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ApoE Mimetic Ameliorates Motor Deficit and Tissue Damage in Rat Spinal Cord Injury

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Apolipoprotein E (apoE), a plasma protein responsible for transporting lipid and cholesterol, modulates responses of the central nervous system to injury. Small peptides derived from the receptor-binding region of apoE can simulate some important bioactivities of apoE holoprotein and offer neuroprotection against brain injury. We tested whether COG1410, an apoE-mimetic peptide, provides protection in a rat model of spinal cord injury (SCI). Traumatic injury was created at T8 by a cortical impact device. Injured rats were randomized to four treatment groups: vehicle, 0.15, 0.3, or 0.6 mg/kg COG1410; sham surgery rats received vehicle. Basso, Beattie, Bresnahan neurological score was evaluated prior to injury and at 1, 3, 7, and 14 days after injury. Histological changes were evaluated at 14 days. All injured rats lost body weight during the first week following injury. Body weight recovery was significantly improved in rats treated with COG1410. Mechanical impact resulted in severe motor deficit, and most animals had a BBB score of 0–1 at 24 hours postinjury. COG1410-treated rats showed significantly improved functional recovery and ameliorated motor deficit at 14 days postinjury. Histological analysis showed that COG1410 groups had a significantly reduced lesion size at the site of injury, a larger preserved luxol fast blue-stained area, and more visible neurons in the surrounding area of injury. Microglial activation was also significantly suppressed. These findings indicate that this apoE mimetic effectively improved neurological and histological outcome following SCI in rats, and the effect was associated with inhibition of microglial activation. © 2014 Wiley Periodicals, Inc.

Key words: apoE; mimetic; outcome; rat; spinal cord injury

Apolipoprotein E (apoE) acts as a vehicle for transporting cholesterol among various cells in the body (Mahley, 1988). ApoE accumulates at the injury site when peripheral nerves are injured, suggesting a role in the injury repair process (Mahley, 1988). Compared with wild-type apoE-sufficient mice, apoE-deficient mice show susceptibility to ischemic insult, with both neurological deficit and infarct size worsened (Laskowitz et al., 1997b). Blood–nerve and blood–brain barriers are also impaired in apoE-knockout mice (Fullerton et al., 2001). These findings indicate that apoE is a key protein involved in maintaining the health of the central nervous system (CNS) and responding to injury. In a mouse model of spinal cord injury (SCI), both mRNA and protein levels of apoE increase following injury (Seitz et al., 2003). The role of apoE in SCI has not yet been determined, and it is not known whether exogenous apoE would provide a beneficial effect on recovery following SCI.

ApoE is a large molecule with 299 amino acids. Smaller apoE-mimetic peptides have been developed to improve permeation of the blood–brain barrier. These smaller peptides, derived from the receptor-binding region of apoE, exhibit anti-inflammatory effects similar to holo-apoE and have proved to be effective in treating brain injury in multiple mouse models such as traumatic brain injury, stroke, subarachnoid hemorrhage, and multiple

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sclerosis (Lynch et al., 2005; Gao et al., 2006; Laskowitz et al., 2006, 2007; Li et al., 2006; Hoane et al., 2007, 2009; Laskowitz et al., 2012; Wang et al., 2013). COG1410 is one such peptide, containing just 12 amino acids derived from apoE residues 138–149 with amino isobutyric acid (Aib) substitution at positions 140 and 145, and has a more potent anti-inflammatory effect. In the present study, we investigated the effect of exogenous COG1410 on neurological deficit and tissue damage in a rat model of spinal cord injury and evaluated the therapeutic potential of this apoE mimetic.

MATERIALS AND METHODS

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The Institutional Animal Care and Use Committee at Duke University approved all aspects of the study design. Experimental protocols adhere to the NIH *Guide for the care and use of laboratory animals*. Male Wistar rats weighing 250–300 g were purchased from Harlan Laboratories (Indianapolis, IN) and housed at the Duke University animal facility with a 12-hour day–night light cycle.

Rats were anesthetized with 5% isoflurane in 30% oxygen balanced with nitrogen. After intraoral trachea intubation, both lungs were mechanically controlled using a rodent ventilator (model 683; Harvard Apparatus, Holliston, MA), and isoflurane was reduced to 1.8% for surgery. Body temperature was maintained at 37°C using a surface heating and cooling system. A 2-cm midline skin incision was made on the surface of the back at the level of T8–9. The paravertebral muscles were dissected and retracted laterally. Laminectomy was performed to create a 4- × 5-mm window. The dura remained intact. Rats were then mounted onto the stereotaxic frame, and a 3-mm-diameter flat metal impact tip was lowered onto the dorsal surface of the spinal cord. The spinal cord contusion was made using a controlled cortical injury device (eCCI model 6.3; Custom Design and Fabrication, Richmond, VA) at a speed of 5 msec, depression of 1 mm, and dwell time of 100 msec. Controlled cortical impact device was previously reported to generate a simple and reproducible injury in rats (Hiruma et al., 1999). The wound was sutured in both muscle and skin layers, and triple antibiotic ointment was applied to the skin surface.

