The neuroprotective function of 2-carba-cyclic phosphatidic acid: Implications for tenascin-C via astrocytes in traumatic brain injury

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A B S T R A C T

We examined the mechanism how 2-carba-cyclic phosphatidic acid (2ccPA), a lipid mediator, regulates neuronal apoptosis in traumatic brain injury (TBI). First, we found 2ccPA suppressed neuronal apoptosis after the injury, and increased the immunoreactivity of tenascin-C (TN-C), an extracellular matrix protein by 2ccPA in the vicinity of the wound region. 2ccPA increased the mRNA expression levels of Tnc in primary cultured astrocytes, and the conditioned medium of 2ccPA-treated astrocytes suppressed the apoptosis of cortical neurons. The neuroprotective effect of TN-C was abolished by knockdown of TN-C. These results indicate that 2ccPA contributes to neuroprotection via TN-C from astrocytes in TBI.

1. Introduction

Traumatic brain injury (TBI) is a serious public health problem caused by physical trauma, such as traffic or sports accidents that disrupt the brain structure due to biomechanical insult to the cranium. Annually, more than 10 million patients suffer from TBI sequelae such as motor dysfunction and language disorders, and TBI causes nearly 50,000 deaths every year (Humphreys et al., 2013). Immediately following TBI, direct trauma to the injured tissue causes necrotic neuronal cell death. Necrosis causes inflammation as a result of primary injury, and apoptotic neuronal cell death occurs hours and days later as secondary injury (Greve and Zink, 2009). The severity of TBI sequelae depends on the extent of induced inflammation and secondary neuronal cell death. However, therapeutic agents targeting inflammation and controlling secondary neuronal death have not yet been developed.

Cyclic phosphatidic acid (cPA), a lipid mediator, is a candidate therapeutic agent for TBI. The molecule of cPA has a unique structure consisting of a cyclic phosphate ring at the sn-2 and sn-3 positions of its glycerol backbone (Murakami-Murofushi et al., 1992; Murakami-Murofushi et al., 2002). In the nervous system, cPA exhibits neuroprotective effects. For example, cPA promotes neuronal survival and neurite outgrowth and exerts neuroprotective effects via signaling pathways of neurotrophin NGF (Fujiwara et al., 2003). Furthermore, it has been reported that cPA protects neuroblastoma Neuro2A cells from hypoxia-induced apoptosis (Gotoh et al., 2012). 2-carba-cyclic phosphatidic acid (2ccPA), a metabolically stabilized derivative of cPA, is one of the compounds in which phosphate oxygen is replaced with a methylene group at the sn-2 position (Baker et al., 2006). 2ccPA protects hippocampal neurons against ischemia-induced delayed neuronal cell death (Gotoh et al., 2010).

After brain injury, neuronal cell death is influenced by neuro-inflammation, and astrocytes, a type of glial cell, adjust their reactivity to varying degrees of inflammation (Early et al., 2020). Tenascin-C (TN-C) is an extracellular matrix protein that is upregulated in coordination with astrocyte activation after a stab wound to the mouse cerebral cortex (Laywell et al., 1992). Furthermore, in TN-C-deficient mice with stab-
wound injuries of the cerebral cortex, the expression of IL-1β, tumor necrosis factor-α, and IL-6 were higher than that in WT mice (Ikeshima-Kataoka et al., 2008). In contrast, TN-C deficiency protects against neuronal cell death in experimental subarachnoid hemorrhage (SAH) in mice (Liu et al., 2018). Therefore, it is possible that the expression of TN-C in astrocytes regulates neuronal cell death after brain injury. It has been reported previously that cPA suppresses the activation of astrocytes and motor dysfunction in the corpus callosum of mice in a multiple sclerosis model (Yamamoto et al., 2014). However, it is not known whether cPA affects neuronal cell death mediated by TN-C.

We recently examined the effect of 2ccPA on the recovery of stab wounds in a TBI mouse model. After the stab wound to the cerebral cortex, 2ccPA significantly suppresses blood-brain barrier (BBB) breakdown and inflammation levels by regulating microglial polarization towards a neuroprotective phenotype in the stabbed cerebral cortex (Hashimoto et al., 2018). These findings suggest that 2ccPA is involved in neuroprotection mediated by microglia. However, it is not known whether 2ccPA regulates neuronal apoptosis and expression of TN-C in astrocytes after the cerebral cortex is stabbed. Accordingly, in the present study, we aimed to investigate the effect of 2ccPA on the neuroprotective function of astrocytes in a stab-wounded cerebral cortex.

In the present study, we found that the administration of 2ccPA suppressed neuronal cell death in a TBI mouse model and caused an increase in the expression level of TN-C in astrocytes, demonstrating that 2ccPA is a promising therapeutic agent for TBI.

2. Materials and methods

2.1. Mice

Female ICR wild-type mice (six weeks old) were obtained from Charles River Laboratory (Kanagawa, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Ochanomizu University, Japan (animal study protocols 18,006, 19,004, and 20,001), and were performed in accordance with the guidelines established by the Ministry of Education, Science, and Culture in Japan.

2.2. Pharmacological agents

2ccPA, a metabolically stabilized derivative of cPA, was chemically synthesized as previously described (Uchiyama et al., 2007) (Fig. 1A). 2ccPA was dissolved in PBS. For in vitro analysis, 2ccPA was dissolved in PBS containing 0.1% BSA (fraction V, fatty acid-free; Merck).

2.3. Stab wound injury and administration of 2ccPA

Stab wound surgery was performed as previously described by Hashimoto et al. (2018). Briefly, a 19-gauge needle was used to penetrate the skull in the occipital region of the right hemisphere, a 27-gauge needle was inserted through this hole along the rostrocaudal axis, and was pulled out gently. The delivery route and dosing regimen of 2ccPA were based on previous studies (Yamamoto et al., 2014, 2017). Wounded or naïve (no injury) mice were randomly assigned to receive either 2ccPA or PBS (control). They received either 500 μg/ml 2ccPA (1.8 mg/kg/day) or PBS by intraperitoneal injection immediately after brain surgery. Successive injections of the same dose of 2ccPA or PBS were administered daily until brain samples were collected (Hashimoto et al., 2018). The cerebral cortices around the stab-wounded regions were collected at baseline for the naïve mice and on day 0 (within 1 h of the surgery), 1, 3, 5, and 7 for wounded mice after the surgery (Fig. 1B).

