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Research Report

The neuroprotective domains of the amyloid precursor protein, in traumatic brain injury, are located in the two growth factor domains

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ABSTRACT

The amyloid precursor protein (APP) is known to increase following traumatic brain injury (TBI). This increase in levels of APP may be deleterious to outcome due to the production of neurotoxic Aβ. Conversely, this upregulation may be beneficial as cleavage of APP via the alternative non-amyloidogenic pathway produces the soluble α form of APP (sAPPα), which is known to have many neuroprotective and neurotrophic functions. Indeed it has previously been shown that treatment with sAPPα following a diffuse injury in rats improves outcome. However, the exact location within the sAPPα molecule which contains this neuroprotective activity has yet to be determined. The sAPPα peptide can consist of up to 6 domains, with the main isoform in the brain missing the 4th and 5th. Of the remaining domains, the D1 and D6a domains seem the most likely as they have been shown to have beneficial actions *in vitro*. This present study examined the effects of *in vivo* posttraumatic administration via an intracerebroventricular injection of the D1, D2 and D6a domains of sAPPα on outcome following moderate-impact acceleration TBI in rats. While treatment with either the D1 or D6a domains was found to significantly improve motor and cognitive outcome, as assessed on the rotarod and Y maze, treatment with the D2 domain had no effect. Furthermore axonal injury was reduced in D1 and D6a domain treated animals, but not those that received the D2 domain. As the D1 and D6a domains contain a heparin binding region while the D2 domain does not, this suggests that sAPPα mediates its neuroprotective response through its ability to bind to heparin sulfate proteoglycans.

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1. Introduction

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality with an estimated 10 million people affected annually by an injury serious enough to result in death or hospitalization (Hyder et al., 2007). Following TBI, cell death is caused by the initial insult and the ongoing contribution of secondary factors such as excitotoxicity, oxidative stress and inflammation (Bramlett and Dietrich, 2004; Enriquez and Bullock, 2004). Although this delayed tissue damage provides a therapeutic window with an opportunity to limit neuronal damage (Vink and Van Den Heuvel, 2004), there are currently no accepted pharmacological interventions available for the treatment of TBI (Maas et al.). As such it seems that the identification of factors within the endogenous neuroprotective and neurotrophic pathways may facilitate the development of novel therapeutic strategies. This is especially important as the upregulation of these pathways appears to be inhibited with more severe injuries (Thompson et al., 2006).

Recent evidence suggests that the amyloid precursor protein (APP) may play a role in these neuroprotective and neurotrophic pathways following TBI, with the metabolite sAPPα shown to improve motor outcome with an associated reduction in axonal injury and apoptotic cell death when administered to rats following TBI (Thornton et al., 2006). Indeed, multiple studies have highlighted the role of sAPPα in providing neuroprotection (Goodman and Mattson, 1994; Masliah et al., 1997), enhancing neurite outgrowth (Ohsawa et al., 1997; Qiu et al., 1995), promoting synaptogenesis (Bell et al., 2006) and increasing neurogenesis (Caille et al., 2004).

sAPPα can consist of up to 6 different domains, although predominant isoform of APP which is present in the central nervous system, APP695, does not contain the 4th (KPI) or 5th (OX-2) domains (Sandbrink et al., 1996). Thus sAPPα from APP695 can be divided into a growth factor like domain (D1), a copper binding region (D2), an acidic region (D3), and a carbohydrate domain (D6), with the carbohydrate domain further divided into an E2 domain (D6a) and a juxtamembrane region (D6b) (Reinhard et al., 2005; Storey and Cappai, 1999). It should also be noted that the combination of the D1 and D2 domains is sometimes referred to as the E1 domain (Soba et al., 2005). Only the D1, D2 and D6a domains participate in secondary structure formation with the D3 and D6b domains providing flexible linkers to connect the individual folding units (Reinhard et al., 2005). The beneficial actions of sAPPα have previously been linked to the D1 and D6a domains (Jin et al., 1994; Ohsawa et al., 1997; Qiu et al., 1995). However, their efficacy *in vivo*, and their ability to improve outcome following TBI, is yet to be determined. As such the present study examined the effects of *in vivo* post-traumatic administration of the D1, D2 and D6a domains of sAPPα on functional outcome following severe impact acceleration TBI compared to that of animals treated with the full length sAPPα.