Experimental Groups and Treatment

After injury, rats were randomly assigned to one of the following four treatment groups: vehicle (lactated Ringer's solution), 0.15, 0.3, or 0.6 mg/kg COG1410 in lactated Ringer's solution, respectively (n = 20 per group). Sham animals were subjected to the same surgical procedure without injury and received Ringer's solution (n = 8). The first treatment was given through the tail vein injection 5 min after injury. The second treatment was injected intraperitoneally at 3 hr postinjury and repeated once per day for 2 weeks. The volume of solution was calculated based on body weight (μl/g). A technician having no responsibility for assessing neurologic score or measuring the lesion was assigned to administer treatment and keep the log until the completion of the experiment.

COG1410 was provided by Cognosci (Research Triangle Park, NC), which was synthesized by NeoMPS (San Diego, CA) with a purity of 95%. This peptide is derived from apoE residues 138–149 with Aib substitutions at positions 140 and 145 with the sequence AS (Aib) LRKL(Aib)KRLL. The injection solution was prepared with lactated Ringer's USP (Hospira, Lake Forest, IL).

Behavioral Tests

Rats were weighed daily before injections to monitor general postsurgical recovery. Neurological deficit was evaluated before surgery and at 1, 3, 7, and 14 days after surgery using a 21-point Basso, Beattle, Bresnahan (BBB) locomotor rating scale (0 = severe and 21 = normal; Basso et al., 1995). An investigator blinded to group assignment performed the neurological tests and assessed histological damage. Rats with the BBB score beyond 2 standard deviations at 24 hr postinjury were considered as outliers and excluded from the study.

Histological Analysis

At the conclusion of the 2-week treatment period, rats were anesthetized and intubated. The chest was opened, and a blunt needle was inserted into the aorta through the left ventricle. Rats were perfused with 100 ml 0.9% sodium chloride solution with 1:250 heparin (2,000 μl/500 ml) followed by 200 ml 10% formalin in PBS, pH 7.4. The spinal cords containing the lesions were carefully harvested and paraffin embedded. A series of 5-μm-thick sections was cut from the injury segment at 250-μm intervals. Four sets of slides (24 sections per animal) were dried in room air and then stained with either hemotoxylin and eosin or luxol fast blue and cresyl violet or were evaluated by GFAP and Iba-1 immunohistochemistry.

The hemotoxylin and eosin (H&E)-stained slides were used to determine the number of surviving neurons in the ventral horn under a light microscope (×40 Leitz DMRB; Leica Microsystems, Buffalo Grove, IL). The number of surviving neurons in each ventral horn was manually counted, and the total number of living neurons at the individual level was analyzed.

Luxol fast blue stains myelin and was used to assess SCI-induced white matter demyelination. The stained area, representing the preserved white matter, was measured from each section. The edge of the stained area was outlined, and the area was automatically computed. The volume of preserved white matter was calculated based on the intervals of two measured sections and used for statistical analysis.

GFAP immunofluorescence staining was used to define the edge of the lesion size. The slides were deparaffinized in xylene; hydrated through 100%, 80%, and 70% alcohol and distilled water; and autoclaved in 0.01 M sodium citrate buffer (pH 6.0) for 2 min. After washing in PBS three times, the sections were blocked by a mixture of 5% normal goat serum, 2% BSA, and 0.1% Triton X-100 in PBS for 2 hr and then incubated in primary antibody polyclonal rabbit antiglial fibrillary acidic protein (GFAP; 1:500; Dako, Glostrup, Denmark) overnight at 4°C. After washing in PBS six times, secondary antibody Alexa Fluor 488 goat-anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA) was added on sections and left at room temperature for 2 hr. After washing in PBS four times, the slides were covered using a Fluoroshield mounting medium

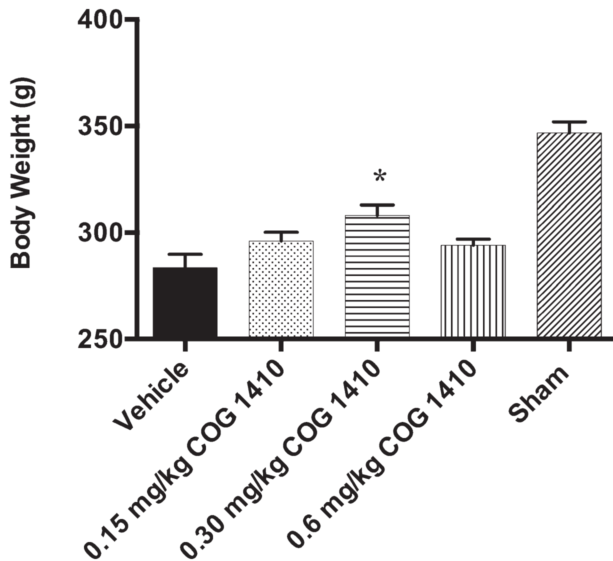

Fig. 1. Body weight at 14 days postsurgery. Rats treated with 0.3 mg/kg COG1410 showed significantly improved body weight recovery compared with vehicle. Data are expressed as mean ± SEM. *$P = 0.03$ vs. vehicle.