2.4. Primary astrocyte cultures

Cortical astrocytes were cultured using a modified version of the procedure described by Milner and Campbell (2002, 2003). Astrocytes were collected from the cerebral cortices of the ICR mice on postnatal day 2. Cultured astrocytes were maintained in T75 flasks in DMEM (Merck) with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin for 14 days. For the experiments, the astrocytes (2.0 × 10⁶ cells/well) were plated on 24-well plates and cultured at 37 °C for 24h. Subsequently, the media was replaced with serum-free astrocyte culture medium (50% Neurobasal Medium [Thermo Fisher Scientific] and 50% DMEM with 1 mM sodium pyruvate, 2 mM L-glutamate, 5 μg/ml N-acetyl-L-cysteine, 100 μg/ml BSA, 100 μg/ml transferrin, 16 μg/ml putrescine, 60 ng/ml progesterone, 40 ng/ml sodium selenite, and 5 ng/ml HB-EGF) to prevent the degradation of 2ccPA. These were then cultured with serum-free astrocyte culture medium at 37 °C for an additional 24 h. After the addition of 1 μg/ml LPS (Merck) with 10 μM 2ccPA or PBS as a vehicle for the cell medium, the astrocytes were incubated for 2–36 h at 37 °C until RNA extraction. To analyze the effect of the secreted materials from astrocytes on the apoptosis of neurons, an astrocyte conditioned medium (ACM) was collected. After incubating the astrocytes at 37 °C for 24 h in a serum-free astrocyte culture medium, astrocytes were treated with LPS or PBS for 6 h and washed with PBS. Then, 2ccPA or 0.1% BSA-containing PBS was added to the astrocytes. After the cells were cultured for 12 h, ACMs were collected. The protein concentrations of the ACMs were measured using a BCA protein assay kit (Takara Bio) and adjusted to 100 μg/well.

2.5. Primary cortical neuron culture

Primary cortical neurons were prepared from the cortices of mouse embryonic brains (embryonic day 15.5; E15.5), which was performed according to a modified version of the procedure previously described...
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Immunohistochemistry of cerebral cortices and cultured neurons was performed as described by Hashimoto et al. (2018). For immunofluorescence staining, the primary antibodies in this study included anti-NeuN (1:500 dilution; Merck Cat# MAB377, RRID: AB_2298772), anti-cleaved caspase-3 (1:1000 and 1:400 dilution; Cell Signaling Technology Cat# 9664, RRID: AB_2070042), anti-GFAP (1:500 dilution; Merck Cat# G9269, RRID: AB_477035), anti-MAP2 (1:200 dilution; Merck Cat# MAB2418, RRID: AB_04856), anti-TN-C (1:200 dilution; Immuno-Biological Laboratories Cat# 10337, RRID: AB_494663), and anti-GFP (1:500 dilution; MBL International Cat# 598-7, RRID: AB_10597267) antibodies. The secondary antibodies were as follows: Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-mouse IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 chicken anti-goat IgG, and Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific). Additionally, DAPI (1:2500 or 1:2000 dilution, Roche Diagnostics) was added 2 μm²-lysine in PBS to stain the nuclei. To detect apoptosis in the culture of neuronal cells, view fields (200 × 200 μm²) around the lesions in the sections of the cerebral cortex or the view fields (300 × 400 μm²) of cultured cortical neurons were chosen randomly in each section or on the cover slip. At least three sections from each mouse cerebral cortex were analyzed.

For immunoenzymatic staining, the sections were incubated with the primary antibody overnight then with secondary biotin-labeled antibodies for 1 h, and additionally incubated with the avidin-biotin complex (Vectorstain Elite ABC Standard Kit; Vector Laboratories) for 1 h followed by 3,3-diaminobenzidine solution to detect signals. For the staining of extravasated serum protein IgG, the sections were incubated with only secondary biotin-labeled anti-mouse IgG antibody for 1 h. Images of the injured cerebral cortices were captured using a microscope (FSX100; Olympus and BZ-X710; Keyence). To determine the number of stained neurons, the fluorescence images of the cerebral cortices were analyzed using Imaris software (Imaris x64 8.4.1, Bitplane), while the number of stained neurons was counted with a confocal microscope (LSM710; Carl Zeiss), and for nuclear staining. The fluorescence images of the cerebral cortices were captured with a confocal microscope (LSM710; Carl Zeiss) following by 3,3-diaminobenzidine solution to detect signals. For the quantification of GFAP staining intensity, mean gray values were quantified using ImageJ in five view fields (8 × 8 μm²), which were selected randomly from immunoenzymatically stained regions in each section of the mouse cerebral cortex. The mean gray value in each contralateral and uninjured region was subtracted from that of the ipsilateral region to correct for background values. At least five sections were analyzed for each mouse.

2.7. Real-time RT-PCR

Whole brains were sliced into 2 mm thick pieces, and cerebral cortices in a 1 mm square containing the injury region were cut out from the brain. RNA was extracted from cerebral cortices. Total RNA was reverse-transcribed using the ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd). The primer sequences are listed in Table 1 and were chosen based on previous studies (Amin et al., 2010; Iketani-Kataoka and Yasui, 2016). mRNA expression levels were quantified using a KOD SYBR qPCR Mix (Toyobo Co., Ltd.) with an ABI 7300 real-time PCR machine (Thermo Fisher Scientific). Relative gene expression levels were analyzed using the 2-Delta-Delta-Ct method.

Table 1. Primer sequences used for real-time RT-PCR.

| Gene     | Primer Sequence          |
|----------|--------------------------|
| Gapdh    | Forward 5'-CGTTTTCTACCCGATCCTG-3'  |
|          | Reverse 5'-TTCGATACCTGTTGCTT-3'  |
| Gfap     | Forward 5'-GAAAGTGGCTTTCCTGGA-3'  |
|          | Reverse 5'-GGCTGGAAAGTGGTTGAG-3'  |
| TnC      | Forward 5'-TCTGACCTGTTTGCGCTC-3'  |
|          | Reverse 5'-CCATGGGAACTTGGTTGTA-3'  |

2.8. Western blot analysis

The condensation of proteins in ACM was performed with cold (−20 °C) acetone and centrifuged for 10 min at 14,000 rpm. The supernatant was discarded and dissolved in the radioimmunoprecipitation assay buffer (BioDynamics Laboratory, Inc.). To detect TN-C protein around the lesion (in 1 mm squares with the injury) after the stab wound in the cerebral cortices, the stab wounded mice at naive and day 0–1 were perfused with PBS to remove blood. Then, the cerebral cortices around stab-wound regions were collected, lysed in sample buffer (25 mM Tris–HCl, 5% glycerol, 1% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue, 1% 2-mercaptoethanol), and heated at 95 °C for 5 min.

The protein concentration of ACM and TN-C protein was measured using a BCA protein assay kit and adjusted to 20 μg, which was loaded on a 10% polyacrylamide gel for electrophoresis and then blotted onto polyvinylidene difluoride membranes (ATTO). The membranes were blocked with 0.3% skim milk overnight and then incubated with primary anti-TN-C (1:1000 dilution; Immuno-Biological Laboratories Cat# 10337, RRID: AB_494663) and horseradish peroxidase-labeled secondary antibodies for 1 h each. The bands were visualized with an enhanced luminescent reagent (Ez WestLumi; ATTO) and captured using an ImageQuant LAS 4000 (GE Healthcare, Little Chalfont). The density of each band was quantified using ImageJ software (National Institutes of Health). To confirm whether each ACM was derived from the same amount of astrocytes, the remaining cells in each well were dissolved in RIPA buffer and subjected to Western blotting for β-actin expression. In case of detecting TN-C protein around the lesion, β-actin was blotted on the same membrane as an internal control.

