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Expression and Cerebral Function of Amyloid Precursor Protein After Rat Traumatic Brain Injury

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1. Introduction

The β-amyloid protein derived from amyloid precursor protein (APP) (Goldgaber et al., 1987; Robakis et al., 1987) is a major component of senile plaque, and β-amyloid protein deposits in Alzheimer disease (AD) (Masters et al., 1985; Selkoe et al., 1986) are thought to be produced by alternative processing of APP (Golde et al., 1992). Therefore, the overexpression of APP is an important factor in the neuronal degeneration of AD (Murakami et al., 1998). The expression of APP has also been shown to increase in swollen axons and neuronal perikarya after neuronal injury, including ischemia (Stephenson et al., 1992) and stab injury (Otsuka et al., 1991). As the pathological findings in dementia pugilistica mimic those in AD (Roberts et al., 1990), it has been posited that traumatic brain injury (TBI) is an epidemiological risk factor for AD (Schofield et al., 1997; DeKosky et al., 2007), although other genetic factors, which include the apoE genotype, might act additively (Nicoll et al., 1995; DeKosky et al., 2007). These findings suggest that the overexpression of APP after TBI may potentiate Alzheimer disease pathology. However, the chronological changes in APP expression have not been evaluated after TBI. Furthermore, the function of APP has not been evaluated after TBI.

In this study,

1. we immunohistochemically investigated chronogical changes in cellular sources and levels of APP production compared to that of mRNA for APP as assessed by RT-PCR in the rat brain following traumatic brain injury.
2. In addition, we investigated the function of APP by assessing water maze and morphological changes following direct infusion of the anti-APP antibody into the damaged brain region following TBI.

2. Materials and methods

2.1 Surgical procedure

Male Wistar rats (8–9 weeks old, 200–250g in weight) were anesthetized by intraperitoneal pentobarbital (50mg/kg) injection. The scalp was incised on the midline and the skull was exposed. A 2–2.5mm hole was drilled (1 mm posterior, + 1 mm right lateral to bregma) in the right parietal calvaria. Brain injury above the dura mater was then induced with a pneumatic control injury device (Itoh et al., 2005; Itoh et al., 2007; Itoh et al., 2009) at an impact velocity of 4m/sec (impact tip diameter of 1mm; fixed impact deformation of 2mm depth from the cerebral surface) (Itoh et al., 2005; Itoh et al., 2007; Itoh et al., 2009). Control rats were subjected to a sham operation, but no cortical penetration injury was inflicted. The contralateral hemisphere was not used as a control, since that area may have been affected by the impact. Rats were placed in a heated cage to maintain their body temperature at 37°C during the recovery from anesthesia.

2.2 Anti-APP antibody infusion

The drug infusion protocol was as previously described (Green-Sadan et al., 2003). Immediately, after TBI, the animals were placed in a stereotactic frame and fitted with a brain infusion cannula (Alzet brain infusion kit 3, 1mm, Alzet, San Diego, CA, USA). The cannula was implanted with the tip inserted into the damaged brain region (1 mm posterior, + 1 mm right lateral to bregma). The cannula was secured with dental cement. A micro-osmotic pump (Model 2001; rate of 1µl/h, Alzet), filled with 300 µl infusion volume kept 37°C, was implanted subcutaneously in the neck and connected to the infusion cannula. Rats received an infusion of either anti-APP antibody (IgG2a isoform, 1µg/ml; anti-APP antibody group; Chemicon, Temecula, CA, USA) diluted in sterilized phosphate buffer saline (0.1M PBS, pH 7.4–7.5; n=20) or sterilized PBS only (PBS group; n=20) for seven days immediately after TBI. For the control of the Morris water maze experiments, ten animals with no operation (sham operation group, no injury) were also collected. In sham operation group, a micro-osmotic pump was implanted subcutaneously in the neck without infusion cannula. Moreover, sham operation group was used only the behavioural experiments. As probe and the extent of APP antibody infusion test, although data not shown in this study, we investigated that the extent of APP antibody diffusion with Evans blue using the osmotic pump and confirmed Evans blue extended whole brain from center of infusion area.

2.3 Morris water maze experiments

A circular, thermostatically regulated, dark gray PVC-plastic water tank (180cm wide, 45cm deep, filled with tap water at 22±1°C), located in the center of the testing room and surrounded by extra-maze cues, was used in the spatial learning task. A constant asymmetrical array of lamps and pictures served as cues for spatial orientation. A circular dark gray platform (15cm wide) submerged 1cm below the water surface served as a platform. The platform was placed in the center of one of the quadrants, i.e., the target quadrant, of the water maze (Elvander et al., 2004). The experiments were monitored using a digital TV system connected to a computer (Elvander et al., 2004). Training took place between 8:00a.m. and 3:00p.m. during seven consecutive days. Each daily training session consisted of four trials with a 120s cutoff time, followed by 30s rest on the platform. Memory was tested in fifteen animals from each of the anti-APP antibody group and the PBS group, and in ten sham operation group animals, starting at day one and continued up to seven days after TBI/sham operation.

2.4 Immunohistochemistry

1, 3, 7, 30 and 90 days after TBI and after the last the Morris water maze test at seven days, rats from each group were perfused intracardially with 300ml of PBS followed by 300ml of 4% paraformaldehyde (PFA; pH 7.4–7.5) in PBS. The brains were then removed and stored in PFA for three days, before the maximum size of the lesion was sliced into serial coronal sections (50μm thick) using a microslicer (Dousaka EM, Kyoto, Japan).

Sections were treated with 3% H_2O_2 in Tris-buffered saline (TBS; 0.1M Tris-HCl, pH 7.5, 0.15M NaCl) containing 0.1% Triton X-100 (TBS-T) for 30min. Next, the sections were washed three times with TBS-T, blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in TBS-T for 30min, and incubated with a mouse monoclonal anti- microtubule associate protein-2 (MAP-2) antibody as a marker of neuronal cell (1:10000 dilution; Sigma-Aldrich), a mouse monoclonal anti-neurofilament (NF) antibody as a marker of neuronal cell (1:10000 dilution; DAKO, Glostrup, Denmark), a rabbit polyclonal anti-Glial Fibrillary Acidic Protein (GFAP) antibody (1:10000 dilution; DAKO, Glostrup, Denmark), a mouse monoclonal anti-APP antibody (1:1000 dilution; Chemicon, Temecula, CA, USA) or a mouse monoclonal anti-CD11b antibody as a marker of microglia/macrophage (1:1000 dilution; Chemicon, Temecula, CA, USA) overnight at room temperature. Following extensive washing, the sections were further incubated with a HISTIFINE Rat-PO (multi)-kit (Nichirei, Osaka, Japan), consisting of a mixed solution of peroxidase-conjugated anti-mouse and rabbit IgG as the secondary antibody, for 60min at room temperature. The HISTIFINE Rat-PO kits contained preabsorbed rat serum, and showed very minimal non-specific binding by rat serum in injured rat tissues. Labeling was visualized using diaminobenzidine (DAB; Vector Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA, USA) for 5min, and the sections were counterstained with hematoxylin.

For TUNEL-staining at three days after TBI, the brains from five rats in each of the anti-APP antibody group and the PBS group were collected as above. Sections were used for TUNEL-staining, a marker of apoptosis. TUNEL-staining was performed using an *in situ* Apoptosis Detection Kit (TaKaRa Biochemicals Co., Kyoto, Japan) according to the manufacturer's instructions. Sections were treated with Proteinase K for 10min, followed by treatment with 3% H_2O_2 in TBS-T for 30min. Next, the sections were washed three times with TBS-T, and incubated with a TdT Enzyme Labeling solution for 90min at room temperature. Labeling was visualized using DAB. The controls for the TUNEL stains were performed without enzyme. Furthermore, to investigate TUNEL-positive cells phenotype, MAP-2 or GFAP immunofluorescence staining was performed following TUNEL-staining. Sections were treated with Proteinase K for 10min. Next, the sections incubated with a fluorescein isothiocyanate (FITC)-conjugated TdT Enzyme Labeling solution for 90min at room temperature. Next, the sections were washed extensively and incubated with an anti-MAP-2 antibody (1:300 dilution; Sigma) or anti-GFAP antibody (1:300 dilution; DAKO) overnight at room temperature. Following extensive washing, the sections were further incubated with Alexa Flour 555 anti-mouse IgG (for anti-MAP-2 antibody) or rabbit IgG (for anti-GFAP antibody, 1:300 dilution; BD Biosciences Pharmingen, San Diego, CA) for 80 min at room temperature. Subsequently, the sections were observed using a confocal laser-scanning microscope (LSM5 PASCAL; Carl Zeiss Jena GmbH, Jena, Germany).