(Sigma-Aldrich, St. Louis, MO). The edge of lesion was easily viewed under a light microscope (Leitz DMRB; Leica). Total area of the spinal cord cross-section and the lesion area were measured separately. The outline was drawn around the edge, and an image analysis (MCID; Imaging Research, St. Catharines, Ontario, Canada) automatically computed the target area. The injury severity was expressed as a percentage of lesion size in spinal cord (lesion area/total area of spinal cord cross-section × 100%).

Iba-1 immunohistochemistry was used to evaluate SCI-induced microglial activation in the 0.6 mg/kg COG1410-treatment group. This group was selected because this dose was previously reported to reduce the inflammatory response significantly (Laskowitz et al., 2007). The slides went through a series of solutions: xylene; 100%, 80%, and 70% alcohol; and distilled water. After washing in 0.01 M PBS, slides were incubated in 0.3% hydrogen peroxide in methanol for 30 min and then washed in 0.01 M PBS again. After incubation with blocking buffer (1.5% normal goat serum and 1% BSA in 0.01 M PBS) at room temperature for 2 hr, the slides were incubated in anti-Iba-1 rabbit primary antibody (0.5 μg/ml, Wako Chemicals, Richmond, VA) overnight at 4°C. The slides were then washed in 0.01 M PBS and incubated with biotonylated anti-rabbit IgG antibody (1:200) at room temperature for 1 hr. After washing three times in 0.01 M PBS, the slides were incubated with Elite ABC reagent (Vector Laboratories, Burlingame, CA) at room temperature for 1 hr. After washing three times again, they were incubated in 0.01% hydrogen peroxidase and 0.05% diaminobenzidine (DAB) in 0.05 M Tris buffer for 10 sec and then placed in distilled water to stop the reaction. The slides were then dehydrated, cleaned, and covered. The number of positive microglia cells was counted in the ventral area of each section.

Statistical Analysis

Data were expressed as mean ± SEM and analyzed using one-way ANOVA or repeated measures for single or multiple time points, respectively. Post hoc tests were performed using Tukey's multiple comparison test in GraphPad Prism 6.0a (GraphPad Software, La Jolla, CA). $P < 0.05$ was considered statistically significant. The sham group served as a normal reference for behavioral and histological changes and was not analyzed along with the injured groups.

RESULTS

Body Weight Loss and Mortality Were Attenuated After ApoE Mimetic Treatment

No body weight difference was observed among groups prior to surgery. In the first week following surgery, all injured rats lost body weight, whereas sham rats gained 4–5 g every day. At 14 days after surgery, COG1410-treated rats demonstrated improved body weight recovery compared with injured rats in the vehicle-treated group ($F[3,64] = 3.82$, $P = 0.01$; Fig. 1). Body weight recovery was significantly improved in rats treated daily with 0.3 mg/kg COG1410 compared with vehicle ($P = 0.03$; Fig. 1). Among the 20 rats in the vehicle group, three died (15% mortality). In COG1410-treated groups, one of 20 rats died in each of the 0.15 mg/kg and 0.3 mg/kg groups (5%), and two of 20 rats died in the 0.6 mg/kg group (10%). Five rats were excluded from the study resulting from insufficient injury because their neurological score was above mean ± 2 SDs in the groups. Thus, the numbers of rats remaining in the study were 15, 19, 18, 16, and eight in the vehicle, 0.15 mg/kg COG1410, 0.3 mg/kg COG1410, 0.6 mg/kg COG1410, and sham groups, respectively.

Motor Function Was Improved Following Treatment With ApoE Mimetic

To assess post-SCI motor deficit, a 21-point BBB score was used (0 = severe deficit, 21 = normal). All rats scored 21 on the BBB scale prior to surgery. At 24 hr postsurgery, the BBB scores had dropped to 0–1 in all injured groups with no significant difference among groups. Motor function was gradually recovered, and the BBB score went up to 5–20 by 14 days postinjury. The 0.3 mg/kg and 0.6 mg/kg COG1410-treated groups showed significant improvement in motor function: 40–50% preinjury level ($F[3,64] = 2.82$, $P < 0.05$; Fig. 2). The 0.15 mg/kg COG1410-treated group had only a transient improvement within 7 days and none at 14 days.