2.9. Detection of apoptotic cells by the TUNEL assay

Brain sections were rinsed in PBS three times and incubated with 3% hydrogen peroxide (H₂O₂) in methanol for 30 min to quench endogenous peroxidase activity. After rinsing in PBS, the sections were subjected to a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using an in-situ apoptosis detection kit (Takara Bio). Colour reactions were developed in 3,3-diaminobenzidine solution to detect apoptotic signals, and counter staining was performed with methyl green (blue) for the nuclei. To detect apoptosis in the culture of cortical neurons, cortical neuron cultures (8.0 × 10⁶ cells/well) were subjected to TUNEL staining for the detection of apoptotic cells. The number of TUNEL-positive neurons was counted in the view fields around the lesions at 20 × magnification, and the ratio of TUNEL-positive cell numbers to total (methyl-green stained) cells was determined.

2.10. Preparation of recombinant Lentivirus for TN-C knockdown

We prepared a shRNA-expressing recombinant lentivirus for the knockdown of TN-C in cultured astrocytes. Lentivirus plasmids expressing shRNA against mouse Tnc (TN-C sh) (TGACCTGGCTACTGACGGGACATTCTTCTC, Origene, Rockville, MD) and negative control shRNA (non-effective scrambled shRNA) (Origene) were used. GFP was inserted into the shRNA. The recombinant virus was prepared

(Beaudoin 3rd et al., 2012). Primary cortical cultures were maintained in 24-well plates (8.0 × 10⁶ cells/well) coated with poly-l-lysine in Neurobasal Medium with 2 mM glutamine and 2% B-27 Supplement (Thermo Fisher Scientific) for 7 days. Ara-C (5μM, Merck) was added 2 days after plating the cells to arrest the proliferation of glial progenitors in neuronal cultures. To examine the effect of ACM on apoptosis, ACM was added to the 7 days cultured neurons, and the neurons were cultured for 24 h. After culturing, the apoptosis level was analyzed.
using a packaging cell, 293 T, and a Lenti-vpak packaging kit (Origene). The recombinant virus was prepared according to the manufacturer's protocol. The 293 T cells (2.5 × 10⁶ cells) were cultured in 10 cm dishes for 1 d with a growth medium (DMEM with 10% fetal bovine serum and penicillin streptomycin), after which the plasmids (5 μg) were transfected into 293 T cells. We collected the conditioned media, including the recombinant virus, and concentrated the virus with a Lenti-X concentrator (Takara Bio).

2.11. Knockdown of TN-C in astrocyte conditioned medium

To knockdown TN-C expression in astrocytes, the concentrated recombinant virus (5 μg) was infected into cultured astrocytes for 24 h in 24-well plates. After an additional 24 h of culture, the medium was

Fig. 2. Effect of 2ccPA on neuronal apoptosis in the stab wounded mouse cerebral cortices. (A) Images of the injured cerebral cortices in PBS (control)-administered mouse brain sections at day 1 after the injury. A circle indicates intracerebral hemorrhage around the stab wound region, which was detected by mouse IgG staining to identify blood-brain barrier (BBB) breakdown. Scale bar: 500 μm. (B) Immunofluorescent images of cerebral cortices in the stab wound region PBS (control) -administered mouse at 3 days after the injury. The section was stained with anti-activated caspase-3 (green) and anti-NeuN (red) antibodies. A white dotted line surrounds the wounded region and its vicinity. Scale bar: 100 μm. (C) High magnification images in the vicinity of the stab wound region. The white dotted lines surround the wound region as shown in (B). Immunofluorescent images of cerebral cortices in the stab wound region of PBS (control)- and 2ccPA-administered mice at 3 days after the injury. Each section was stained with anti-activated caspase-3 (green) and anti-NeuN (red) antibodies, while DAPI (blue) was used for the nuclei. The caspase-3 antibody-stained (Caspase-3), anti-NeuN antibody-stained (NeuN), and merged (Merge) images are shown. Scale bar in the left panels: 100 μm. The white square in left panel magnifies to the image of the right panel, respectively. The arrowheads indicate caspase-3+/NeuN+ cells. Scale bar in the right panel: 50 μm. (D) Percentage of cleaved caspase-3+ cell numbers in NeuN+ cell numbers in the vicinity of the stab wounded region in naïve mice and on days 0–7 in PBS (control)- or 2ccPA-administered mice (n = 3 female mice/group). The number of each positive cell was counted in each view field (100 × 100 μm²). *p < 0.05, **p < 0.01, ***p < 0.001 vs. control groups, two-way ANOVA and Tukey–Kramer test. Data represent the mean ± SEM of three independent experiments using three pairs of mice each specified day. Four fields per section and three sections of mouse brain were analyzed. A total of 36 female mice (6 weeks old) were subjected to this experiment. (E) Images of cerebral cortices in the stab wound regions of PBS (control)- and 2ccPA-administered mice at day 3 after injury with TUNEL assay. Methyl green (blue) was used for the nuclei. The red arrowheads indicate TUNEL+ cells. Scale bar: 100 μm. The high magnification images show the apoptotic cell. Scale bar: 50 μm. (F) The percentage of TUNEL+ cell numbers in total (methyl green stained) cell numbers in the vicinity of the stab wound region in naïve mice and on days 0–7 in PBS (control)- and 2ccPA-administered mice (n = 3 female mice/group). The cell numbers were counted in each view field (100 × 100 μm²). * p < 0.05, **p < 0.01, ***p < 0.001 vs. control groups, two-way ANOVA and the Tukey–Kramer test. Data represent the mean ± SEM of three independent experiments using three pairs of mice on each specified day. Four fields per section and three sections of the mouse brain were analyzed. A total of 36 female mice (6 weeks old) were subjected to this experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
replaced with serum-free astrocyte culture medium and LPS or PBS was added to the cells. The cells were then cultured for 6 h and washed. Then, 2ccPA or 0.1% BSA-containing PBS was added to the cells and cultured for 12 h. Subsequently, the astrocyte conditioned medium (ACMs) were collected. ACM was added to cultured cortical neurons to analyze the effect of TN-C knockdown in ACM. To analyze the infection efficiency of the lentivirus, the cultured astrocytes were stained with anti-GFP (1:500 dilution; Merck Cat# G9269, RRID: AB_477035) antibody. To perform the rescue experiments for knockdown of TN-C, 1 μg/ml TN-C, which was derived from a human glioma cell line, was added with ACM from TN-C knockdowned astrocytes. (Rescue experiment).

