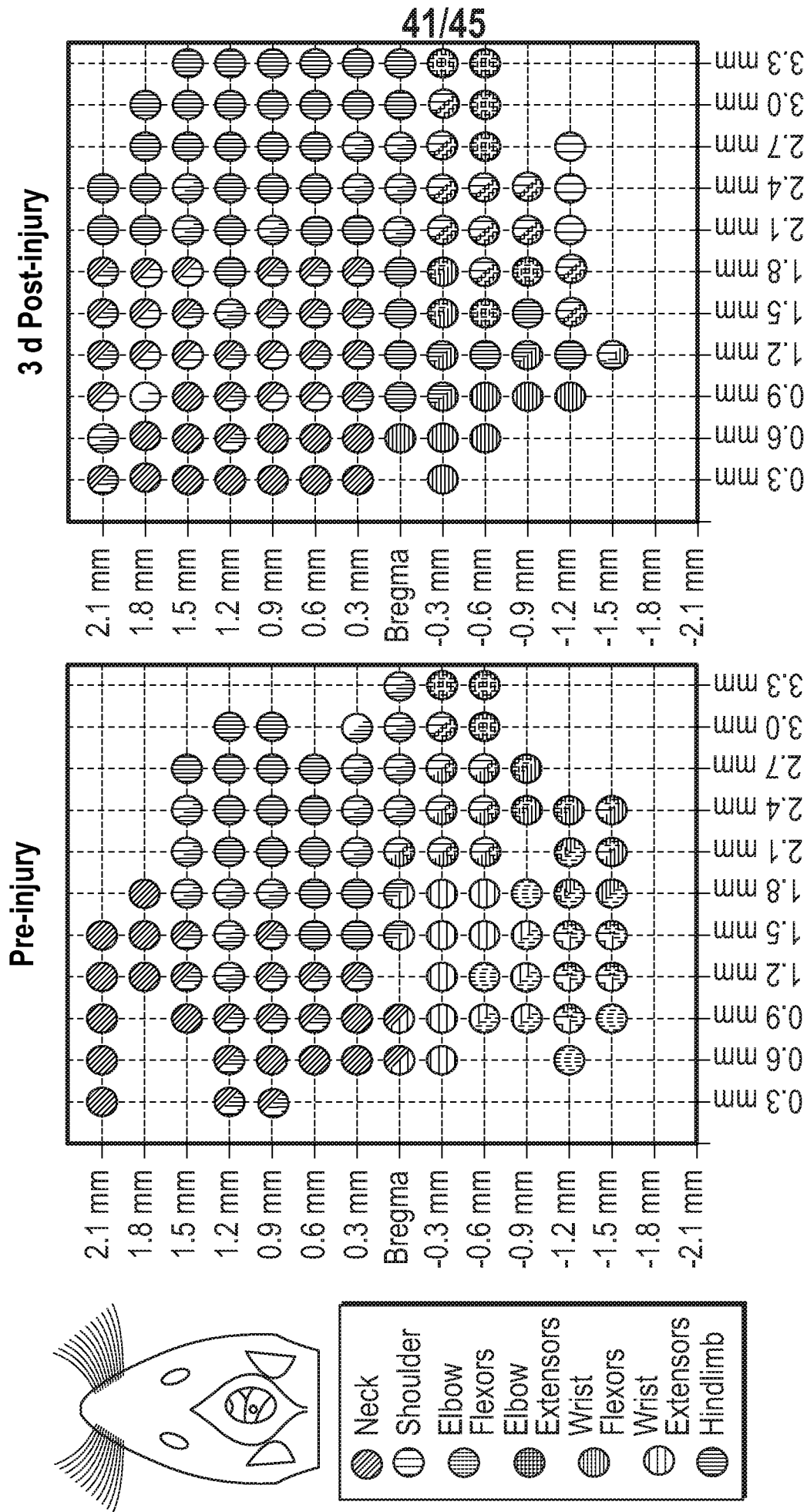


FIG. 18



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