2.5 Quantification

To determined the number of APP -positive neurites and cells, MAP-2-, GFAP-, or TUNEL-positive cells, each DAB-positive cells around the damaged area (cortex) without white matter after TBI were observed and DAB-labeled cells were counted in three serial sections

(each section was 50 µm in thickness) under a Nikon E 1000M microscope (Nikon Corporation, Tokyo, Japan) using a 20x objective. To determine the measured area of DAB-positive cells, an image of the measured area was captured under the Nikon E 1000M microscope at 1x magnification using a CCD camera (ACT-2U; Nikon Corporation). The measured area in each image was traced and measured using a computer (Power Macintosh G3; Apple Computers, Cupertino, CA) and the NIH Image 1.6 software (NIH, Bethesda, MD). The number of DAB-positive cells was expressed as positive cell number/$100\mu m^3$.

2.6 Double-immunofluorescence staining

Each serial section was washed with TBS-T, blocked with 3% BSA in TBS-T for 30 min, and incubated with a polyclonal rabbit anti-APP antibody (1:300 dilution; Chemicon) overnight at room temperature. Following extensive washing, the sections were further incubated with a polyclonal rhodamine-conjugated anti-rabbit IgG antibody (1:300 dilution; DAKO) for 80 min at room temperature. Next, the APP-stained sections were washed extensively and incubated with a monoclonal mouse anti-NF antibody (1:300 dilution; DAKO), a marker for neurons and nerve fiver; a monoclonal mouse anti-MAP-2 antibody (1:300 dilution; Sigma), a marker for neurons; a monoclonal mouse anti-CD11b antibody (1:300 dilution; AbD Serotec, Raleigh, NC), a marker for macrophages/microglia or a monoclonal mouse anti-GFAP antibody (1:300 dilution; DAKO), a marker for astroglia overnight at room temperature. Following extensive washing, the sections were further incubated with a polyclonal fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (1:300 dilution; DAKO) for 80 min at room temperature. Subsequently, the sections were observed by fluorescence microscopy (Nikon E-800; Nikon, Tokyo, Japan).

2.7 Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression levels were investigated by RT-PCR at 1 to 90 days after TBI. Five rats were used for each time point. Briefly, total RNA was isolated from an area of the cerebral cortex (without the corpus callosum and hippocampus) with a diameter of 2 mm from the center of the lesion using RNA-Bee (Tel-Test Inc., Friendswood, TX) and redissolved in water, before the concentration was determined photometrically using the wavelength ratio of 260 nm/280 nm. The two oligonucleotide primers used for APP mRNA were: (accession No X07648): sense primer: 5 '-GGA TGC GGA GTT CGG ACA TG -3 'antisense primer: 5 '-GTT CTG CAT CTG CTC AAA G -3 'Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CLONTECH, No5507, BD Biosciences Pharmingen, San Diego, CA) was used as an internal control.

RT-PCR was performed using an mRNA PCR Kit (TaKaRa Biochemicals Co., Kyoto, Japan) according to the manufacturer's instructions. RT was performed at 42°C for 25 min, followed by heat inactivation at 99°C for 5 min. PCR amplification was carried out in a PCR EXPRESS (Hybaid US, Franklin, MA) for 30 cycles of 2 min at 94°C, 30 s at 94°C, 30 s at 55°C and 1.5 min at 72°C. The final step was extended to 5 min at 72°C. After separation by electrophoresis in 1.5% agarose gels, the products were stained with ethidium bromide and located by fluorescence using UV light (3UV Transilluminator; UVP, Upland, CA). Bio Max 1D TM1.5.1 (Kodak, Tokyo, Japan) was used to evaluate the band intensities of the PCR bands. The signals were normalized to the corresponding GAPDH signal in the same RNA sample.

2.8 Enzyme-linked immunosorbent assay (ELISA) for APP

The APP protein levels after TBI were investigated by ELISA using extracts of the TBI area. Five rats were used for each time point. Briefly, brain tissue from the above-described TBI

area was isolated at each time point, and homogenized (10% w/v) in homogenizing buffer (PBS containing 0.25 mol/L sucrose, 5 mmol/L ethylene glycol bis(2-aminoethyl ether)-tetraacetic acid (EGTA), 25 µg/ml leupeptin, 25 µg/ml aprotinin) at 4°C using a hand homogenizer. After treatment of the homogenates with ultrasonic waves at 4°C for 100 s and centrifugation at 20,000 g for 30 min at 4°C, the resulting supernatants were collected. The protein concentrations of the supernatants were determined using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, and then adjusted to 1 µg/µl. The brain tissue samples were stored at -80°C until analysis by ELISA.

ELISAs were performed as previously described. Briefly, flat-bottomed microtiter plates (type H for ELISA; Sumitomo Bakelite Co., Tokyo, Japan) were coated with a rabbit polyclonal anti-APP antibody (diluted to 0.5 µg/ml in PBS containing 3% BSA (PBS-B); Chemicon) and blocked with 300 µl of PBS containing 3% BSA, 1% sucrose and 0.05% Tween 20 for 120 min at room temperature. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T). Recombinant APP (Chemicon) was serially diluted with PBS-B (0.001-12 ng/ml) and used to construct a standard curve. Aliquots (100 µl) of the diluted samples were incubated in the wells at 4°C overnight. After washing, 100 µl of a mouse monoclonal anti-APP antibody (1:10000 dilution in PBS-B; Chemicon) was added to each well and incubated for 2 h at room temperature. After washing, 100 µl of simple-stain MAX-PO (mouse)-kit (Nichirei) consisting of a peroxidase-conjugated anti-mouse IgG was added to each well. Color development was carried out using an Enzyme Reaction Kit Type T (Sumitomo Bakelite Co., Tokyo, Japan), and the absorbances at 490 nm were measured using a microplate reader (Model 3550UV; Bio-Rad Laboratories). The detection limit of the assay for APP was 0.005 ng/ml. The measurements were made in duplicate.

2.9 Area measurement of damaged brain region

To determine the area of the damage region at seven days after TBI, after the last the Morris water maze five rats per group were collected as above, and the anteroposterior of the maximum size of the lesion was sliced into forty serial coronal sections (50µm thick). Anteroposterior sections 1–40 were stained with hematoxylin and eosin (HE). Images of the HE stain in the forty anteroposterior serial sections were captured using a Nikon E 1000M microscope at 1X magnification and a CCD camera. The area of the damaged region in each image was traced and measured by computer, and the average area of the damaged region calculated over the forty serial sections.

2.10 Statistical analysis

Water maze data were expressed as mean±SE. Data were analyzed using ANOVA and Fisher's PLSD-test (Stat View®; SAS Institute Inc, Cary, NC. USA). Other data were expressed as mean±SD and analyzed using ANOVA. $p<0.05$ was considered statistically significant.

3. Results

3.1 Double-immunofluorescence of amyloid precursor protein (APP) and neurofilament (NF), microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP) or CD11b

The neuronal or microglial localizations of APP protein in the cortical traumatic injured rats at 1 day, 3 days and 7 days post-lesion are illustrated (Fig.1-3). Neurons, astroglia or microglia were identified by immunohistochemical staining using anti-NF, anti-MAP-2, and anti-GFAP or anti-CD11b, respectively. Double labeling immunohistochemistry with anti-

NF (Fig. 1A) and anti-APP (Fig. 1B) antibodies clearly demonstrated the co-localization of NF and APP proteins in swollen and dystrophic nerve fibers at 1day after TBI (Fig. 1C). At 7 days after cortical traumatic injury, MAP-2-positive nerve cell cytoplasm (Fig. 1D) was positive for APP (Fig. 1E&F).