2. Results

2.1. *The D1 and D6a domains of sAPPα are as effective as the full length peptide at improving motor outcome post-injury*

Following TBI, motor outcome was determined using the rotarod (Figs. 1A–C), with sham rats performing at close to the maximum time of 120 s, ranging from 111.5 s to 118.7 s over the testing period. The vehicle animals were significantly impaired on all days following injury ($p<0.01$), and although they did improve from 45 s on day 1 to 85.5 s on day 7 post-injury, they never returned to sham level. Similarly, the D2 treated rats (Fig. 1C) were significantly worse than sham rats

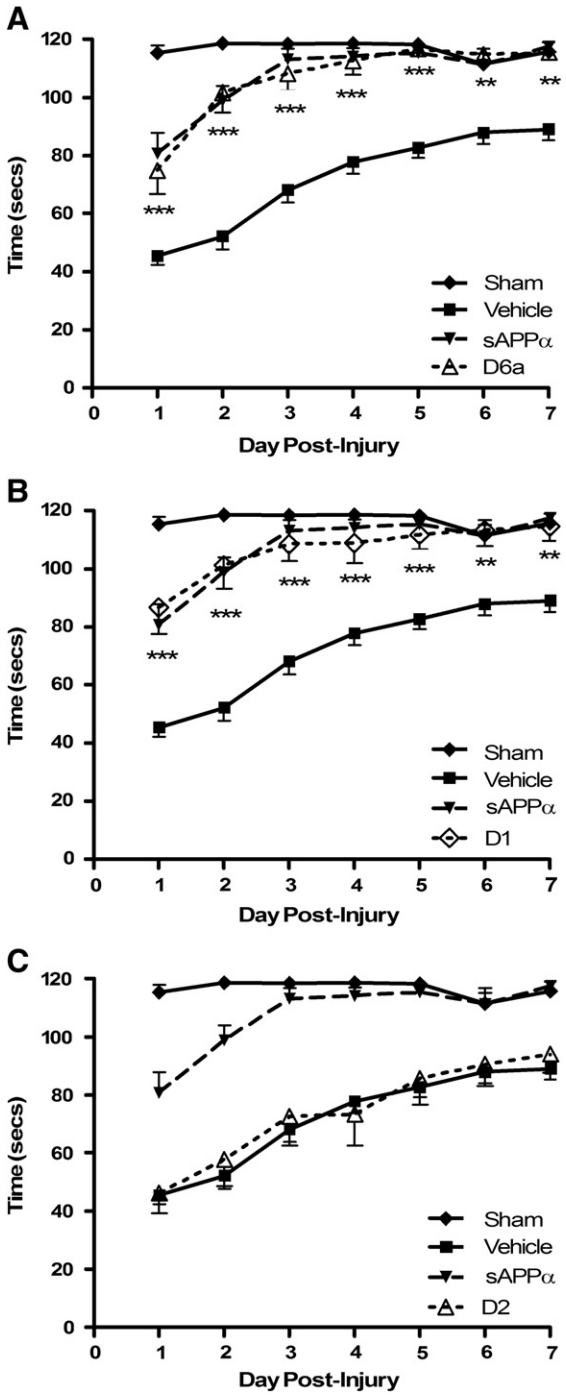

Fig. 1 – Motor (rotarod) scores for rats following TBI. The D1 (1A), D6a (1B) and D2 (1C) treated groups are compared to the performance of the sham, sAPPα and vehicle control groups. Results are expressed as means ± SEM (n = 10 per group) (*$p<0.001$, **$p<0.01$ compared to vehicle controls).**

on days 1 to 5 following injury ($p<0.001$), with scores from 46.2 s on day 1 to 94 s on day 7. In contrast the D1 (Fig. 1A) and D6a (Fig. 1B) groups were almost identical to the sAPPα treatment group, being significantly different from shams only on day 1 post-injury. At this time point they were, however, still performing significantly better than the vehicle ($p<0.001$) and D2 ($p<0.01$) treated animals with scores of 80 s (sAPPα), 86 s (D1) and 75 s (D6a). By day 3 the times for the sAPPα, D1 and D6a groups were similar to those for sham animals at 113.7 s, 108.6 s and 108.2 s respectively, and remained at this level for the week of assessment. Indeed, they were significantly different to vehicle treated animals on all days (1 to 7) post-injury ($p<0.01$). In contrast treatment with D2 was ineffective, with these animals showing no significant difference as compared to the vehicle treated rats on all days tested.