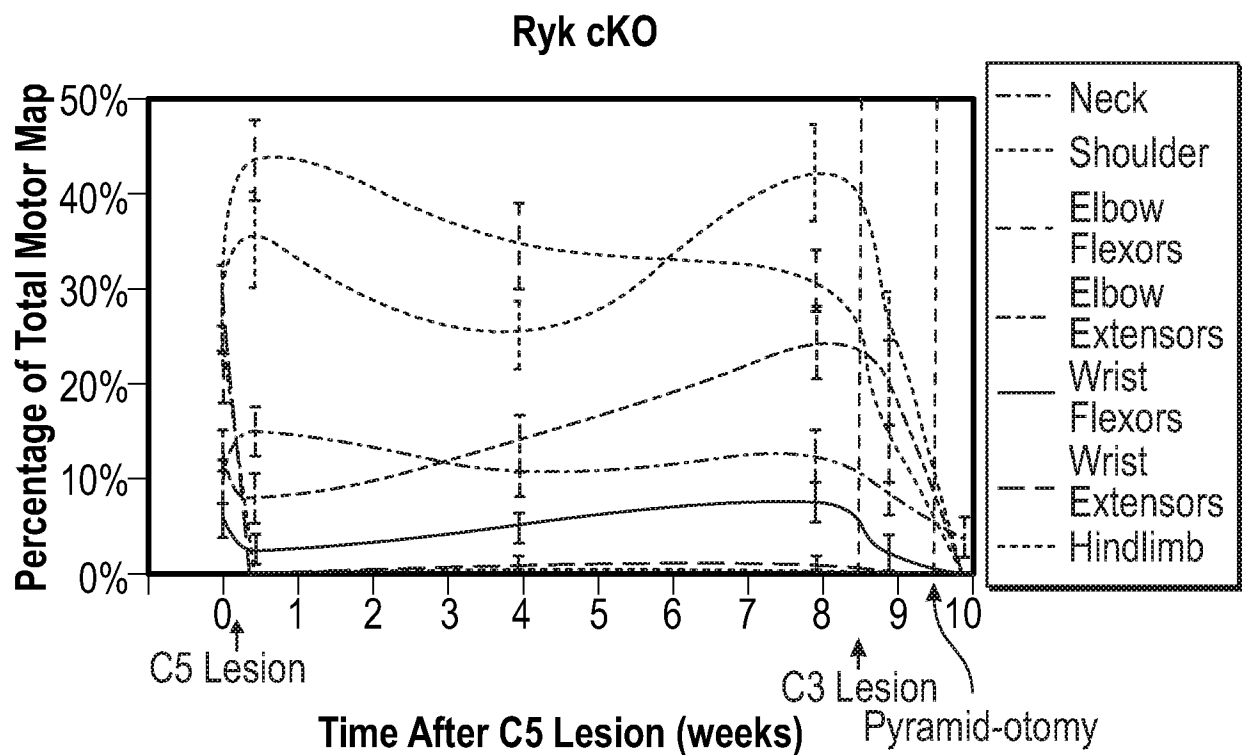
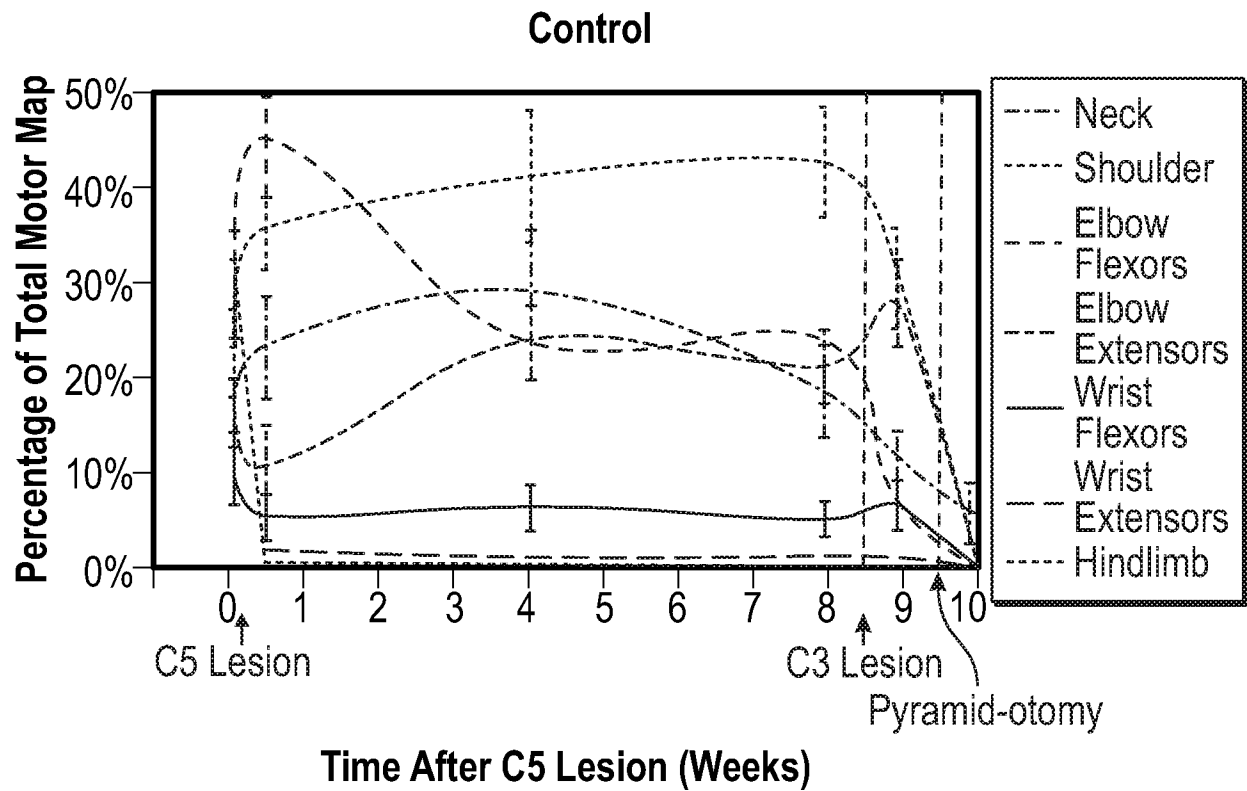