Anti-GFAP (Fig. 2A) or anti-CD11b (Fig. 2D) and anti-APP (Fig. 2B&E) antibodies did not demonstrate co-localization in the same cells at 3 days after cortical traumatic injury (Fig. 2C&F). At 7 days after traumatic injury, a few CD11b-positive cells (Fig. 2G) expressed weakly positive APP immunoreactivity (Fig. 2H&I).
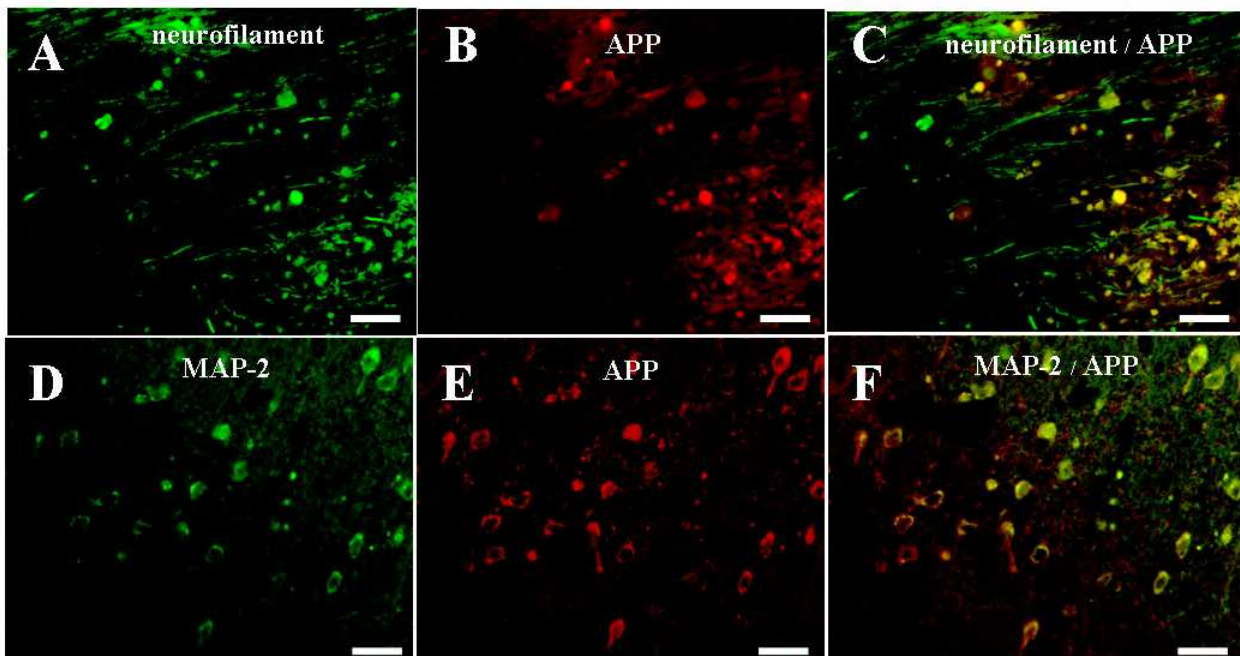
Fig. 1. Double-immunofluorescence staining of localized neurofilaments (A) or MAP-2 (D) and APP (B, E) around the damaged area after traumatic brain injury. At 1 day after injury, damaged dystrophic and swollen neurites (A, green) in the cortex were APP-positive (B, red). The merged image (C) of panels (A) and (B) reveals colocalization of these proteins (yellow). At 7 days after injury, neurons near the injured portion were positive for MAP-2 (D, green) and APP (E, red). The merged image (F) of panels (D) and (E) reveals colocalization of these proteins (yellow). Scale bar = 50 μm.
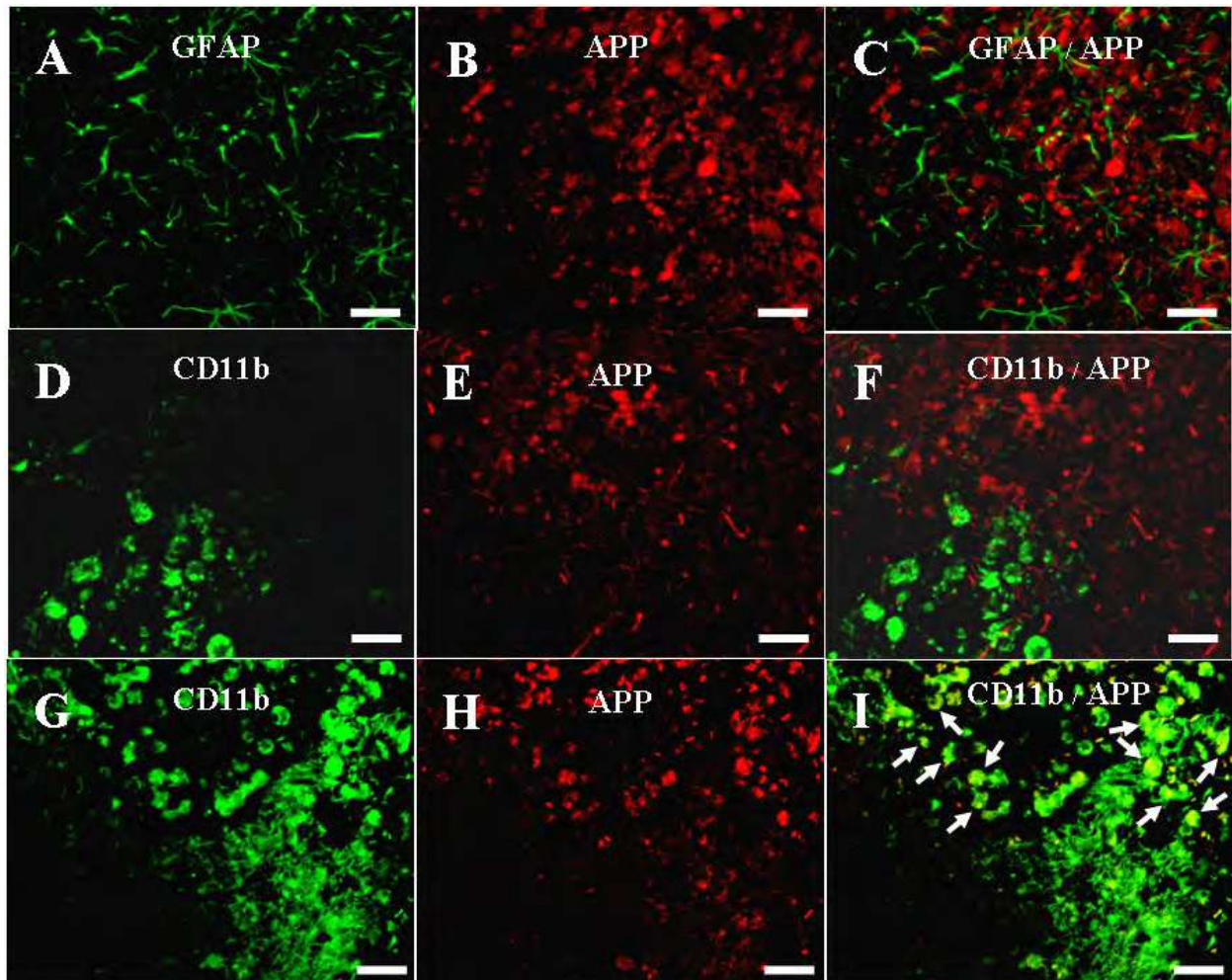
Fig. 2. Double-immunofluorescence staining for GFAP (A) or CD11b (D, G) and APP (B, E, H) around the damaged area after traumatic brain injury. At 3 days after the injury, GFAP-immunopositive (A, green) and APP-immunopositive (B, red) cells are observed. The merged image (C) of panels (A) and (B) does not show any colocalization. At 3 days after the injury, CD11b-immunopositive (D, green) and APP-immunopositive (E, red) cells are observed. The merged image (F) of panels (D) and (E) does not show any colocalization. However, at 7 days after the injury, CD11b-immunopositive (G, green) and APP-immunopositive (H, red) cells are colocalization (I, arrows). Scale bar = 50 μm.