Lesion Size, Neuronal Cell Death, and White Matter Demyelination Were Ameliorated Following ApoE-Mimetic Treatment

To assess the severity of tissue damage, lesion size and number of surviving ventral horn neurons were evaluated. The edge of the lesion was easily identified in these GFAP-stained slides. We measured both lesion area and total area of spinal cord cross-section and then calculated

the percentage of the lesion size in the total area of spinal cord section. In the vehicle group, the average lesion size was 74.62% ± 3.54%; in the COG1410-treatment groups, the lesion size was significantly reduced (F[3,64] = 3.28, $P = 0.03$; Fig. 3). No lesions were found in sham surgery rats. The number of surviving neurons in the ventral horn was counted at the epicenter of the injury and at 400, 800, 1,200, 1,600, and 2,000 μm away from the epicenter. Most of the neurons died at the epicenter, and there was no intergroup difference. However, postinjury neuron survival was significantly improved at 2,000 μm from the injury center in the groups that received 0.3 mg/kg or 0.6 mg/kg COG1410 treatment (F[3,64] = 8.48, $P < 0.01$; Fig. 4).

Luxol fast blue stains normal myelin in the white matter of the spinal cord. Thus, the unstained area represents white matter demyelination induced by impact injury. In all of the injured rats, the stained area was decreased. In the COG1410-treated groups, the stained area was larger compared with vehicle (F[3,64] = 4.62, $P < 0.01$; Fig. 5).

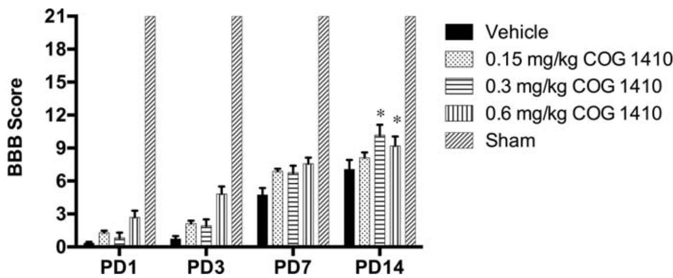

Fig. 2. Neurological deficit at postsurgery days 1, 3, 7, and 14 using 21-point BBB score. All injured rats had a pronounced motor deficit that was significantly improved in the 0.3 and 0.6 mg/kg COG1410-treated groups on PD 14. PD, postsurgery day. *$P < 0.05$ vs. vehicle.

Microglia Activation Was Inhibited in the Group Treated With 0.6 mg/kg ApoE Mimetic

ApoE suppresses the inflammatory response and microglial activation (Laskowitz et al., 1997a, 1998, 2000, 2001). Here we tested whether COG1410 inhibits microglial activation following spinal cord injury. Again, the 0.6 mg/kg COG1410-treatment group was selected for this experiment because this dose was previously reported to significantly reduce the inflammatory response (Laskowitz et al., 2007). A few positive cells were seen in the sham group, which were probably a consequence of the laminectomy surgical procedure. In the vehicle group, there was a significant increase in positive microglia cells,