FIG. 20

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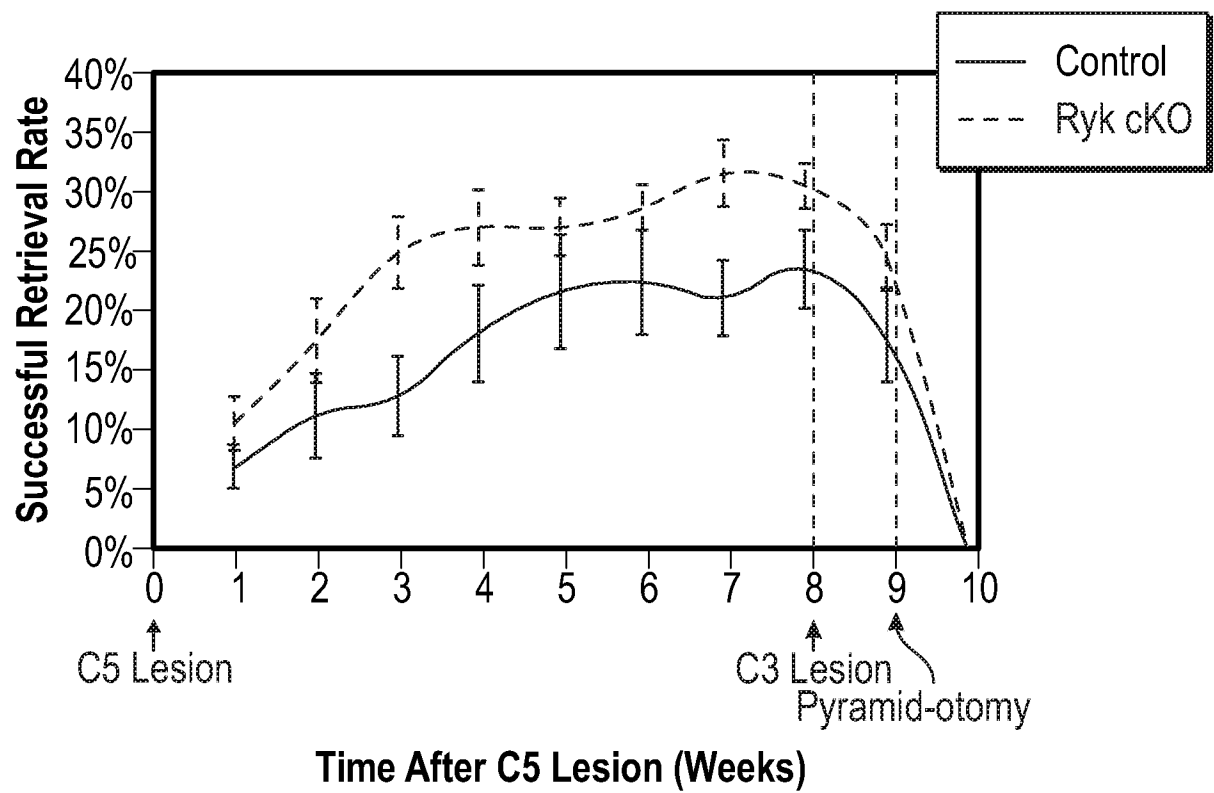