2.2. *The D1 and D6a domains of sAPPα are as effective as the full length peptide at improving cognitive outcome post-injury*

Cognitive outcome was tested using the Y Maze on days 5 (Fig. 2A) and 10 (Fig. 2B) post-injury. At both days 5 and day 10 post-injury, the sAPPα, D1 and D6a treated rats were similar to sham animals in that they spent significantly more time ($p<0.01$) in the novel arm than either the start or other arm. Indeed the amount of time spent in the novel arm was similar in these groups with sham animals spending 80.33 s on day 5 and 84.4 s on day 10 in the novel arm compared to 83.22 and 81.67 s for the sAPPα treated group, 89.1 and 75.91 s in the D6a treated group and 85.4 and 83.4 s for the D1 treated group. In contrast the vehicle and D2 treated animals did not spend significantly more time in any of the arms. On day 10 the vehicle treated animals spent 58.3 s in the novel arm, 64.17 s in the start arm and 57.5 s in the other arm. Similarly at day 10 the D2 treated animals spent 64.3 s in the novel arm, 54.1 s in the start arm and 61.5 s in the other arm. There was no significant difference between the groups in number of arm entries on either day 5 or 10 post-injury (data not shown).

2.3. *The D1 and D6a domains of sAPPα are as effective as the full length peptide at reducing axonal injury post-injury*

To determine if the improvement in motor function and cognition correlated to the neuroprotective activity of APP, axonal injury was determined by counting the numbers of APP immunopositive lengths within the corpus callosum (Fig. 3). The vehicle and D2 treated animals had a significant increase ($p<0.05$) in the number of injured axons when compared to sham animals. In contrast, the sAPPα, D1 and D6a treated animals were not significantly different to sham controls and had significantly less injured axons than the vehicle ($p<0.05$) or D2 treated animals ($p<0.05$).

3. Discussion

This study found that the D1 and D6a, but not the D2 domain of sAPPα were as effective as the full length peptide at improving outcome following TBI. Animals treated with the

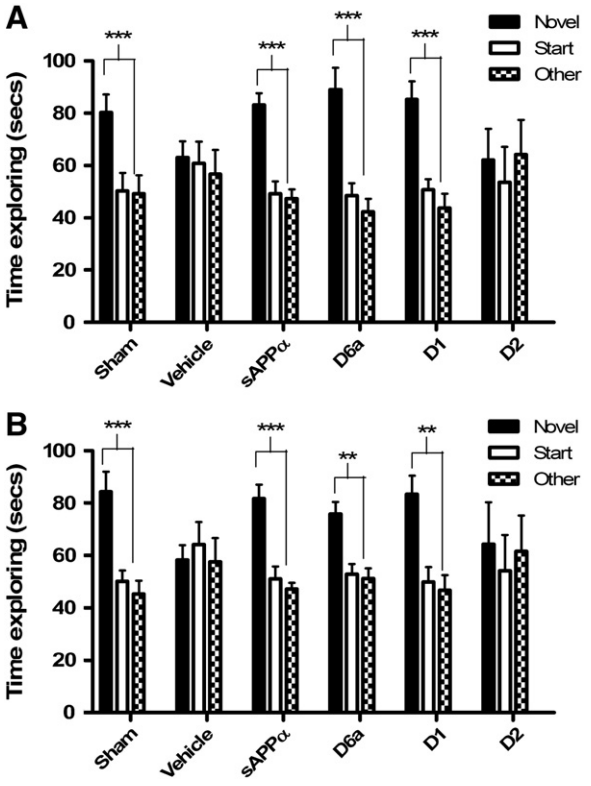

Fig. 2 – A) Cognitive outcome (Y Maze) on day 5 following injury. Results are expressed as means ± SEM (Vehicle, sAPPα and D6a groups n = 10, Sham, D1 and D2 groups n = 9) (*$p<0.001$ compared to start and other arms). B) Cognitive outcome (Y Maze) on day 10 following injury. Results are expressed as means ± SEM (Vehicle, sAPPα and D6a groups n = 10, Sham, D1 and D2 groups n = 9) (***$p<0.001$ compared to start and other arms).**

D1 and D6a domains showed a significant improvement in motor and cognitive outcome, with a corresponding decrease in axonal injury that was identical to that seen in those treated