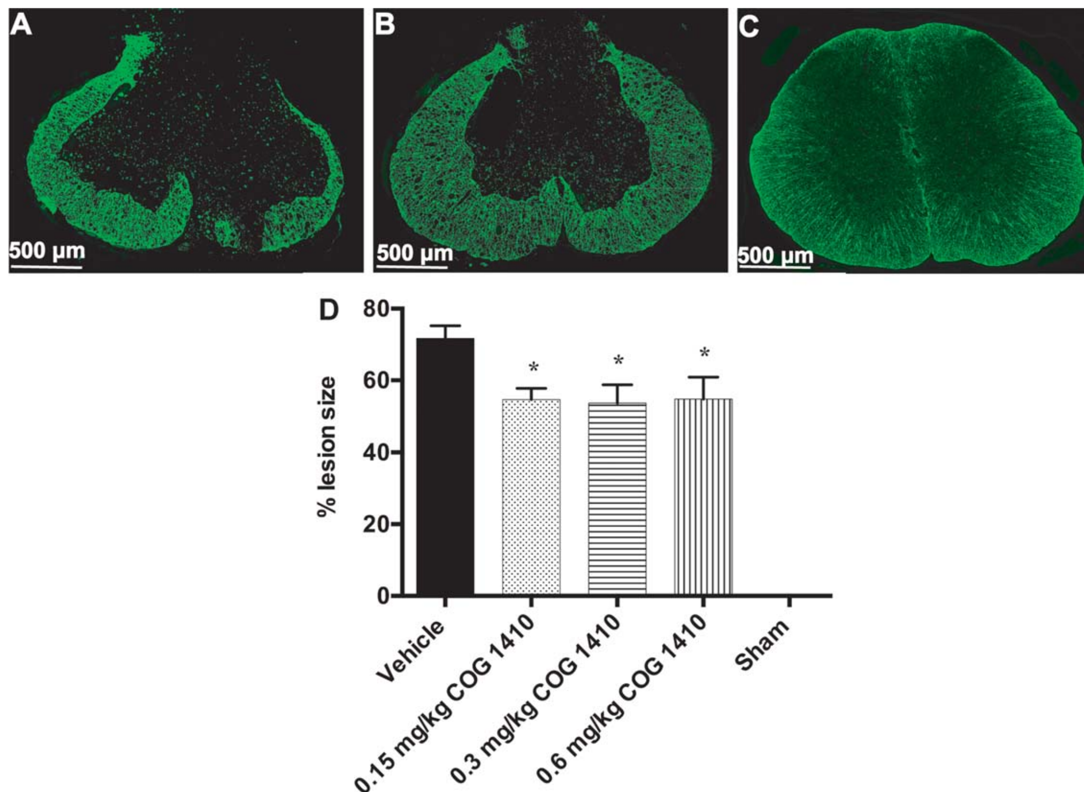

Fig. 3. Lesion size at the injury center was defined by GFAP immunohistochemistry and expressed as percentage of the total area of spinal cord cross-section. **A–C** represent vehicle, 0.3 mg/kg COG1410, and sham, respectively. **D:** Lesion size was significantly reduced in all COG1410-treatment groups. *$P < 0.05$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

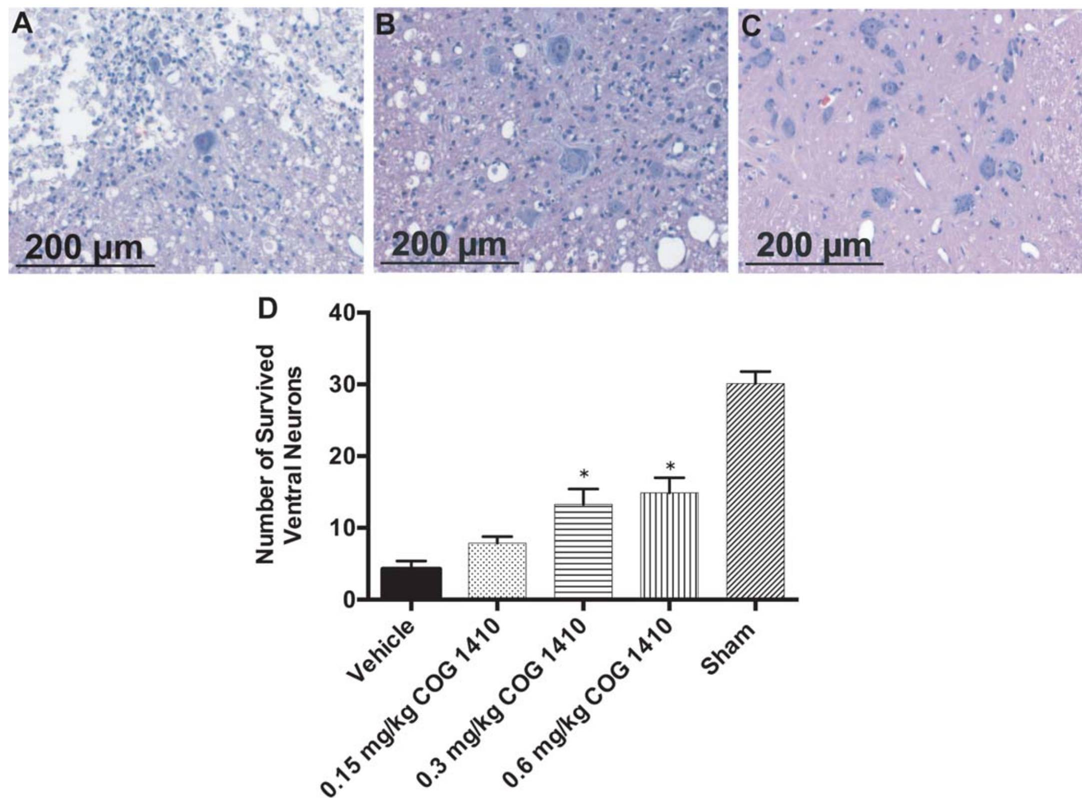

Fig. 4. H&E staining shows surviving neurons in the ventral horn at 2,000 μm from injury center. **A–C** represent vehicle, 0.3 mg/kg COG1410, and sham, respectively. **D:** Neuronal survival was increased significantly in 0.3 mg/kg and 0.6 mg/kg COG1410-treatment groups. $*P < 0.05$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and the COG1410-treated group showed attenuated microglial activation by 50% ($P < 0.01$; Fig. 6).