FIG. 21

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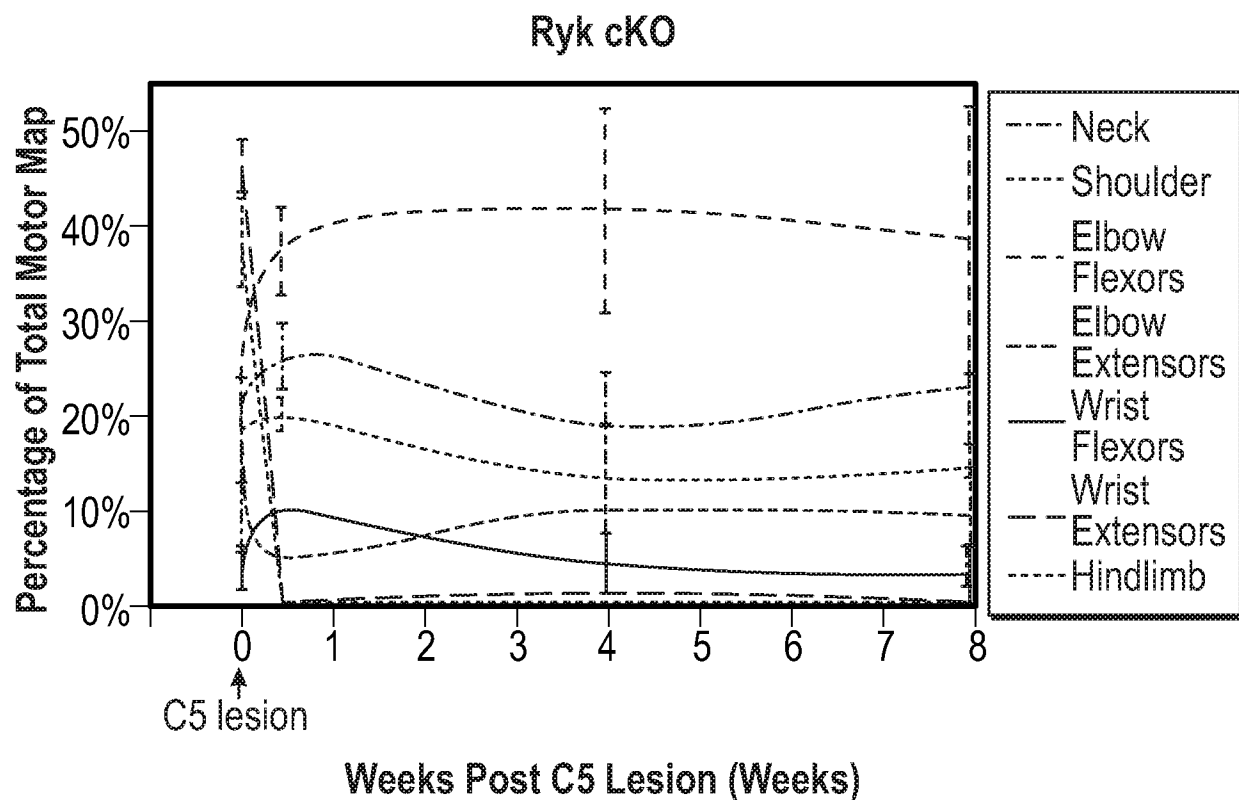
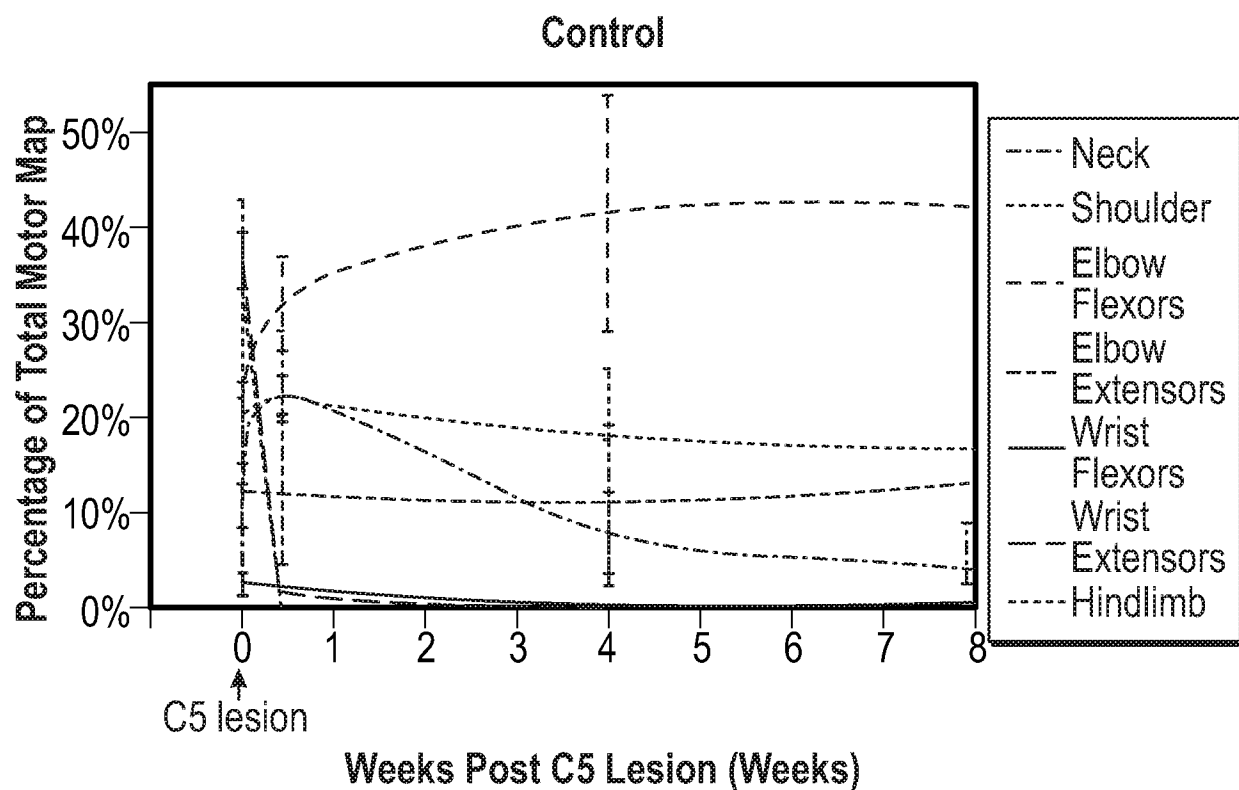