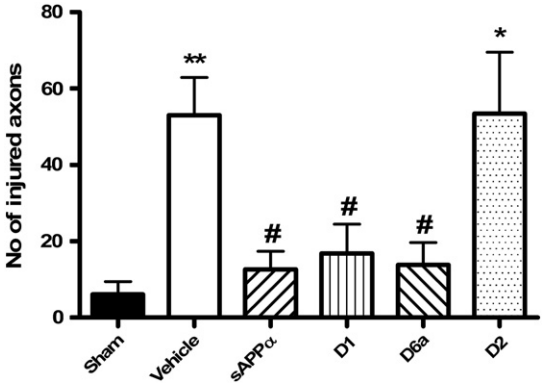

Fig. 3 – Number of injured axons within the corpus callosum. Results are expressed as means ± SEM (n = 5 per group) (*$p<0.05$, **$p<0.01$ compared to sham animals; #$p<0.05$ compared to vehicle controls).

with sAPPα. Indeed these animals returned to a sham level of performance on the rotarod by day 3 post-injury and were indistinguishable from shams in their performance in the Y maze. In contrast treatment with the D2 domain had no effect, with these animals performing in an identical fashion to vehicle controls.

The neuroprotective properties of the D1 domain most likely relate to its heparin binding site, which spans residues 96–110 and contains a disulphide bridge (Rossjohn et al., 1999). Although this region has not previously been linked specifically to neuroprotective actions, it is known that the binding of this region to heparin sulfate proteoglycans (HSPGs) promotes neurite outgrowth from central and peripheral neurons (Small et al., 1994). Furthermore, an antibody that binds to this region inhibits functional synapse formation (Morimoto et al., 1998) and completely abolishes depolarization induced neurite outgrowth (Gakhar-Koppole et al., 2008).

Similar to D1, the neuroprotective properties of the D6a domain may relate to its heparin binding site which encompasses six basic residues from helices αC and αD of one monomer (Arg-375, His-382, Arg-424, Lys-428, His-432 and His-439) and a conserved Arg-448 from helix αB′ of the second monomer (Wang and Ha, 2004). This region may regulate intracellular calcium since sAPPα reduces neuronal excitability, stabilizes calcium homeostasis and protects neurons against excitotoxicity, most likely through activation of high conductance potassium channels which hyperpolarize the cell; with these actions were blocked by an antibody that partially covers the heparin binding site (Mattson et al., 1993). Excessive calcium influx seen with excitotoxicity following TBI activates a number of destructive enzymes which can worsen secondary axotomy (Buki and Povlishock, 2006). Thus any reduction in calcium concentration due to the actions of this region would help maintain cytoskeletal integrity and reduce axonal injury. Furthermore, this region also has neurotrophic properties, whereby deletion of amino acids 305–591 abolished the promotion of neurite extension in cells exposed to APP (Qiu et al., 1995).

Alternatively, the D6a active site may involve the pentapeptide motif 'RERMS' (a.a residues 328–332) which was originally found to be responsible for the growth regulating activity of APP in fibroblasts (Ninomiya et al., 1993). The same sequence can promote survival of rat cortical cells (Ohsawa et al., 1997; Yamamoto et al., 1994). An extended 17 mer peptide enhances synaptic density in the frontoparietal cortex of rats with an associated increase in memory retention (Roch et al., 1994). The surrounding amino acids may contribute to the biological activity of the RERMS sequence, as neurite extension activity in B103 cells was enhanced with longer sequences, than RERMS alone (Jin et al., 1994). However, other studies have found that small peptides encompassing the RERMS sequence did not protect cells against glutamate or Aβ toxicity (Furukawa et al., 1996) or promote neurite outgrowth in cultured rat neurons (Ohsawa et al., 1997).

The D2 domain, which does not contain a heparin binding site, had no effect on functional outcome or the amount of injured axons following TBI. This supports the model that the protective properties of sAPPα may relate to its heparin binding sites contained within the D1 and D6a domains. Heparin and heparin sulfate are components of the glycosaminoglycan sidechains of proteoglycans which are present in cell membranes, extracellular matrices and basement membranes. The majority of binding sites for APP in the extracellular matrix are these HSPGs (Ninomiya et al., 1994), with the interaction of APP with HSPGs promoting cell adhesion, neuron–cell or cell–matrix interactions (Reinhard et al., 2005), which are important for a number of functions including cell signaling and growth. The role of heparin binding sites in facilitating the neuroprotective activities of sAPPα were highlighted by the ability of heparinases to prevent the ability of sAPPα to protect cultured cells from a number of insults including glutamate toxicity and glucose deprivation (Furukawa et al., 1996). In addition, binding of the heparin like molecules glypican and perlecan was capable of inhibiting APP-induced neurite outgrowth, presumably by competing with endogenous proteoglycans which mediate this activity (Williamson et al., 1996).