DISCUSSION

In the present study, we found that COG1410, a small peptide derived from the receptor-binding domain of apoE, reduced SCI-induced neurological deficit, neuronal cell death in the ventral horn, and white matter tissue damage in rats. COG1410 also significantly suppressed microglial activation in our injury model. These results are consistent with other reports that this apoE mimetic provides neuroprotection against brain injury (Gao et al., 2006; Laskowitz et al., 2007, 2012; Wang et al., 2013). This neuroprotective effect has now been demonstrated in two species and in multiple models of CNS injury, suggesting that COG1410 has therapeutic potential for treatment of SCI.

This preliminary efficacy data on the SCI model also support our original hypothesis that apoE represents a therapeutic target for development of SCI therapy. The accumulating data from research on apoE in the last 3 decades show that apoE possesses neuroprotective and neuroregenerative properties, which are most the desirable feature for SCI therapy. In support of this notion, apoE was found to be abundantly expressed in the brain (Koch et al., 2001) and has been associated with the neurobiology of acute and chronic human neurological diseases (Bu G, 2009). Specifically, the presence of the apoE4 allele has been associated with increased susceptibility of developing late-onset familial and sporadic Alzheimer's disease (AD) (Strittmatter et al., 1993) and with poor outcome after acute brain injuries such as stroke, intracranial hemorrhage, and traumatic brain injury (Sheng et al., 1999; Lynch et al., 2002). More recently, apoE was found to be robustly increased during acute SCI and is also present in the recovery stage, when its ability to deliver cholesterol to sites of damage is needed to promote remodeling and repair (Seitz et al., 2003, 2005). Extensive screening for genes changed after SCI showed that apoE is one of a few genes that are most significantly upregulated after SCI (Resnick et al., 2004), suggesting that intrinsic enhancement of apoE expression might contribute an autoreparative instrumental role in the body's response to injury. ApoE was found to accumulate at the injury site following peripheral nerve damage (Ignatius et al., 1986; Snipes et al., 1986; Mahley, 1988). These findings indicate that apoE is important for maintaining CNS health and that it is involved in the mobilization and reutilization of lipids for the repair process.

In the CNS, apoE was demonstrated to exert neuroprotective effects by interfering with several signal