3.2 Immunostaining and APP-immunopositive cell and neurites count after TBI

There were many APP-positive damaged swollen and dystrophic neurites at 1 day after TBI (Fig. 3A). At 7 days after TBI, there were many APP-positive neurons and a few APP-positive damaged swollen and dystrophic neurites (Fig. 3B). However, there were no APP-immunopositive cells and fivers in the sham-operated cerebral cortex (Fig. 3C). The number of APP-positive neurites ($p<0.001$, Fig. 3D) and cells ($p<0.001$, Fig. 3E) was increased in the cortex as early as 1 day following injury, with the elevation becoming significant from 1 day to 90 days($p<0.001$, Fig. 3E). There were no such changes in the sham group (Fig. 3D&E).
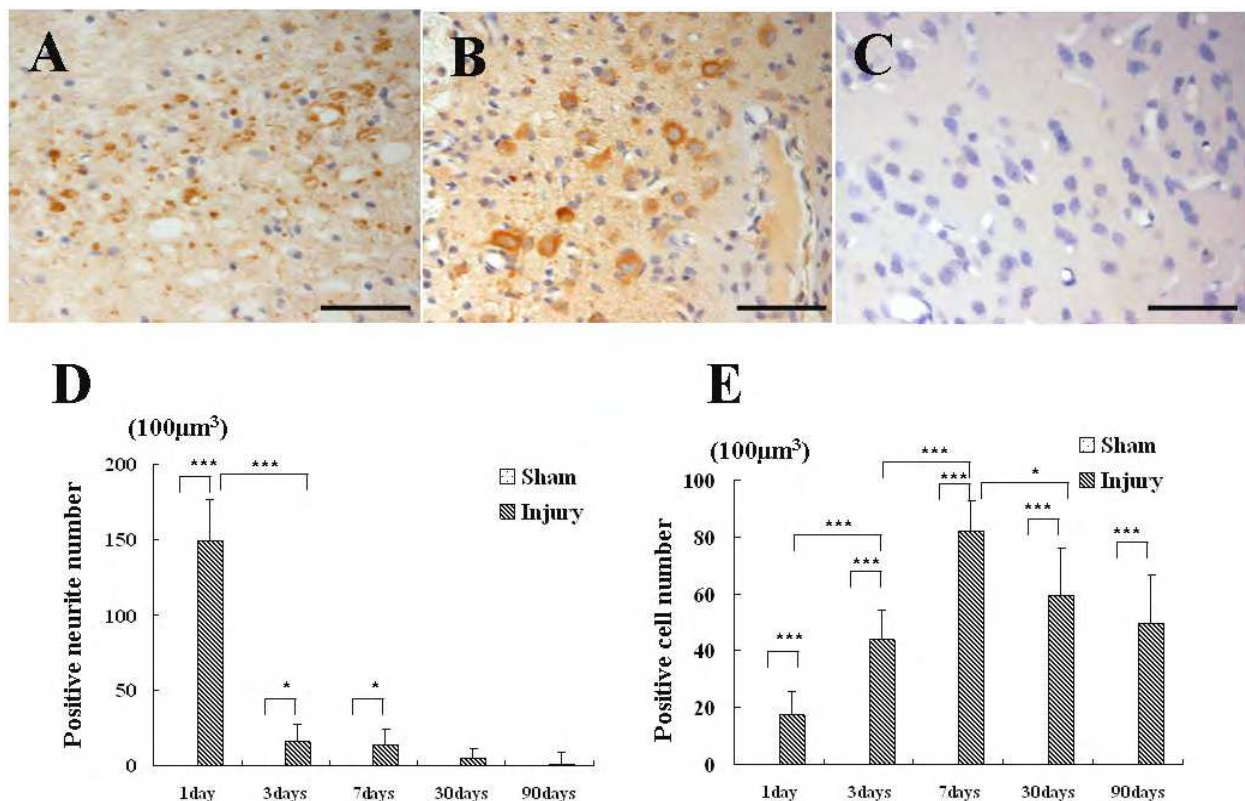
Fig. 3. Immunostaining for APP around the damaged cerebral cortex after traumatic brain injury. At 1 day after injury, there are many APP-positive damaged dystrophic and swollen neurites (A). At 7 days after injury, there are many APP-positive neurons and a few APP-positive damaged dystrophic and swollen neurites (B). However, the sham-operated control cortex does not contain any APP staining or APP-immunopositive cells (C). Scale bar = 50 μm. Graph showing the numbers of APP-immunopositive neurites (D) and cells (E) around the damaged cerebral cortex after traumatic rat brain injury. The results are shown as the mean ± SD. $*P < 0.05$, $***P < 0.001$, $n = 5$.

3.3 RT-PCR analysis of APP

As determined by RT-PCR, the mRNA levels for APP were elevated after cortical injury. The APP transcript level displayed an increase in the cortex as early as 1 day following injury, with the elevation becoming significant between 1 day and 30 days and returning to the control level by 90 days ($p<0.05$, Fig. 4A) . One peak of elevation was determined between 1 day and 30 days after injury. There were no such changes in the sham group (Fig. 4A).

3.4 APP protein in brain tissue after TBI

As determined by ELISA, the levels of APP were elevated after cortical injury. ELISA demonstrated a pattern similar to that showing by RT-PCR. APP levels displayed an increase in the injured side as early as 1 day following injury, with the elevation becoming significant between 1 day and 90 days ($p<0.001$, Fig. 4B). One peak of elevation was determined between 1 day and 90 days after injury (Fig. 4B). There were no such changes in the sham group (Fig. 4B).
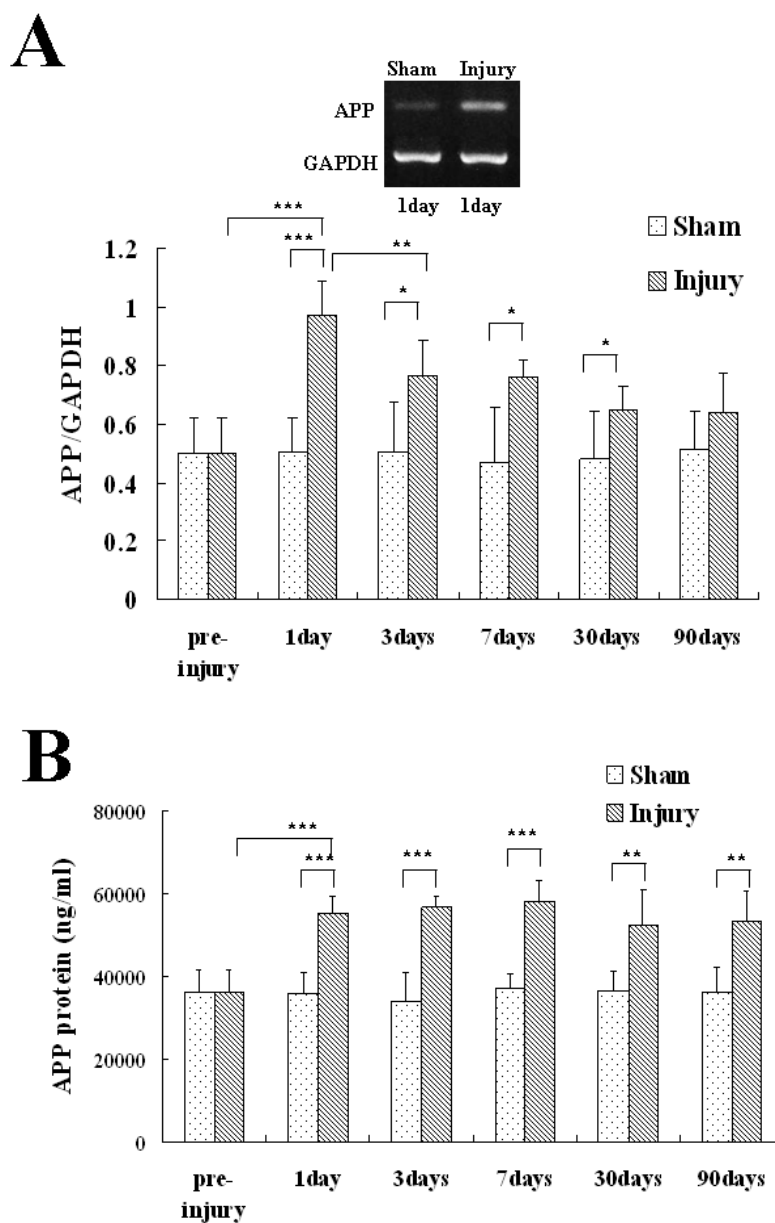
Fig. 4. Graphs show the optical density units of ethidium bromide-stained RT-PCR products for APPmRNA expression (A) and the level of APP protein (B) after traumatic brain injury. The results are shown as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $n = 5$.