2.12. Statistical analyses

In the experiments using tissue sections, three pairs of 2ccPA-administered mice and control mice were used. For real-time RT-PCR analysis, at least four pairs of mice were used. For Western blot analysis, three pairs of datasets were used. Results are reported as the mean ± SEM. Gray levels were detected using ImageJ software. Differences were considered statistically significant if the p-value obtained from the two-way ANOVA followed by the Tukey–Kramer test or Student’s t-test using Prism Software 9.00 (GraphPad Software) reached one of the following significance levels: *p < 0.05, **p < 0.01, and ***p < 0.001.

3. Results

3.1. 2ccPA suppresses neuronal cell death after the stab wound injury

To investigate the effect of 2ccPA on the neuroprotective function after TBI, the cerebral cortex in the right hemisphere was injured by a stab wound in a TBI mouse model. The wounded region on day 1 demonstrated extravasation of serum IgG (Fig. 2A). We analyzed the percentage of cleaved caspase-3-positive [Caspase-3+] cell numbers in NeuN-positive [NeuN+] in the stab-wounded cortices (Fig. 2D). The administration of 2ccPA to injured mouse brains significantly suppressed the percentage of neuronal death at day 0 (p = 0.005), day 1 (p = 0.004), and day 3 (p < 0.001) after the injury in the vicinity of the stab wound regions compared with the control (stab wound regions in PBS-administered mouse) cortices (Fig. 2D). Apoptosis was evaluated using the TUNEL assay to confirm the neuroprotective effect of 2ccPA (Fig. 2E). We analyzed the percentage of TUNEL-positive [TUNEL+] in methyl green-stained [total] cells in the stab-wounded cortices (Fig. 2F). The administration of 2ccPA to injured mouse brains significantly suppressed the percentage at day 0 (p < 0.001), day 1 (p < 0.001), day 3 (p < 0.001), day 5 (p = 0.006), and day 7 (p = 0.02) after the injury in the vicinity of the stab wound regions compared to that in control cortices (Fig. 2F). These results indicate that 2ccPA suppresses stab-wound-induced neuronal cell death in the mouse brain.

3.2. 2ccPA suppresses the activation of astrocyte in cerebral cortex after the stab wound injury

To determine whether 2ccPA treatment regulates the activation of astrocytes in the cerebral cortex after TBI, the mRNA expression level of glial fibrillary acidic protein (GFAP) around the lesion (in 1 mm squares with the injury) was examined after the stab wound in the cerebral cortices. Administration of 2ccPA significantly suppressed the Gfap mRNA expression level at day 3 (p = 0.002) after the injury compared to that of the control cortices (Fig. 2A). Next, the immunoreactivity of GFAP around the lesion was quantified using immunoenzymatically stained mouse brain sections (Fig. 2B). The administration of 2ccPA significantly decreased GFAP immunoreactivity in the vicinity of the stab wound regions at day 3 (p = 0.004) compared to that in the control cortices (Fig. 2C). Additionally, astrocytes in the vicinity of the stab-wounded region were analyzed using an antibody against GFAP to examine the effect of 2ccPA on the number of astrocytes. The administration of 2ccPA decreased the ratio of GFAP-positive [GFAP+] cells in the vicinity of the stab wound region especially at day 3 (p = 0.007) after inflicting the wound compared to that in the control cortices (Fig. 3D). Moreover, to confirm the effect of 2ccPA on the activity of astrocytes, GFAP+ cells were immunostained with an antibody against an activated astrocyte marker called Sox9 (Fig. 3E). The ratio of Sox9 and GFAP double-positive [Sox9+; GFAP+] cell numbers to GFAP+ cells significantly decreased at day 3 (p = 0.006) after inflicting the wound by the administration of 2ccPA compared to that in control cortices (Fig. 3F).

3.3. 2ccPA increases the number of TN-C-positive astrocytes in the early stages of injury

The expression of TN-C, an extracellular matrix protein, has been reported to be upregulated in astrocytes in injured mouse brains (Laywell et al., 1992; Ikeshima-Kataoka and Yuasa, 2008). Then, the effect of 2ccPA on the expression of TN-C was examined in the cerebral cortices using immunohistochemistry with an antibody against TN-C after inflicting the stab wound (Fig. 4A). TN-C deposition was observed in astrocytes and in the vicinity of astrocytes on days 0 and 1. It was observed that the amount of TN-C in the stab-wound region of 2ccPA-administered mice was larger than that of control mice on day 0 and that the amount of TN-C in both mice was reduced at day 7 (Fig. 4A). The Imaris 3D images indicated that TN-C was localized to the surrounding astrocytes, suggesting that TN-C was secreted from astrocytes (Fig. 4B). The administration of 2ccPA significantly increased the percentage of TN-C and GFAP-double-positive [TN-C+; GFAP+] cell numbers in GFAP+ cells at days 0 and 1 (p < 0.001) but decreased the percentage after day 3 in the vicinity of the stab wound regions compared to that in control cortices (Fig. 4C). To determine whether 2ccPA administration regulates the expression of TN-C in the cerebral cortex after TBI, the mRNA and protein expression level of TN-C around the lesion were examined in TBI model mice (Fig. 5). As a result, the administration of 2ccPA significantly suppressed the Tnc mRNA expression level at day 5 (p = 0.03) after the injury compared to that of the control cortices in PBS-administered mice, but the Tnc mRNA expression level at day 0 and 1 were no significant difference than that of control mice (Fig. 5A). On the other hand, the TN-C protein level of 2ccPA-administered mice was significantly higher than that of control mice on day 0 and 1 (p < 0.05) (Fig. 5B and C).

3.4. 2ccPA increases the expression of TN-C in lipopolysaccharide (LPS)-stimulated astrocytes

To examine the direct effect of 2ccPA on the expression of TN-C in activated astrocytes, a bacterial endotoxin, LPS, was exposed to astrocytes as an in vitro model of inflammation after injury. First, primary cultured astrocytes were pre-cultured for 24 h and replaced with serum-free media. After an additional 24 h of culture, 2ccPA and/or LPS were added to astrocytes, and the astrocytes were cultured for 2–36 h (Fig. 6A). The addition of LPS only and 2ccPA only increased the mRNA expression levels of Tnc in a time-dependent manner and peaked at 4 and 6 h of culture, respectively, compared with the control (not including LPS and 2ccPA) (Fig. 6B). Moreover, LPS + 2ccPA synergistically increased the expression levels of Tnc mRNA (Fig. 6B). To confirm the upregulation of Tnc mRNA levels by LPS and 2ccPA, the secreted TN-C protein levels were analyzed in the astrocyte-conditioned medium (ACM) of LPS-, 2ccPA-, and LPS + 2ccPA treated astrocytes using Western blotting (Fig. 6C). LPS ACM and 2ccPA ACM increased the expression of TN-C protein levels in a time-dependent manner (6–24 h), and the secreted TN-C protein expression levels of LPS + 2ccPA ACM were higher than those in LPS or 2ccPA ACM at 6–24 h (Fig. 6D).
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Figure A: Graph showing GFAP mRNA level (G6PD/GAPDH) over days 0-7 for control and 2ccPA groups.