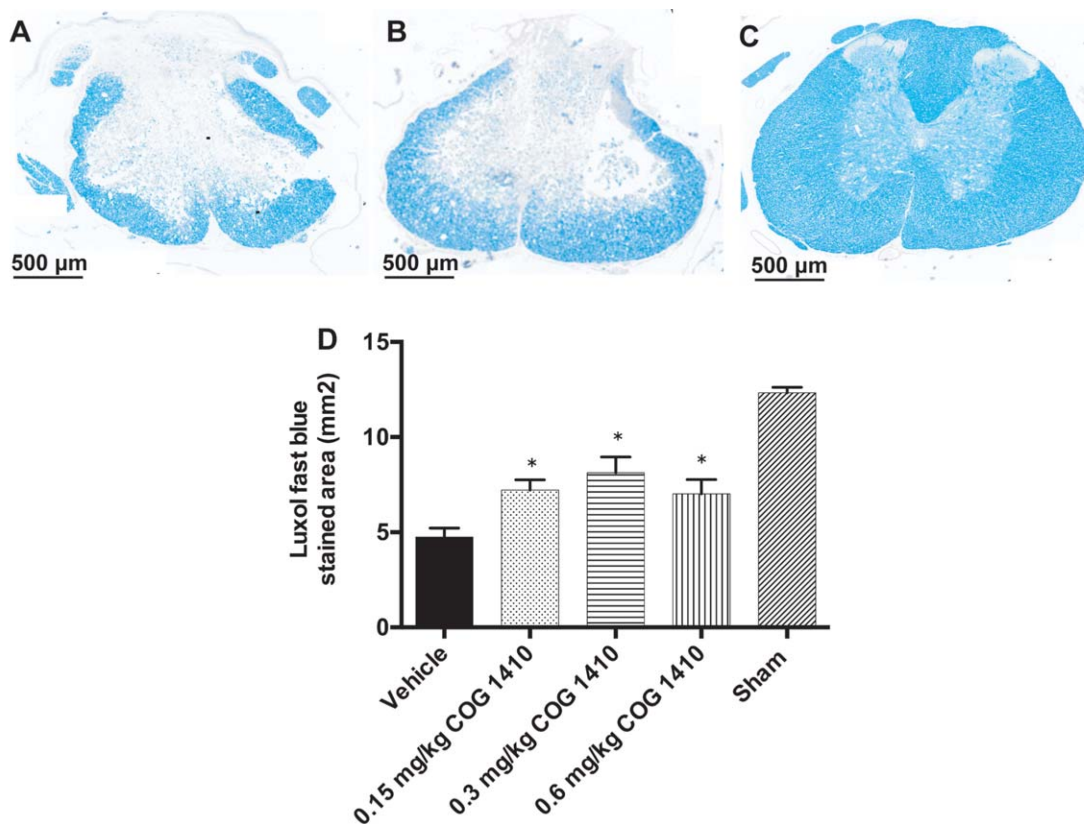

Fig. 5. White matter damage after SCI. Luxol fast blue stains normal myelin in white matter of the spinal cord. **A–C** represent vehicle, 0.3 mg/kg COG1410, and sham, respectively. **D:** Area of LFB-stained tissue was decreased in injured rats, indicating damage to white matter. COG1410 treatment significantly attenuated this decrease of LFB-stained tissue area compared with vehicle. LFB, luxol fast blue. $*P < 0.05$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pathways of neural cell death. In vitro studies have shown that apoE inhibits NMDA toxicity and regulates nitric oxide (Aono et al., 2002; Colton et al., 2002). Other experiments have demonstrated that apoE attenuates the production of tumor necrosis factor-α (TNF-α) and other inflammatory cytokines (Lynch et al., 2001) through suppressing microglial activation (Laskowitz et al., 1998). Intraventricular infusion of apoE significantly reduces hippocampal cell death in apoE-deficient mice subjected to global ischemia (Horsburgh et al., 2000). These findings together support the idea that apoE possesses antioxidant, anti-inflammatory, antiexcitotoxic, and neurotrophic properties and therefore may represent an ideal target for development of SCI therapy. ApoE-mimetic peptides were then developed to translate the concept into potentially therapeutic agents for clinical practice.

COG1410 has been developed for therapeutic purpose with demonstrated permeability across the blood–brain barrier (Laskowitz et al., 2007). The sequence of COG1410 is acetyl-AS-Aib-LRKL-Aib-KRLL-amide and is derived from apoE residues 138–149 with Aib substitution at positions 140 and 145. An in vitro study showed that this peptide has a more potent anti-inflammatory effect than apoE or the parent peptide apoE mimetic 133–149 (Gao et al., 2006). In vivo experiments also demonstrated that it significantly suppresses postinjury inflammatory responses, i.e., microglial activation and subsequent TNF-α release, in TBI and intracerebral hemorrhage (Laskowitz et al., 2007, 2010, 2012).

The potential utility of COG1410 on treatment of CNS disorder or injury has been tested in a variety of animal models. Using a mouse model of subarachnoid hemorrhage (SAH), Gao et al. (2006) reported that both 0.6 mg/kg and 1.2 mg/kg COG1410 increased postinjury survival and rotarod latency, and reduced SAH-induced vasospasm. Wang et al. (2013) reported that 0.6 mg/kg COG1410 did not cause hypotension in mice subjected to middle cerebral artery occlusion (MCAO) but did reduce water edema and infarct volume when administered 2 hr after onset of cerebral ischemia. The effect was absent when COG1410 was given 4 hr postischemia. A small MRI study confirmed that infarct volume in animals treated with COG1410 was not significantly different at the early phase of cerebral ischemia but was approximately 70% smaller at 24 hr compared with controls. In a mouse model of closed head injury, Laskowitz et al.