FIG. 22

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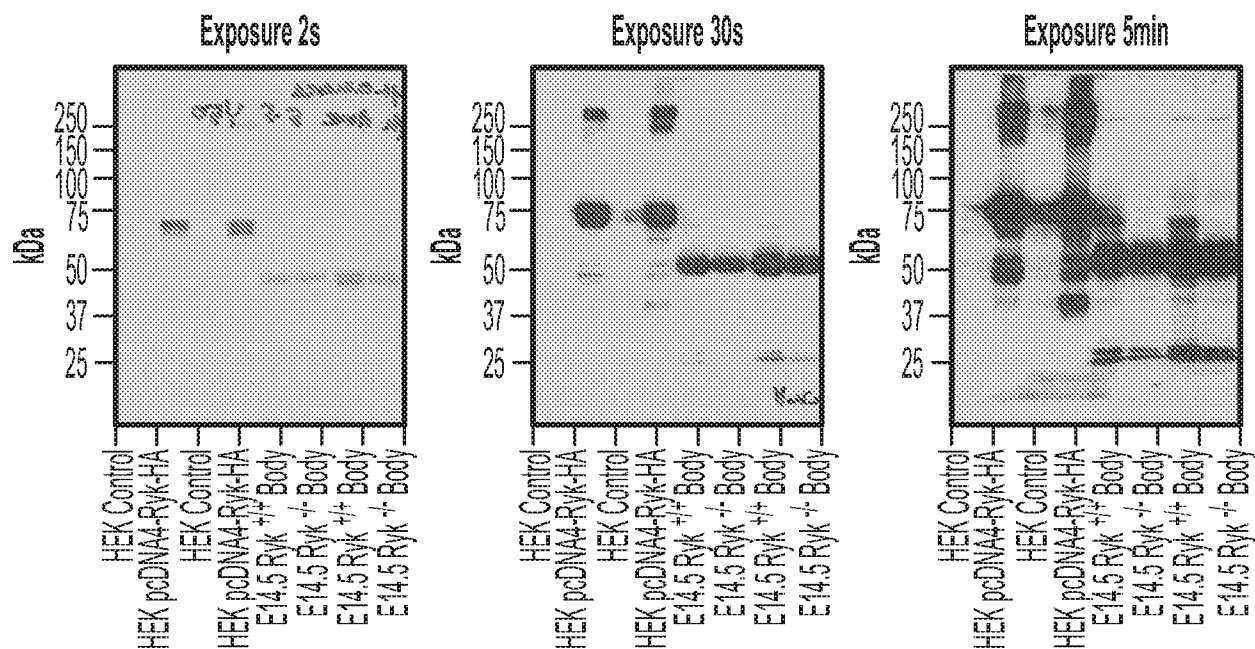