Binding to heparin also appears to induce dimerization, with this phenomenon occurring with an extended version of the D1 domain (D1 and D2 domains) (Dahms et al.; Soba et al., 2005) and the D6 domain (Wang and Ha, 2004). This could potentially initiate intracellular signaling cascades important for physiological cellular events, although these have yet to be fully elucidated (Reinhard et al., 2005). Indeed, the effects of sAPPα may be mediated in part through an interaction with full length membrane bound APP (Young-Pearse et al., 2008; Gralle et al., 2009), with heparin induced dimerization a possible mechanism of action.

This study shows that the D1 and D6a domains are as effective as full length sAPPα at improving functional outcome and reducing axonal injury following TBI. As the D1 and D6a domains contain heparin binding sites, but the inactive D2 does not, this suggests the neuroprotective ability of sAPPα may relate to its ability to bind HSPGs. Further research on the exact mechanisms on how the binding of sAPPα to HSPGs provides neuroprotection may allow the development of exogenous agents to improve functional outcome following TBI.

4. Experimental procedures

4.1. *Induction of traumatic brain injury*

A total of 90 adult male Sprague–Dawley rats weighing between 390 and 460 g were randomly assigned into two broad groups, outcome studies (n=60) or histological studies (n=30). These animals were then further randomly assigned into sham, vehicle control, sAPPα, D1, D2 or D6a treatment groups. Animals were injured using the impact–acceleration model of diffuse traumatic brain injury as described previously (Marmarou et al., 1994). Briefly, animals were anaesthetized with isoflurane and the skull exposed by a midline incision so that a stainless steel disc (10 mm in diameter and 3 mm in depth) could be fixed rigidly with polyacrylamide adhesive to the animal's skull centrally between lambda and bregma. The rats were subsequently placed on a 12-cm foam bed and subjected to brain injury by dropping a 450-g brass weight a distance of 2 m onto the stainless steel disc. Previous studies have shown that this level of injury results in moderate to severe functional deficits

(Heath and Vink, 1999). Sham operated animals were surgically prepared but were not injured.

Animals were treated with either the D1 (APP28–123), D2 (APP124–189), D6a (APP316–498) domains of sAPPα, the full length peptide (APP18–611) or artificial CSF vehicle (Fig. 4). sAPPα, the D1, D2 and D6a peptides were expressed as a secreted protein in the methylotrophic yeast, *Pichia pastoris* as previously described (Henry et al., 1997). They were then purified from the media by anion exchange chromatography using a Hiload Q sepharose column (26×12 mm, GE Healthcare, Sweden), followed by affinity chromatography using a Hitrap heparin HP column (5 mL, GE Healthcare, Sweden). 150 μl of either D1, D2, D6a or sAPPα (25 μM) was then mixed with 75 μL of artificial CSF vehicle (Roch et al., 1994; Thornton et al., 2006) prior to administration. This was based on a dose–response study which showed that 25 μm of sAPPα was more effective than 2.5, 0.25 or 0.025 μM at improving motor outcome (data not shown).

Following injury, and after the animal was stable, the steel disc was removed from the skull and a 0.7 craniotomy performed at the stereotaxic coordinates relative to the bregma: posterior 0.6 mm, lateral 1.5 mm (Paxinos, 1998; Thornton et al., 2006). A 30-gauge needle attached to a 5-μL syringe was then stereotaxically lowered 4.0 mm then retracted 0.5 mm to facilitate administration into the lateral ventricle. At 30 min post-injury, 5 μL of the appropriate treatment was administered at a rate of 0.5 μL/min. The method of administration was selected on the basis of our previous work which showed that administering sAPPα via an intracerebroventricular (ICV) injection improved outcome following TBI (Thornton et al., 2006), in line with previous research which used autoradiography to show that ICV administration results in a homogenous distribution of sAPPα throughout the rat brain and a higher concentration compared to shams (Roch et al., 1994). Following the injection, the animal was removed from the stereotaxic device, and the midline incision closed using 9-mm surgical clips. Animals were maintained at a rectal temperature of 37 °C throughout surgery, recovery, and drug administration using a thermostatically controlled heating pad.