Figure B: Images showing GFAP expression over days 0-7 for control and 2ccPA groups.

Figure C: Graph showing intensity of GFAP immunoreactivity for control and 2ccPA groups.

Figure D: Graph showing ratio of GFAP+ cells (200 x 200 μm²) for control and 2ccPA groups.

Figure E: Images showing Merge, Sox9, and GFAP expression on Day 3 for control and 2ccPA groups.

Figure F: Graph showing ratio of Sox9+ cells (200 x 200 μm²) on Day 3 for control and 2ccPA groups.
cultured cortical neurons, as shown in the step of cultured astrocytes for 12 h, as shown in the step of + staining for cleaved caspase-3 and MAP2 (Fig. 7 B).

LPS and 2ccPA, respectively (Fig. 7 A). The neurons were cultured for 24 " levels peaked at 6 h of culture by LPS stimulation (Fig. 6 B). LPS was increases the survival of neurons. To test this hypothesis, we examined the effect of the conditioned medium of 2ccPA treated astrocytes on neuronal apoptosis. First, we treated cells with LPS for 6 h, as shown in the step of “Pretreatment of astrocyte,” because Tnc mRNA levels peaked at 6 h of culture by LPS stimulation (Fig. 6B). LPS was removed by washing with PBS (Fig. 7A). Then, 2ccPA was applied to the cultured astrocytes for 12 h, as shown in the step of “Treatment of astrocytes.” Subsequently, the collected ACMs were applied to the primary cultured cortical neurons, as shown in the step of “Treatment of neurons.” PBS and 0.1% BSA-containing PBS were used as the vehicles for LPS and 2ccPA, respectively (Fig. 7A). The neurons were cultured for 24 h after the addition of ACM to the cortical neurons. The effect of each ACM on the apoptosis of cortical neurons was examined by immunostaining for cleaved caspase-3 and MAP2 (Fig. 7B). LPS, 2ccPA, and Direct 2ccPA ACM remarkably decreased the number of MAP2-positive [MAP2+] cells compared to control ACM (Fig. 7B), suggesting that LPS-pretreated astrocytes secreted neurotrophic factors, and that the toxic factors accumulated in LPS, 2ccPA, and Direct 2ccPA ACM. Therefore, these ACMs remarkably decreased the number of cultured cortical neurons. Therefore, we examined the effect of ACMs on the apoptosis of cortical neurons. As a result, LPS ACM increased the percentage of caspase-3-positive [Caspase-3+] cell numbers in MAP2-positive [MAP2+] cortical neurons compared to that in control ACM (Fig. 7C). Compared to the percentage of [Caspase-3+] in [MAP2+] with LPS ACM, 2ccPA ACM significantly suppressed the percentage of [Caspase-3+] in [MAP2+] cortical neurons (p = 0.042) (Fig. 7C). However, 2ccPA ACM did not suppress apoptosis to the same level as the control ACM. Furthermore, to examine the effect of the remaining 2ccPA in 2ccPA ACM on the apoptosis of cortical neurons, the same amount of 2ccPA in 2ccPA ACM was added to cortical neurons with LPS ACM (Direct 2ccPA ACM) (Fig. 7A). Even though the percentage of [Caspase-3+] in [MAP2+] with Direct 2ccPA ACM was lower than that of LPS ACM, the percentage of Direct 2ccPA ACM was significantly higher than that of 2ccPA ACM (p = 0.047) (Fig. 7C), indicating that 2ccPA contributes to neuroprotection not only directly but also via astrocytes.

To analyze whether TN-C could serve as a neuroprotective factor in 2ccPA ACM, we examined the secreted TN-C protein levels in each ACM (Control, LPS, 2ccPA) (Fig. 7D). As a result, the TN-C protein level of LPS ACM was remarkably higher than that of control ACM and the TN-C protein level of 2ccPA ACM was significantly higher than that of LPS ACM (p = 0.024) (Fig. 7E). Thus, the TN-C protein levels of the ACMs correlated with the effects of the ACMs on the apoptosis of cortical neurons, suggesting that the secretion of TN-C by 2ccPA in the ACMs may contribute to the suppression of neuronal cell death.

3.6. Knockdown of astrocyte-derived TN-C increases the ratio of apoptotic neurons

Knockdown of TN-C in primary cultured astrocytes was performed to clarify whether 2ccPA regulates neuronal cell death through TN-C secreted from astrocytes. The lentivirus for TN-C knockdown (TN-C sh) or control negative shRNA [Scrambled shRNA] was transfection into astrocytes in serum-free medium and the astrocytes were cultured for 24 h as shown in the step of “Pretreatment of lentivirus.” The efficiency of the lentivirus infection was approximately 98% (scrambled sh) and 99% (TN-C sh) while the knockdown of TN-C reduced the mRNA expression levels by 75% compared to the control (scrambled shRNA) level (p = 0.002) (Fig. 8B). After that, astrocytes were treated as shown in Fig. 7 (6 h pretreatment of astrocytes and 12 h treatment of astrocytes), and the TN-C sh and scrambled sh ACMs were collected. ACMs were added to the cortical neurons (Fig. 8A). The effect of the ACMs on neuronal apoptosis was detected by immunostaining for caspase-3 and MAP2 (Fig. 8C). The addition of TN-C sh ACM increased the percentage of cleaved caspase-3-positive [Caspase-3+] cell numbers in MAP2-positive [MAP2+] cell numbers in cortical neurons compared with scrambled sh ACM (p = 0.024) (Fig. 8D), indicating that TN-C knockdown abolished the neuroprotective effect of 2ccPA. The addition of TN-C protein (Rescue) to TN-C sh ACM decreased the percentage of [Caspase-3+] to the same level as scrambled sh-ACM (p = 0.022) (Fig. 8D), indicating that the increase in neuronal apoptosis by TN-C knockdown was rescued by the addition of TN-C protein.