3.5 Water maze experiments

When compared with the sham operation group, the arrival time to platform was significantly increased at seven days after TBI in the PBS group ($p<0.001$, Fig. 5). However, at seven days after TBI, the arrival time to platform was significantly decreased in the anti-APP antibody group versus the PBS group ($p<0.001$, Fig. 5), and was not different from the sham operation group at seven days after TBI (Fig. 5).
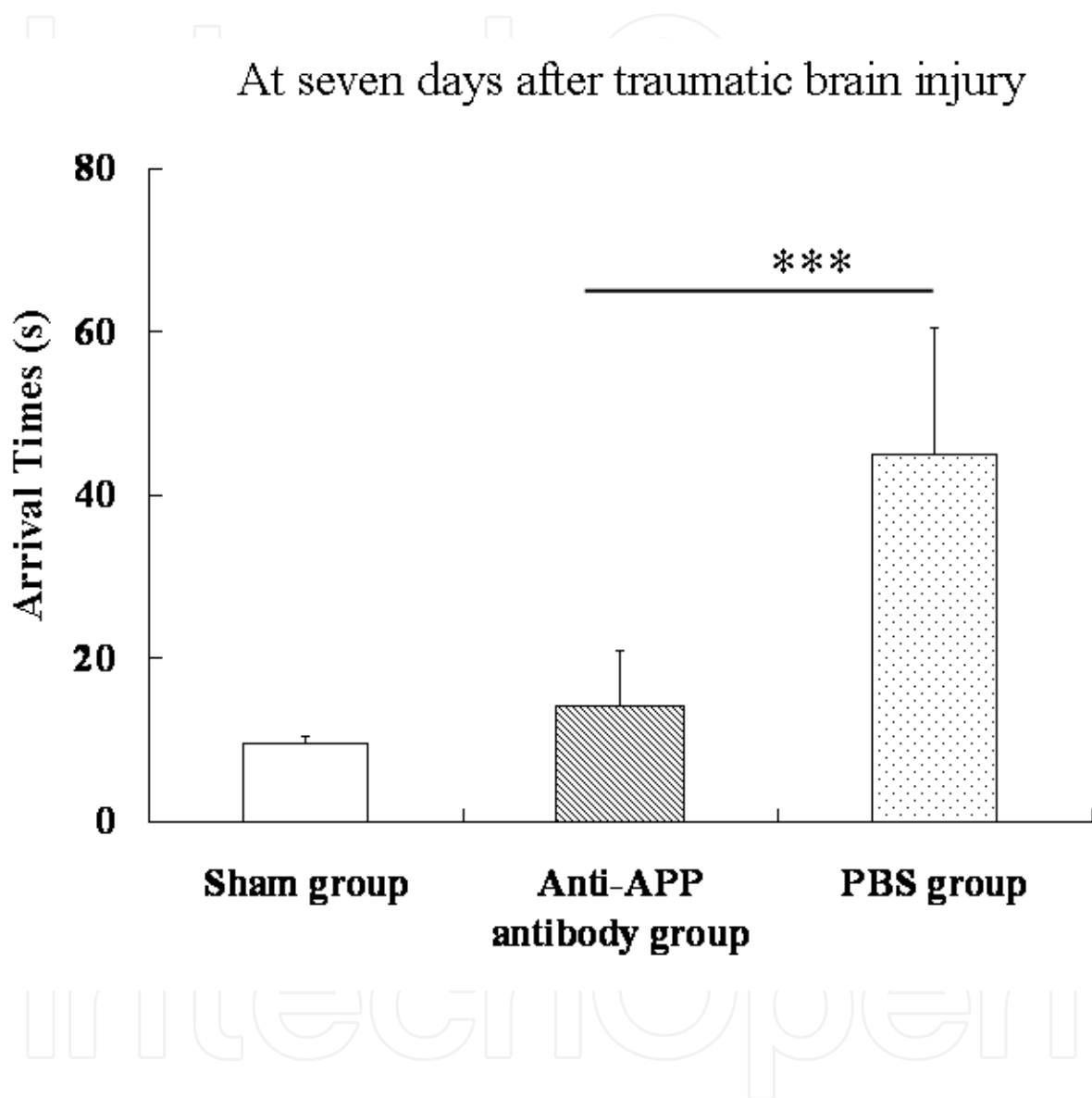
At seven days after traumatic brain injury

Fig. 5. The effect of anti-APP antibody on cerebral function at seven days after TBI. Anti-APP antibody (1µg/ml) or PBS was infused over seven days by osmotic pump (1µl/hr). The sham operation group had no injury. The effects of treatment group on arrival time to platform are shown. Values represent mean±SE (n=10/group). PBS group *vs.* anti-APP antibody group *vs.* sham operation group. ***p<0.001

3.6 Area of the damaged brain region after TBI

HE images of the damaged brain region at seven days after TBI can be seen in Figure 6A-B. The area of the damaged brain region was significantly decreased in the anti-APP antibody group versus the PBS group (p<0.001, Fig. 6C).