FIG. 23A

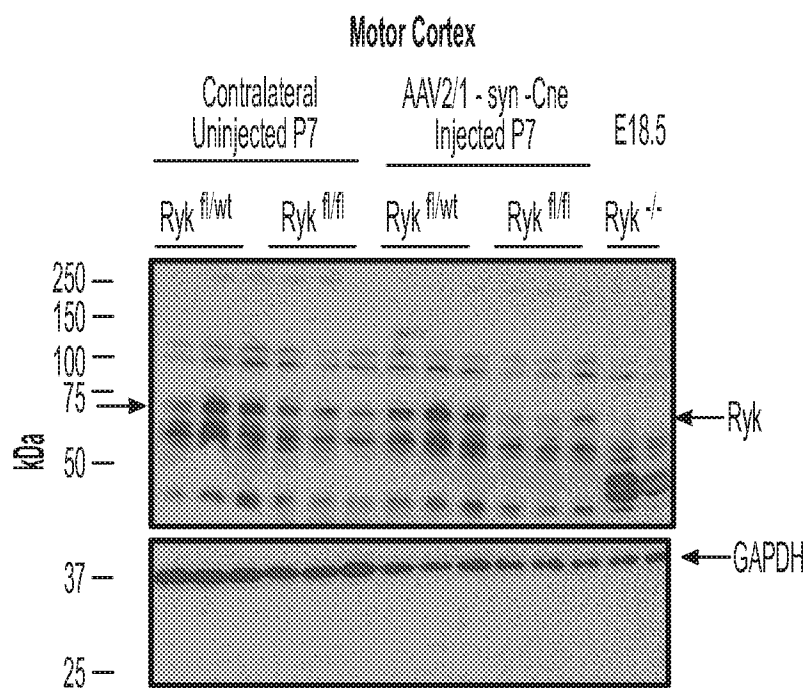


FIG. 23B

20378-201389_SL.TXT
SEQUENCE LISTING

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<120> ANTI-RYK ANTIBODIES AND METHODS OF USING THE SAME
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<150> 62/314,025
<151> 2016-03-28
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<210> 2
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Arg Ala Asn
1

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20378-201389_SL.TXT

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35 40 45

Tyr Arg Ala Asn Arg Leu Val Asp Gly Val Pro Ser Arg Phe Ser Gly
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Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Glu Tyr
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Glu Asp Met Gly Ile Tyr Tyr Cys Leu Gln Tyr Asp Glu Phe Pro Leu
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Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr Ser Met Ser
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Phe Asn Arg Asn Glu Cys
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<212> PRT

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1 5

<210> 6
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
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<210> 7
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Ser Ser Ala Lys
20

<210> 8
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35 40 45

Ala Tyr Ile Ser Asn Gly Gly Gly Gly Thr Tyr Tyr Pro Asp Thr Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
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Leu Gln Met Asn Ser Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys
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20378-201389_SL.TXT

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 130 135 140
 Val Lys Gly Tyr Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly
 145 150 155 160
 Ser Leu Ser Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Asp
 165 170 175
 Leu Tyr Thr Leu Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro
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 Ser Glu Thr Val Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys
 195 200 205
 Val Asp Lys Lys Ile Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile
 210 215 220
 Cys Thr Val Pro Glu Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro
 225 230 235 240
 Lys Asp Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val
 245 250 255
 Val Asp Ile Ser Lys Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val
 260 265 270
 Asp Asp Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln
 275 280 285
 Phe Asn Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Met His Gln
 290 295 300
 Asp Trp Leu Asn Gly Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala
 305 310 315 320
 Phe Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro
 325 330 335
 Lys Ala Pro Gln Val Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala
 340 345 350
 Lys Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu
 355 360 365

Asp Ile Thr Val Glu Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr
370 375 380

Lys Asn Thr Gln Pro Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr
385 390 395 400

Ser Lys Leu Asn Val Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe
405 410 415

Thr Cys Ser Val Leu His Glu Gly Leu His Asn His His Thr Glu Lys
420 425 430

Ser Leu Ser His Ser Pro Gly Lys
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<210> 9
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 9
Gln Asp Ile Asn Arg Tyr
1 5

<210> 10
<211> 214
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polypeptide

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Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Ala Ser Leu Gly
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Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Asn Arg Tyr
20 25 30

Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys Ser Pro Glu Thr Leu Ile
35 40 45