Next, to confirm the neuroprotective effect of 2ccPA via TN-C, the effect of TN-C knockdown on apoptosis was examined using the TUNEL assay (Fig. 9A). LPS ACM increased the percentage of TUNEL-positive [TUNEL+] in methyl green-stained [total] cell numbers compared to control ACM, while the increase in the ratio was suppressed by 2ccPA (p = 0.001) (Fig. 9B). To confirm the neuroprotective effect of 2ccPA via astrocytes, 2ccPA was added to LPS ACM (Direct 2ccPA) just before the ACM was added to neurons. The percentage of TUNEL-positive cells in Direct 2ccPA ACM was significantly lower than that in LPS ACM (p = 0.002) but was significantly higher than that in 2ccPA ACM (p = 0.008) (Fig. 9B), indicating that 2ccPA suppresses apoptosis via astrocytes. Furthermore, to confirm the effect of TN-C on apoptosis, we knocked down TN-C in ACMs, as shown in Fig. 8. TN-C sh ACM increased the percentage of TUNEL-positivity [TUNEL−] in methyl green-stained [total] cells in cortical neurons compared with scrambled shACM (p = 0.003) (Fig. 9B), indicating that TN-C contributes to the suppression of apoptosis. Then, for the rescue experiment, TN-C was added to the TN-C sh ACM. The ratio of apoptosis in Rescue was significantly lower than that in TN-C sh ACM (p = 0.03) (Fig. 9B).
Fig. 4. Effects of 2ccPA on the TN-C deposition in the astrocytes in the vicinity of the stab wound region. (A) Immunofluorescence staining of cerebral cortex sections in the PBS (control)- and 2ccPA-administrated mice at day 0 and 7 after the injury using anti-TN-C (green) and anti-GFAP (red) antibodies, as well as DAPI (blue). Images of TN-C deposition were constructed three-dimensionally in astrocytes by confocal and Imaris 3D software with 2.5 μm z-stack thickness. Each white square region was magnified in the bottom panels of (B). The arrowheads indicate TN-C+ cells. Scale bar: 100 μm. (B) Highly magnified images of deposited TN-C in the vicinity of astrocytes. The highly magnified images (Merge) of deposited TN-C (green) were observed in the vicinity of the astrocytes (red). Scale bar: 50 μm. (C) The effect of 2ccPA on the percentage of TN-C positive cells to GFAP+ numbers (TN-C deposit around astrocyte) in the vicinity of the stab wound region in naïve mice and on days 0–7 after the injury in PBS (control)- and 2ccPA-administrated mice (n = 3 female mice/group). The cell numbers were counted in view fields (200 × 200 μm²). Three sections were analyzed from each mouse. ** p < 0.01 vs. control groups, two-way ANOVA and the Tukey–Kramer test. Data represent the mean ± SEM of three independent experiments using three pairs of mice at each specified day. Four fields per sections and three sections of mouse were used. A total of 36 female mice (6 weeks old) were subjected to this experiment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

So far, we have studied the effect of 2ccPA on the recovery of brain injury in a model of TBI and stab wound injury. Our previous results showed that 2ccPA suppresses extravasation, attenuates inflammation, and modulates microglial polarization towards the neuroprotective phenotype M2 in the area of the stab wound injury (Hashimoto et al., 2018). However, we did not examine the effects of 2ccPA on neuronal death in the vicinity of the stab wound injury or on astrocyte activity, which is closely related to the repair of TBI (Burda et al., 2016). In the current study, we analyzed the neuroprotective effect of 2ccPA and the involvement of 2ccPA in astrocyte activity after a stab wound injury. The present study clarified the following. First, 2ccPA suppressed neuronal death in the vicinity of the stab wound (Fig. 2). Second, 2ccPA significantly attenuated astrocyte activity in the vicinity of the lesions by the stab wound at day 3 after injury (Fig. 3). To examine the mechanism by which 2ccPA regulates neuronal death after the stab wound, we focused on an extracellular matrix protein, TN-C, which is upregulated in astrocytes after brain injury, and confirmed that the TN-C deposition in astrocytes in the 2ccPA-administered mice (Fig. 4). In addition, 2ccPA increased the TN-C protein levels in primary cultures of astrocytes (Fig. 6). Our study revealed that the astrocyte conditioned medium (ACM) of 2ccPA-treated astrocytes suppressed the apoptosis of cortical neurons (Fig. 7), and that the ACM from the knockdown of TN-C in astrocytes abolished the neuroprotective effect of 2ccPA (Figs. 8 and 9). These data indicate that 2ccPA suppressed neuronal death via TN-C from astrocytes, and that 2ccPA could serve as a neuroprotective factor after stab wounds are inflicted to the mouse brain.

Astrocyte activity in the vicinity of the stab wound region drastically increased within 1 h after injury. This astrocytic activity was downregulated by 2ccPA. GFAP, an astrocyte marker, is known to be upregulated by astrocyte activation (Eddleston and Mucke, 1993; Ridet et al., 1997). To analyze the effect of 2ccPA on the activity of astrocytes, GFAP expression levels in astrocytes and Sox9-positive activated astrocytes were examined in the regions around the stab wound. Administration of 2ccPA significantly decreased GFAP immunoreactivity and activated astrocyte numbers as well as mRNA expression levels of Gfap in the stab wound regions at day 3 (Fig. 3). We previously revealed that 2ccPA suppresses the mRNA levels of pro-inflammatory cytokines and microglial activity in the vicinity of the stab wound regions at day 3 after injury. This suppression of inflammation by 2ccPA may indirectly contribute to the suppression of astrocyte activity.

To analyze the mechanism by which 2ccPA contributes to the suppression of apoptosis via astrocytes, we focused on the extracellular matrix proteins. Recently, the protein levels of several extracellular matrix proteins, fibronectin, TN-C, and reelin have been reported to be changed in the acute phase of rat brains using experimental diffuse TBI model (Griffiths et al., 2020) and the modulation of extracellular matrix is a potential target of TBI treatment (George and Geller, 2018). Reactive astrocytes are known to express TN-C at extremely high levels when the brain is damaged (Laywell et al., 1992; Ikeshima-Kataoka and Yuasa, 2008), and there are many reports of TN-C expression in astrocytes around the injury site. Our data showed the TN-C protein level of 2ccPA-administered mice was significantly higher than that of PBS-administered mice (Fig. 5B and C), and our results are consistent with these previous reports. However, it had no effect on the TN-C mRNA levels between 2ccPA-administered mice and PBS-administered mice on day 0 and 1 (Fig. 5A). This discrepancy could be attributed to the TN-C degrading inhibitory effect of 2ccPA. Increased expression of matrix metalloproteinases (MMPs) has been described in TBI (Minta et al., 2020), and TN-C degrading MMP-1 and -3 are known to be expressed in the healing wounds (Saraihako-Kere et al., 1993; Imai et al., 1994). In addition, it is reported that 2ccPA suppresses the expression of MMP-1 and -3 (Gotoh et al., 2014). This report suggests that 2ccPA may suppress the degradation of TN-C protein in TBI model. Moreover, we found that TN-C expression levels in primary cultured astrocytes were remarkably higher for both LPS and 2ccPA treatment and LPS + 2ccPA than in LPS- or 2ccPA-treated astrocytes (Fig. 6), indicating that LPS and 2ccPA synergistically increased TN-C expression. It was suggested that the upregulation of TN-C expression by 2ccPA is effective in activated astrocytes. This suggestion is supported by the fact that the reduction of TN-C expression was observed at day 3 after injury, which was when the number of activated astrocytes (Sox9 positive) was decreased (Fig. 3).
Our study revealed that TN-C is a neuroprotective factor in stab wound regions. 2ccPA administration increased the percentage of TN-C-positive astrocytes making up the total astrocyte cell number in the acute phase (within 1 h and 1 d) after the stab wound compared to that of the control (Fig. 4) while simultaneously suppressing neuronal death (Fig. 2). In addition, 2ccPA ACM suppressed the percentage of apoptosis in cortical neurons compared to LPS ACM, and the secreted TN-C protein expression level in 2ccPA ACM was higher than that in LPS ACM (Fig. 7). These results suggest that TN-C in ACM may be a neuroprotective factor for cortical neurons. To confirm this hypothesis, ACM from the