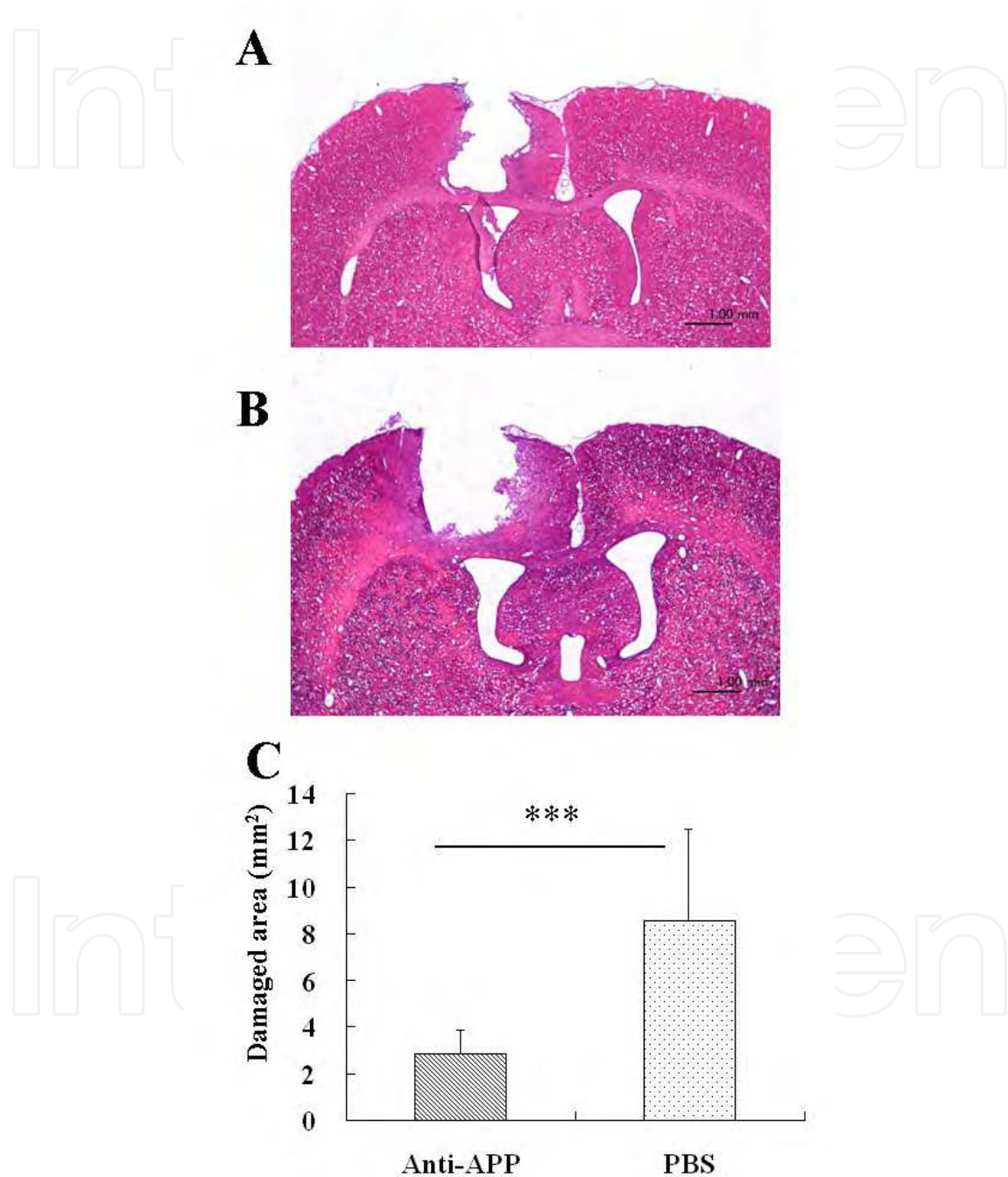Fig. 6. The size of the damaged brain region at seven days after TBI. HE stains show the anti-APP antibody group (1µg/ml) (A) and the PBS group (B). Scale bar = 1mm. (C) Measurement results of each group (n=5/group). Anti-APP antibody group *vs*. PBS group. ***p<0.001.

3.7 Immunostaining and GFAP-immunopositive cell counts after TBI

Immunostaining results for GFAP expression and the numbers of GFAP-positive cells around the damaged brain region at seven days after TBI can be seen in Figure 7A-E. In the anti-APP antibody group there were many larger GFAP-positive cells with GFAP staining in their cytoplasm and long elongated projections around the damaged region (Fig 7. A-B). Furthermore, there were many GFAP-positive fibers that formed glial scars after TBI, and these fibers were enriched at the damaged brain region (Fig. 7A). In contrast, in the PBS group there were only a few small GFAP-positive cells which possessed a small GFAP-positive cytoplasm and a few projections (Fig. 7C-D). There was a significant increase in the number of GFAP-positive cells in the anti-APP antibody group versus the PBS group (p<0.001, Fig. 7E).
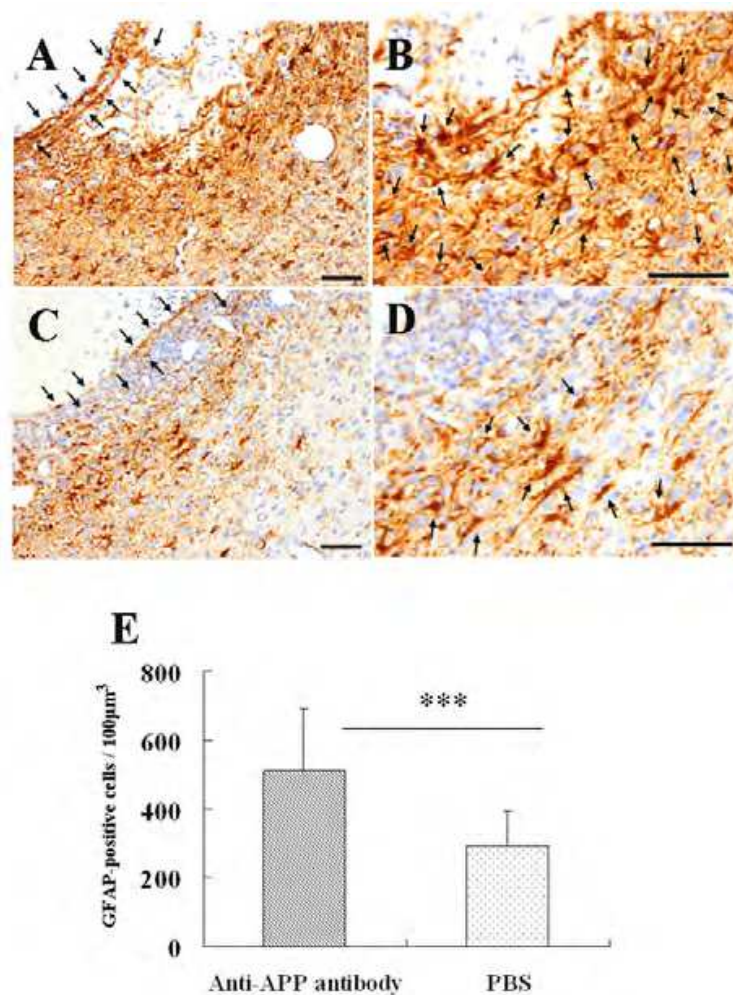

Fig. 7. GFAP expression around the damaged region at seven days after TBI. Note that there were many glial scars (A, arrows) and many large GFAP-positive cells and projections (B, arrows) in the anti-APP antibody group (1μg/ml; A: low power, B: high power), while glial scars (C, arrows), only a few small GFAP-positive cells, and a few projections (D, arrows) were observed in the PBS group (C: low power, D: high power). Scale bar = 50μm. (E) Number of GFAP-positive cells in each group (n=10/group). Anti-APP antibody group *vs*. PBS group. ***p<0.001.

3.8 Immunostaining and MAP-2-immunopositive cell counts after TBI

Immunostaining results for MAP-2 expression and the numbers of MAP-2-positive cells around the damaged region at seven days after TBI can be seen in Figure 8A-C. In the anti-APP antibody group there were many large MAP-2-positive nerve cells (cytoplasm stained) and MAP-2-positive fibers (Fig. 8A). In contrast, in the PBS group there were only a few small MAP-2-positive cells which possessed a small MAP-2-immunopositive cytoplasm and a few MAP-2-positive fibers (Fig. 8B). There was a significant increase in the number of MAP-2-positive cells in the anti-APP antibody group versus the PBS group (p<0.001, Fig. 8C).
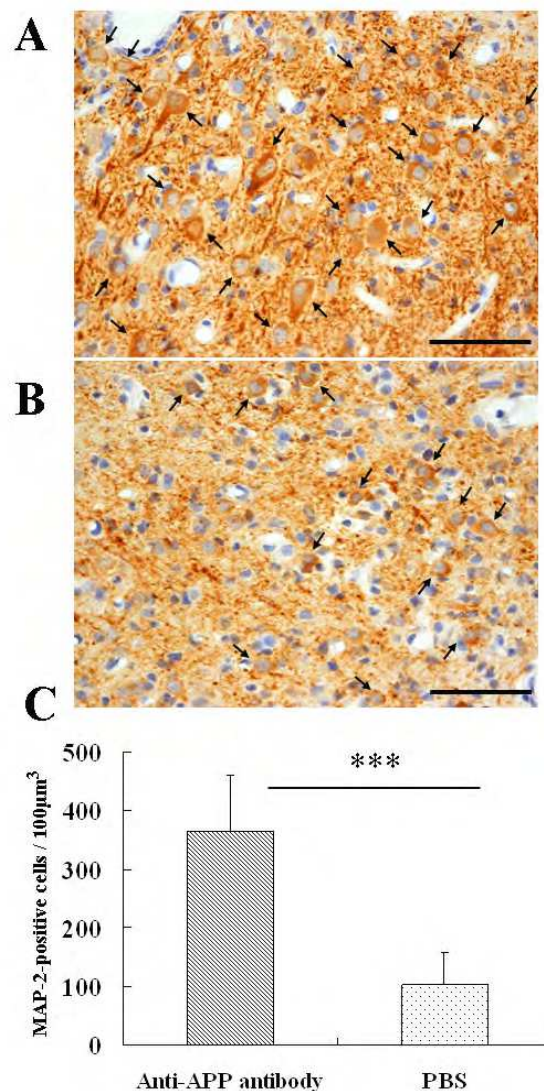