4.2. *Motor outcome*

Motor deficits produced by TBI were assessed using the rotarod, which is used extensively within our laboratory and has been described as the most sensitive test for the detection of motor deficits in rodents (Hamm et al., 1994). The rotarod device consists of a motorized rotating assembly of eighteen 1 mm metal rods, which introduces a grip test component to the assessment. Rotational speed of the device was increased from 6 to 36 revolutions per minute (rpm) in intervals of 3 rpm every 10 s. The duration in seconds, up to a maximum of 120 s, was recorded at the point when animals had either completed the task, clung to the rods for 2 consecutive rotations without actively walking or had fallen off. All animals were pre-trained daily on the device for 1 week prior to injury and assessed daily for 7 days after injury.

4.3. *Cognitive outcome*

Cognitive deficits produced by TBI were assessed using the Y maze, which tests spatial and recognition memory and has been shown to be a sensitive test for detecting hippocampal damage in rats (Conrad et al., 1996). The maze consists of three arms with a different object placed at the end of each arm. Distinct spatial clues are located around the maze and kept constant throughout the study in order to allow the rats to orientate themselves and thus differentiate between the arms without examining the objects. Addition of objects provides stimulus for the rats to continue to explore the maze. The arms are arbitrarily designated as the start, other and novel arms, with this randomly alternated among the rats.

In the first phase of the experiment, rats are introduced into the maze in which one of the arms (novel) has been closed and allowed to explore for 3 min. This means that the animals were able to explore the start and other arms, but not the novel arm. An hour later the animals are placed back into the maze in their start arm with all the arms (start, other and novel) open and allowed to explore for another 3 min. This test works on the basis that an uninjured animal will spend more time exploring the novel arm to which they have not been previously exposed, rather than the other two arms. In contrast an injured animal will spend an equal amount of time in each of the arms as they will not remember the maze. In order to remove scent trails the maze was wiped thoroughly with 70% alcohol after each trial. The experimenter was not in the room during the trials, with all trials captured on video, and the number of times each arm was visited and the time spent in each arm analyzed. Animals that did not enter the novel arm during the first part of the experiment were not included.

4.4. *Histological analysis*

Rats were sacrificed by perfusion at day 3 post-injury. For perfusion, animals were anaesthetized with Isoflurane and given 1 ml of 5000 U heparin IP to prevent coagulation of blood. The thorax was opened to expose the heart and a blunt

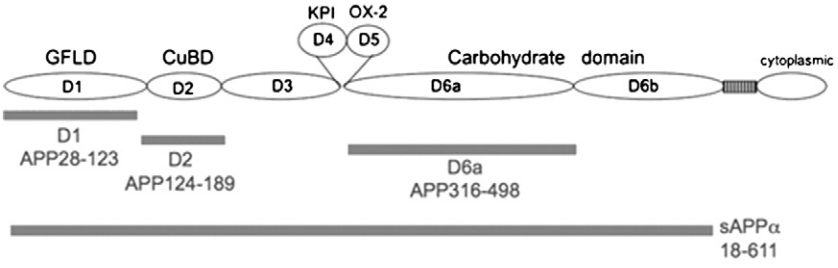

Fig. 4 – Schematic of the different peptides administered to rats post-injury.

19 G needle inserted into the aorta, with the right ventricle then cut. The animal was transcardially perfused with 10% formalin (pH 7) at room temperature. Upon completion of perfusion the fixed brains were removed and stored in 10% formalin for 3 days. Following this they were cut into 7×2 mm coronal slices, embedded in paraffin and sectioned into 5 μm coronal sections. For each animal a section was cut from the region −4.5 mm from the bregma, as this was located directly underneath the impact site. These sections were incubated overnight with a 1:1000 dilution of a biotinylated monoclonal antibody specific to APP (Novocastra 122703), followed by secondary antibody (1:250 Sigma-Aldrich) for 30 min at room temperature. Bound antibody was detected with 3,3-diaminobenzidine tetrahydrochloride (Sigma) and sections were counterstained with haematoxylin. All slides were scanned (Nanozoomer, Hamamatsu, Hamamatsu City, Japan) and viewed with the associated software to allow the number of APP immunopositive lengths along the corpus callosum to be counted on 1 slide per animal.

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The authors declare that they have no conflict of interest.

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