Fig. 6. Expression of TN-C in LPS-stimulated cultured astrocytes. (A) Experimental scheme for analyzing the expression of TN-C in LPS-stimulated astrocytes. First, primary cultured astrocytes (Div 14) were incubated with growth medium for 24 h. The medium was replaced with serum-free medium, and cells were cultured for 24 h. Next, the cells were treated with LPS and/or 2ccPA from 2 h to 36 h. Then, RNAs were extracted from each cell. (B) The effect of 2ccPA on the Tnc mRNA expression levels in LPS- and 2ccPA- treated cultured astrocytes for each time course (2, 4, 6, 12, 16, 24, 36 h). Each mRNA expression level of Tnc was normalized to that of Gapdh. Each mRNA level was normalized to that of cultured astrocytes without LPS and 2ccPA (control) (n = 8 cultures/condition). * p < 0.05, ** p < 0.01 vs. LPS condition, t-test and # p < 0.05, ## p < 0.01 vs. LPS + 2ccPA condition, t-test. (C) Western blotting analyses of secreted TN-C protein levels in LPS- and/or 2ccPA- treated astrocytes conditioned medium (ACM) for each time course (6, 12, 16, 24 h). To adjust the amount of ACM protein and load, the same amount of proteins for Western blotting analysis was done to measure the protein concentration of each ACM. To confirm the number of cells after the collection of ACMs from astrocyte culture, the remaining cells were lysed and subjected to Western blotting for β-actin expression. (D) Quantitative analysis of secreted TN-C protein expression levels in LPS- and/or 2ccPA- treated ACM for each time course (6, 12, 16, 24 h). Each secreted TN-C protein level was normalized with that in ACM of primary astrocytes without LPS and 2ccPA (control) (n = 3 cultures/condition). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. LPS ACM, t-test and # p < 0.05, ## p < 0.01 vs. LPS + 2ccPA ACM, t-test.
Fig. 7. Effect of 2ccPA on the neuroprotective function for cortical neurons via astrocytes.

(A) Experimental scheme for analyzing the effect of 2ccPA treated astrocyte conditioned medium (ACM) on the apoptosis of cortical neurons. First, primary cultured astrocytes (Div 14) were incubated with growth medium for 24 h. The medium was replaced with serum-free medium, and cells were cultured for 24 h. The cells were then treated with LPS or PBS for 6 h as shown in the step of “Pretreatment of astrocyte” and washed out. Then, 2ccPA or 0.1% BSA-containing PBS was added to the astrocytes (treatment), and the cells were cultured for 12 h as shown in the step of “Treatment of astrocyte.” In the LPS-pretreated astrocytes, 2ccPA-treated ACM (2ccPA) or PBS-treated ACM (LPS) was added to primary cultured cortical neurons. After PBS pretreatment, PBS-treated ACM was used as control ACM (Control). To confirm the effect of 2ccPA on the apoptosis of cortical neuron via astrocytes, 2ccPA was directly added to cortical neurons right before the addition of LPS (Direct 2ccPA).

(B) Immunofluorescence images of cortical neurons with each ACM. Each neuron was stained with anti-cleaved caspase-3 (green), anti-MAP2 (red), and DAPI (blue) for the nuclei. The arrowheads indicate caspase-3+ / MAP2+ cells. Scale bar: 100 μm.

(C) The percentage of caspase-3+ cell numbers in MAP2+ cell numbers were examined in cortical neurons at 24 h after each ACM was added (n = 3 cultures/condition). *p < 0.05 vs. LPS, t-test and #p < 0.05 vs. 2ccPA, t-test.

(D) Western blotting analyses of secreted TN-C expressions levels in each ACM. To confirm the cell number of astrocytes after the collection of ACMs, the remaining cells were lysed and subjected to Western blotting analysis for β-actin detection.