Fig. 8. MAP-2 expression around the damaged region at seven days after TBI. Note that numerous large MAP-2-positive cells and fibers were observed in the anti-APP antibody group (1μg/ml; A, arrows), while only a few small MAP-2-positive cells and a few fibers were observed in the PBS group (B, arrows). Scale bar = 50μm. (C) The number of MAP-2-positive cells in each group (n=10/group). Anti-APP antibody group *vs*. PBS group. ***p<0.001.

3.9 TUNEL-staining and TUNEL-positive cells counts after TBI

TUNEL-staining results and the numbers of TUNEL-positive cells around the damaged region at three days after TBI can be seen in Figure 9A-E. In the anti-APP antibody group there were only a few TUNEL-positive cells which possessed a small cytoplasm, such as glial cells, or a large cytoplasm, such as neural cells (Fig. 9A). In contrast, in the PBS group there were many TUNEL-positive cells which possessed small or large cytoplasm's (Fig. 9B). Furthermore, Almost TUNEL-positive cells in PBS group colocalize with the MAP-2 staining (Fig. 9C). A few TUNEL-positive cells in PBS group colocalize with the GFAP staining (Fig. 9D). In addition, the number of TUNEL-positive cells in the anti-APP antibody group was significantly decreased versus the PBS group ($p<0.001$, Fig. 9E).
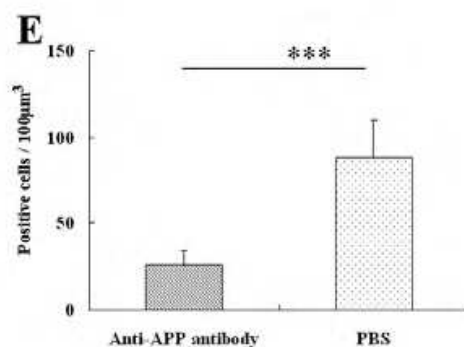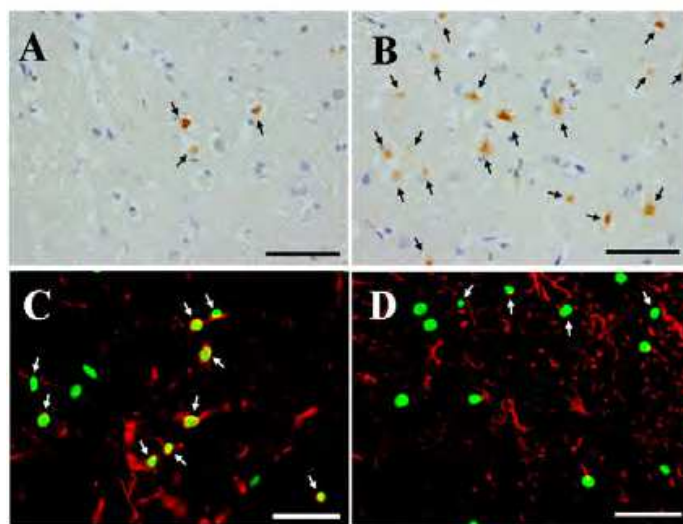

Fig. 9. TUNEL-staining around the damaged region at three days after TBI. Note that there were only a few TUNEL-positive cells, which possessed small or large cytoplasms, in the anti-APP antibody group (1µg/ml; A, arrows), while many small or large cytoplasm TUNEL-positive cells were observed in the PBS group (B, arrows). (C) and (D) were indicated TUNEL-staining (green) and MAP-2 (C, red) or GFAP (D, red) immunofluorescence staining in PBS group. Many TUNEL-positive (C, green) cells showed MAP-2-immunopositive (C, red). A few TUNEL-positive (D, green) cells showed GFAP-immunopositive (D, red). The merged image of TUNEL- and MAP-2- or GFAP-positive cells showed arrows (C&D). Scale bar = 50µm. (E) Number of TUNEL-positive cells in each group (n=5/group). Anti-APP antibody group *vs.* PBS group. ***$p<0.001$.

4. Discussion

The hypothesis that head trauma is a risk factor associated with subsequent development of Alzheimer disease is based on the neuropathology of dementia (Roberts 1988; Roberts et al., 1994). Some reports have shown that the expression of APP is enhanced by various forms of brain injury, including traumatic (Otsuka et al., 1991), chemical (Nakamura et al., 1992) , and ischemic injury (Stephenson et al., 1992). Further, the overexpression of APP suggests the possibility of an Alzheimer disease-like pathology after traumatic brain injury (Rumble et al., 1989). Previously, the appearance of APP in the brain during pathological events was usually attributed to its synthesis by neurons, macrophages, microglia or astrocytes (Otsuka et al., 1991). However, APP was synthesized by neurons, as showing by immunohistochemical, ELISA and RT-PCR obtained performed in this study. In the present report, double labeling immunohistochemistry with anti-NF or anti-MAP-2 and anti-APP antibodies demonstrated the co-localization. Furthermore, we showed that the expression of APP in neurons and neurites was significantly increased in the cerebral cortex after traumatic brain injury. It has been pointed out that increased APP immunoreactivity occurrs in damaged axons due to disturbance of fast anterograde axonal transport (Koo et al., 1990)by destroyed cytoskeletal proteins, including neurofilaments (Posmantur et al., 1994) and MAP-2 (Taft et al., 1992). These results suggested that APP synthesis was increased from the early phase after brain injury and it lasted for a long period during the experiment.

In the present study, the mRNA and protein levels for APP were elevated after TBI. It was previously reported that APP mRNA and protein levels were increased in neurons after TBI (Van den Heuvel et al., 1999; Ciallella et al., 2002). Furthermore, expression of APP was increased in neurons after ischemia and axonal injury (Stephenson et al., 1992; Xie et al., 2003). Taken together, these results suggest that APP is synthesized by neurons, rather than glial cells and macrophages/microglia, after TBI. In future studies, it will be necessary to further clarify the cell type producing APP after TBI.

TBI occurs as the result of a direct mechanical insult to the brain, and induces degeneration and death in the central nervous system (CNS) (Chirumamilla et al., 2002; Rice et al., 2003). Following the initial mechanical insult, secondary pathways are activated that contribute to the ischemic damage induced by circulatory disturbance, blood-brain barrier disruption and excitotoxic damage (Kawamata et al., 1995; Azbill et al., 1997; Xiong et al., 1997). The expression of APP has also been shown to increase in swollen axons and neuronal perikarya after neuronal injuries, including ischemia (Stephenson et al., 1992) and stab injuries (Otsuka et al., 1991). CNS disorders can be caused by the widespread neuronal and axonal degeneration induced by TBI (Chirumamilla et al., 2002; Rice et al., 2003). Taken together, these results suggest that APP leaks out from damaged and necrotic axons and neuronal cytoplasm after TBI. In the present study, CD11b-positive cells were colocalized with APP protein at 7 days after TBI. It appeared that macrophages/microglia engulfed and digested the APP that leaked from axons and neurons during this phase. Therefore, at 7 days after TBI, both neurons and macrophages/microglia expressed APP.