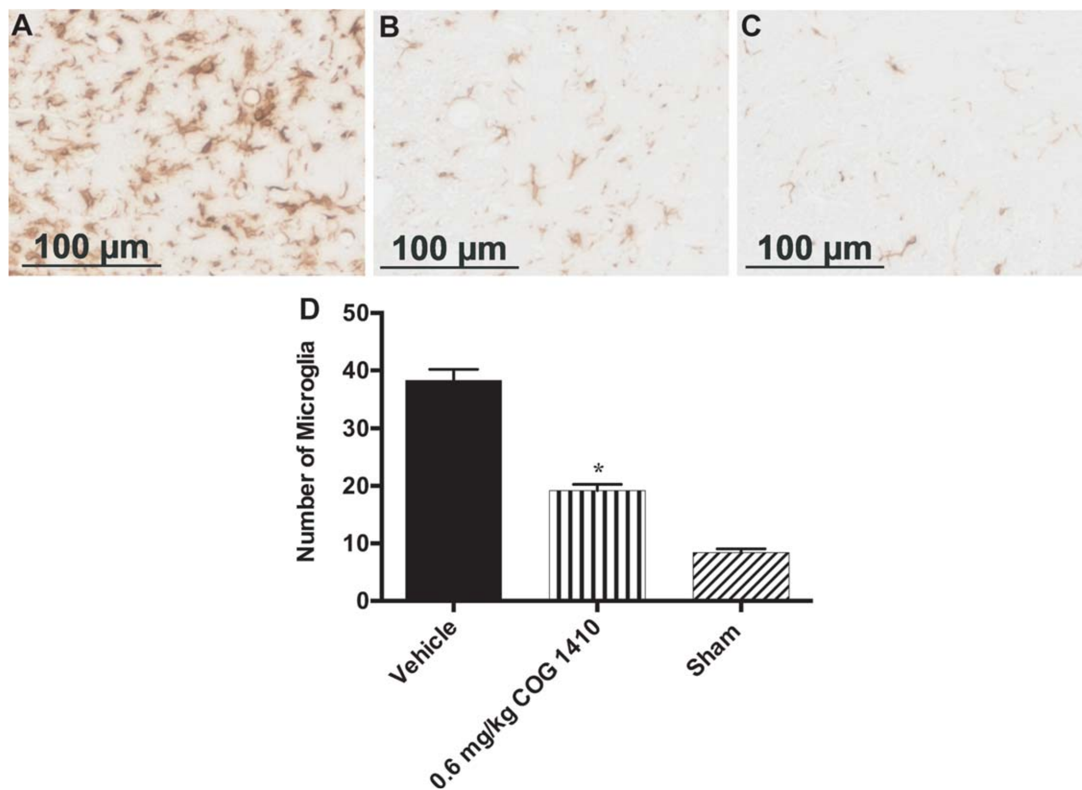

Fig. 6. Microglial activation was examined by Iba-1 immunohistochemistry at 14 days postsurgery. **A–C** represent vehicle, 0.6 mg/kg COG1410, and sham, respectively. **D:** Injury resulted in an increase of the number of positive microglia cells in the vehicle group. This increase was significantly attenuated by COG1410 treatment. $^{\star}P < 0.01$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(2007) reported that both 0.3 mg/kg and 0.6 mg/kg COG1410 improved rotarod latency and learning and memory when given within 120 min post-TBI. Fewer hippocampal cells are Fluoro-Jade B positive in animals treated with COG1410. TBI exacerbates neurodegenerative pathology, but this is also improved by this apoE mimetic (Laskowitz et al., 2010). In a mouse model of intracerebral hemorrhage, 2 mg/kg COG1410 decreased brain edema and effectively improved rotarod latency when given within 120 min of injury (Laskowitz et al., 2012). All of these findings support the notion that COG1410 is neuroprotective and provides a strong rationale to test the therapeutic efficacy of COG1410 in a rat model of spinal cord injury. Again, our data provide support for the hypothesis that this apoE mimetic has therapeutic potential for SCI. This is the first study to test this compound in rats. We tried to explore the dose–response curve on the SCI contusion model based on the effective doses on other CNS disease models in both mouse and rat (Hoane et al., 2007, 2009; Laskowitz et al., 2007, 2012; Kaufman et al., 2010). However, although our results demonstrated the efficacy of this peptide, there was not a large dose-dependent effect. SCI leads to gastrointestinal motility abnormalities (de Assis Gondim et al., 2002), which may affect the absorption of the peptide when given by intraperitoneal injection. Therefore, the blood concentration likely varied widely even within the same dose group, resulting in overlaps among treatment groups, and the dose-dependent effect was lost. To overcome this limitation, we will use an osmotic pump in future animal studies to allow intravenous administration.

There are several issues to be addressed in future studies. First is the molecular and pharmacological mechanism of COG1410 on SCI. Christensen et al. (2011) recently reported that apoE protein and its mimetic peptides, including COG1410, bind to a target protein SET, which is an endogenous inhibitor for protein phosphatase 2A and is also know as I_2PP2A. After binding to the C-terminal region of SET, apoE and its mimetic peptides can release PP2A from the PP2A/SET complex and therefore increase PP2A activity. With a murine microglia cell line BV2, COG1410 and other apoE mimetics reduced LPS-induced hyperphosphorylation of signaling MAPKs, such as p38 and JNK, as well as I-κB kinase and NF-κB activation. Because phosphorylated MAPK and NF-κB are well-known inflammatory signaling pathways and are the substrates of PP2A, binding of apoE and its mimetics to SET may underlie a molecular mechanism of the anti-inflammatory activity of apoE and its mimetic peptides. The second issue is the neuroregenerative

potential to be investigated in other SCI models. We have observed that COG1410 can promote axonal outgrowth in primary neuronal culture (Li et al., unpublished data). Thus, we speculate that COG1410 may support axonal regeneration in the spinal cord following SCI and will study this in a spinal cord transection model. The third issue involves the therapeutic time window and long-term effect, which are important for clinical development. The results we have indicate that this is a promising agent for SCI treatment.

CONCLUSIONS

The results in this study are consistent with previous reports and demonstrate in a second species and injury model that apoE mimetic promotes tissue repair and functional recovery.

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