Tyr Arg Ala Asn Arg Leu Val Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Glu Tyr
65 70 75 80

Glu Asp Met Gly Ile Tyr Tyr Cys Leu Gln Tyr Asp Glu Phe Pro Leu
85 90 95

Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Ala
100 105 110

Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu Thr Ser Gly
115 120 125

Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro Lys Asp Ile
130 135 140

Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn Gly Val Leu
145 150 155 160

Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr Ser Met Ser
165 170 175

Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His Asn Ser Tyr
180 185 190

Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro Ile Val Lys Ser
195 200 205

Phe Asn Arg Asn Glu Cys
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<210> 11
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 11
Ile Ser Thr Gly Gly Gly Ser Thr
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<210> 12
<211> 19
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 12
His Gly Glu Phe Asn Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser
1 5 10 15

Ala Ala Lys

<210> 13
<211> 439

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polypeptide

<400> 13

Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
 20 25 30

Thr Met Ser Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val
 35 40 45

Ala Tyr Ile Ser Thr Gly Gly Gly Ser Thr Tyr Tyr Pro Asp Thr Val
 50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80

Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met Tyr Tyr Cys
 85 90 95

Ala Arg His Gly Glu Phe Asn Tyr Trp Gly Gln Gly Thr Leu Val Thr
 100 105 110

Val Ser Ala Ala Lys Thr Thr Pro Pro Ser Val Tyr Pro Leu Ala Pro
 115 120 125

Gly Ser Ala Ala Gln Thr Asn Ser Met Val Thr Leu Gly Cys Leu Val
 130 135 140

Lys Gly Tyr Phe Pro Glu Pro Val Thr Val Thr Trp Asn Ser Gly Ser
 145 150 155 160

Leu Ser Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Asp Leu
 165 170 175

Tyr Thr Leu Ser Ser Ser Val Thr Val Pro Ser Ser Thr Trp Pro Ser
 180 185 190

Glu Thr Val Thr Cys Asn Val Ala His Pro Ala Ser Ser Thr Lys Val
 195 200 205

Asp Lys Lys Ile Val Pro Arg Asp Cys Gly Cys Lys Pro Cys Ile Cys
 210 215 220

Thr Val Pro Glu Val Ser Ser Val Phe Ile Phe Pro Pro Lys Pro Lys
 225 230 235 240

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Asp Val Leu Thr Ile Thr Leu Thr Pro Lys Val Thr Cys Val Val Val
 245 250 255
 Asp Ile Ser Lys Asp Asp Pro Glu Val Gln Phe Ser Trp Phe Val Asp
 260 265 270
 Asp Val Glu Val His Thr Ala Gln Thr Gln Pro Arg Glu Glu Gln Phe
 275 280 285
 Asn Ser Thr Phe Arg Ser Val Ser Glu Leu Pro Ile Met His Gln Asp
 290 295 300
 Trp Leu Asn Gly Lys Glu Phe Lys Cys Arg Val Asn Ser Ala Ala Phe
 305 310 315 320
 Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys Gly Arg Pro Lys
 325 330 335
 Ala Pro Gln Val Tyr Thr Ile Pro Pro Pro Lys Glu Gln Met Ala Lys
 340 345 350
 Asp Lys Val Ser Leu Thr Cys Met Ile Thr Asp Phe Phe Pro Glu Asp
 355 360 365
 Ile Thr Val Glu Trp Gln Trp Asn Gly Gln Pro Ala Glu Asn Tyr Lys
 370 375 380
 Asn Thr Gln Pro Ile Met Asp Thr Asp Gly Ser Tyr Phe Val Tyr Ser
 385 390 395 400
 Lys Leu Asn Val Gln Lys Ser Asn Trp Glu Ala Gly Asn Thr Phe Thr
 405 410 415
 Cys Ser Val Leu His Glu Gly Leu His Asn His His Thr Glu Lys Ser
 420 425 430
 Leu Ser His Ser Pro Gly Lys
 435