The overexpression of APP suggests the possibility of an Alzheimer disease-like pathology after TBI (Rumble et al., 1989; Itoh et al., 2009). Previously, the appearance of APP in the brain during pathological events was usually attributed to its synthesis by neurons, macrophages, microglia, or astrocytes (Otsuka et al., 1991). Sun *et al.* reported that overproduction of APP induced expression of the apoptosis-related Fas antigen in cultured neural, astrocytes, and microglia (Sun et al., 2004). Furthermore, APP induced nitric oxide

synthetase (iNOS) and nitric oxide (NOS) production, and induced neural and glial cell apoptosis(Sun et al., 2004). In the present study, a continuous infusion of the anti-APP antibody infusion inhibited neural and glial apoptotic cell death at three days after TBI. This neuroprotection was associated with numerous GFAP-positive cells and glial scars, suggesting that the anti-APP antibody inhibited APP-induced glial cell apoptosis.

It was previously reported that APP which was expressed on the cell membrane of cultured cortical neural cells controlled intracellular Ca^{2+} entry (Bouron et al., 2004). Intracellular entry of Ca^{2+} activates Ca^{2+}-dependent signal pathways and can induce neural cell death. Additionally, choline acetyltransferase (ChAT) activity was decreased in the frontal cortex and hippocampus of APP 695 transgenic mice, an Alzheimer model overexpressing APP, and increased the number of apoptotic neurons(Feng et al., 2004). These APP 695 transgenic mice exhibit increased learning and memory impairment (Feng et al., 2004). In the present study, in the anti-APP antibody group there was an increase in the number of MAP-2-positive cells and a reduction in the arrival time to platform versus the PBS group. These data suggested that the anti-APP antibody increased ChAT activity and inhibited neural degeneration induced by overproduced APP in the frontal cortex and hippocampus after TBI, while cerebral function may be improved. As such, measurement of ChAT activity in the anti-APP antibody and the PBS groups after TBI will form the basis of future research.

Recent evidence suggests that APP, a ubiquitously expressed, highly conserved integral membrane glycoprotein, is not only a sensitive marker of axonal injury (Gentleman et al., 1993; Blumbergs et al., 1995), but may also have an important functional role following TBI. APP is upregulated acutely in injured neurons and reactive astrocytes following TBI (Pierce et al., 1996; Van den Heuvel et al., 1999), and this upregulation has been associated with increase hippocampal cell death (Murakami et al., 1998). Although the mechanisms leading to cell death are unknown, as the precursor to the neurotoxic Aβ protein, the conversion of APP to Aβ may have detrimental effects including an increased risk for development of Alzheimer's disease (Chen et al., 2004). Indeed, there are numerous clinical studies demonstrating substantial Aβ deposition and the formation of amyloid plaques (Smith et al., 2003; Ikonomovic et al., 2004). Furthermore, following TBI in the rat there is an increased β-secretase enzyme expression (BACE 11) that could potentially facilitate Aβ production (Blasko et al., 2004), a direct link between increase APP levels and increased Aβ deposition and toxicity (Stone et al., 2002).

In contrast to the potential deleterious effects of APP, an early acute rise in APP during the reparative phase of injury has resulted in the hypothesis that APP may actually serve a neuroprotective function (Van Den Heuvel et al., 2004). APP has been shown to be both beneficial and detrimental depending on its method of posttranslational processing within cells. The beneficial, secreted α form of APP (sAPPα) is generated by α-secretase cleavage, whereas the secreted APPβ (sAPPβ) and deleterious Aβ are generated from cleavage by β- and γ-secretases (Hardy 1997). sAPPα has been reported to have many neuroprotective and neurotrophic functions within the central nervous system (CNS) (Mattson et al., 1993); for instance, sAPPα administration reduced neuronal injury and improved functional outcome following rat TBI (Thornton et al., 2006). The α-secretase pathway is a non-amyloidogenic pathway in which the majority of APP is normally processed (Suh and Checler 2002), while after brain injury the β- and γ-secretase pathways are activated and are responsible for producing the toxic sAPPβ (Matrone et al., 2008; Sola Vigo et al., 2008) and Aβ (Mills and

Reiner 1999; Stone et al., 2002; Blasko et al., 2004). For instance, sAPPβ was shown to induce apoptotic cell death in PC 12 cells and neuronal degeneration in rat hippocampal neurons (Matrone et al., 2008; Sola Vigo et al., 2008). Nakagawa et al. reported that the APP overexpression of ST6Gal-1 in Neuro2 enhanced producing sAPP and the level of the extracellular sAPP β form is increased the level of sAPPα form(Nakagawa et al., 2006). In AD brain, increase of the extracellular sAPP beta level could affect the pathology of AD(Nakagawa et al., 2006). In the present study, anti-APP antibody treatment resulted in a reduction of neuronal and glial apoptotic cell death and recovery of memory and learning function to sham operation group levels, after TBI. These data suggest that toxic sAPPβ is produced more than sAPPα after TBI, and that toxic sAPPβ may be responsible for the neuronal and glial cell degeneration and death. Additionally, overproduction of sAPPβ after brain injury may facilitate Aβ production and deposition.

In the adult CNS, TBI results in a rapid response from resident astrocytes, a process often referred to as reactive astrocytosis or glial scarring (Davies et al., 1999; Jurynec et al., 2003). Glial scars have been reported to inhibit neurite elongation of damaged neurons and axonal regeneration, and thus prevent functional recovery (Davies et al., 1999; Jurynec et al., 2003). Furthermore, neurite outgrowth of cultured rat hippocampal neurons was found to be inhibited by glial scars *in vitro* (Rudge and Silver 1990). However, gliosis and glial scars protect against secondary insults that contribute to the ischemic damage induced by circulatory disturbance, blood-brain barrier disruption, excitotoxic damage, and free radicals (Pekny and Nilsson 2005). Moreover, reactive astrocytes secrete neurotrophic factor (NTF), nerve growth factor (NGF), and extracellular matrix which induce axonal outgrowth and regeneration of the neural network (Bechmann and Nitsch 2000; Deller et al., 2000). In addition, the formation of glial scars prevents leakage of secreted factors, and separates non-damaged areas from damaged areas, thereby maintaining normal CNS homeostasis (Silver et al., 1997; Gallo and Chittajallu 2001). Therefore, although the role of gliosis and glial scars in neuronal regeneration after brain injury remains controversial, the results from the present study suggest that gliosis and glial scars after TBI might be beneficial.

The results from the present study also demonstrated that infusion of the anti-APP antibody into the damaged region following TBI inhibited degeneration of neuronal and glial cells induced by overproduced APP after brain injury, with a significant reduction in injury size. This may be attributed a protective effect of the anti-APP antibody against neural and glial cell death induced by overproduced APP after TBI.

5. Conclusion

In conclusion, the results of the present study have demonstrated that TBI induces long-term increases in APP overexpression in the neuronal perikarya and neurites. In addition, endogenous overproduced APP after TBI inhibited astrocyte activity around the damaged brain region and induced neural cell degeneration. On the basis of these findings, we speculate that overexpression of APP after TBI is related to Alzheimer type dementia, and is an important risk factor for this disease.

6. Acknowledgments

7. References

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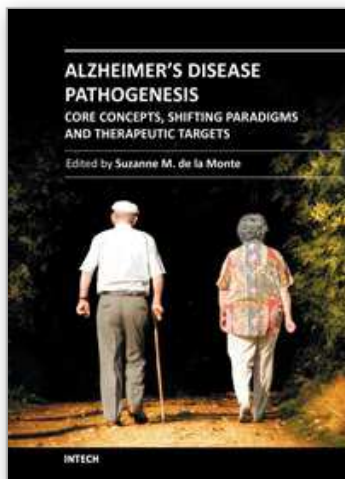
Alzheimer's Disease Pathogenesis-Core Concepts, Shifting Paradigms and Therapeutic Targets

Edited by Dr. Suzanne De La Monte

Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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