(E) Relative protein expression levels of secreted TN-C in each ACM. The values of secreted TN-C protein levels were normalized to that in Control (n = 3 cultures/condition). *p < 0.05 vs. LPS, t-test. Data represent the mean ± SEM of three independent datasets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
knockdown of TN-C in astrocytes was analyzed. ACM from knockdown by TN-C sh in astrocytes increased the percentage of neuronal cell death in cortical neurons compared to that of Scrambled sh. Furthermore, the increase in apoptosis in cortical neurons by the ACM from TN-C knockdown was rescued by the addition of TN-C protein (Figs. 8 and 9), indicating that TN-C serves as a neuroprotective factor in the ACM of 2ccPA-treated astrocytes. However, it remains possible that the other secreted factors serve as neuroprotective factors from astrocytes because Rescue ACM did not fully recover to the same level of apoptosis as 2ccPA ACM (Fig. 9). Taken together, TN-C expression induced by 2ccPA in
Fig. 8. Effect of TN-C on the neuroprotective function for primary cortical neurons (A) Experimental scheme for analyzing the effect of secreted TN-C in ACM on the apoptosis of cortical neurons. For analysis of the effect of secreted TN-C, after the culture of astrocytes for 24 h in growth medium, astrocytes were infected with TN-C knockdown (KD) shRNA or control negative KD shRNA lentivirus in serum-free medium and cultured for 24 h as shown in the step of “Pretreatment of lentivirus.” Then, the cells were treated with LPS or PBS for 6 h as shown in the step of “Pretreatment of astrocyte” and washed out. Next, 2ccPA or 0.1% BSA-containing PBS was added to the astrocytes, and the cells were cultured for 12 h as shown in the step of “Treatment of astrocyte.” The 2ccPA-treated ACM were added to primary cultured cortical neurons, which were infected with control negative shRNA (Scrambled sh) and TN-C KD shRNA (TN-C sh) lentivirus. PBS-treated ACM (Control) was used as control condition. To confirm the effect of TN-C on the apoptosis of cortical neurons, TN-C was added to cortical neurons after the TN-C knockdown treated ACM was done as a rescue experiment (Rescue). (B) The efficiency of lentivirus infection into primary cultured astrocytes and the efficiency of TN-C KD. Fluorescence images of lentivirus-infected astrocytes using anti-GFP antibody. The infected cells were identified by the staining of anti-GFP antibody. The staining showed that the efficiency of infection by lentivirus was about 98% (Scrambled sh) and 99% (TN-C sh). The efficiency of TN-C KD in astrocytes by the infection of lentivirus. Tnc mRNA expression levels were examined in lentivirus-infected astrocytes using real time RT-PCR. The mRNA expression level of Tnc was normalized to that of Gapdh. The Tnc mRNA level in Scrambled sh-infected astrocytes was regarded as 1, while the Tnc mRNA level in TN-C sh-infected astrocytes was normalized to that of cultured astrocytes infected with Scrambled sh (n = 3 cultures/condition). ** p < 0.01, t-test. (C) Immunofluorescence images of cortical neurons with each ACM. Each neuron was stained with anti-cleaved caspase-3 (green), anti-MAP2 (red), and DAPI (blue). The arrowheads indicate caspase-3 normalized to that of cultured astrocytes infected with control negative shRNA (Scrambled sh) and TN-C KD shRNA (TN-C sh) lentivirus. PBS-treated ACM (Control) was used as control condition. To confirm the effect of TN-C on the apoptosis of cortical neurons, TN-C was added to cortical neurons after the TN-C knockdown treated ACM was done as a rescue experiment (Rescue). (B) The efficiency of lentivirus infection into primary cultured astrocytes and the efficiency of TN-C KD. Fluorescence images of lentivirus-infected astrocytes using anti-GFP antibody. The infected cells were identified by the staining of anti-GFP antibody. The staining showed that the efficiency of infection by lentivirus was about 98% (Scrambled sh) and 99% (TN-C sh). The efficiency of TN-C KD in astrocytes by the infection of lentivirus. Tnc mRNA expression levels were examined in lentivirus-infected astrocytes using real time RT-PCR. The mRNA expression level of Tnc was normalized to that of Gapdh. The Tnc mRNA level in Scrambled sh-infected astrocytes was regarded as 1, while the Tnc mRNA level in TN-C sh-infected astrocytes was normalized to that of cultured astrocytes infected with Scrambled sh (n = 3 cultures/condition). ** p < 0.01, t-test. (C) Immunofluorescence images of cortical neurons with each ACM. Each neuron was stained with anti-cleaved caspase-3 (green), anti-MAP2 (red), and DAPI (blue). The arrowheads indicate caspase-3 normalized to that of cultured astrocytes infected with Scrambled sh (n = 3 cultures/condition). * p < 0.05 vs. Scrambled sh, t-test and ** p < 0.01 vs. Rescue, t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Effect of 2ccPA and TN-C on the apoptosis in cortical neurons. (A) Images of TUNEL assay in cortical neurons. Scale bar: 100 μm. The arrowheads indicate TUNEL+ in methyl green stained cells. Scale bar: 100 μm. (B) The percentage of TUNEL+ cell numbers in total (methyl green stained cells) cell numbers in cortical neurons at 24 h after each ACM was added to the cortical neurons (n = 3 cultures/condition). ** p < 0.01 vs. LPS, t-test and *** p < 0.01 vs. 2ccPA, t-test. (C) Immunofluorescence images of cortical neurons with each ACM. Each neuron was stained with anti-cleaved caspase-3 (green), anti-MAP2 (red), and DAPI (blue). The arrowheads indicate caspase-3 normalized to that of cultured astrocytes infected with Scrambled sh (n = 3 cultures/condition). * p < 0.05 vs. Scrambled sh, t-test and ** p < 0.01 vs. Rescue, t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Astrocytes could contribute to the suppression of apoptosis in the vicinity of the stab wound region within 1 h and 1 d after injury. This notion is supported by reports that acute changes in the levels of TN-C may indicate a restorative or regenerative response associated with recovery from TBI. (Griffiths et al., 2020), and that TN-C promotes locomotor recovery after spinal cord injury (Chen et al., 2010). TN-C deposition promotes fibrin accumulation in fibrosis, asthma, and cancer (Breilier et al., 2011). Furthermore, we found that 2ccPA significantly attenuated the extent of BBB breakdown caused by a stab wound (Hashimoto et al., 2018). This will be of interest to investigate in the context that upregulation of TN-C expression by 2ccPA could promote recovery from injury-induced breakdown of the BBB.

A previous study reported that TN-C modulates the balance between neuroprotective and neurotoxic inflammatory cytokine expression in the injured brain (Ikeshima-Kataoka et al., 2008). On the other hand, it has been reported that the deficiency of TN-C attenuates the expression of IL-1β and IL-6 and prevents BBB disruption following experimental SAH in mice (Fujimoto et al., 2016), and TN-C in cerebrospinal fluid concentration has been shown to positively correlate with subarachnoid hemorrhage severity (Suzuki et al., 2015). Moreover, increased serum and cerebrospinal fluid levels of TN-C have been reported in human TBI, they are closely related to trauma severity and clinical outcomes after
TBI (Zhao et al., 2017; Minta et al., 2019). These findings suggest that TN-C can serve as a neurotoxic factor after the brain injury. Moreover, Liu et al. found that TN-C deficiency protects against neuronal cell death in a mouse model of SAH (Liu et al., 2018). In this SAH model, the increase in oxygen hemoglobin due to hemorrhage is involved in apoptosis in the SAH lesions, and recent silencing of TN-C inhibits inflammation and apoptosis via the PI-3 K/Akt/NF-kB signaling pathway in an SAH cell model (Tong et al., 2020). However, 2ccPA suppressed hemorrhage in the stab wound region. Thus, it is suggested that 2ccPA suppresses the neurotoxic effects of TN-C. It remains to be seen whether this apparent discrepancy results from the use of different approaches for brain injury models or other factors. However, other studies indicate that α9 integrin promotes neurite outgrowth in the injured CNS, in which TN-C is upregulated (Andrews et al., 2009). Additionally, TN-C interactions with αvβ3 integrin can promote cell proliferation and inhibit apoptosis (Tucker and Chiquet-Ehrismann, 2015). The other possibility for the discrepancy of TN-C effects may be caused by splice variant of TN-C. The injury-induced splice variant of TN-C may well influence axonal regeneration and repair processes in the damaged CNS (Dobbertin et al., 2010). In this study, the high (250 kDa) and low (210 kDa) molecular weight of TN-C was observed in Figs. 6 and 7. These different molecular weights seem to be generated by alternative splicing. The low molecular weight of TN-C has not been shown, when the neurotoxic effect of TN-C is observed in injured brain (Liu et al., 2018; Griffiths et al., 2020; Wiemann et al., 2021). These reports support the notion that the low molecular weight would exert neuroprotective function. In a recent study, it was suggested that TN-C is involved in a restorative or regenerative response associated with recovery from TBIs (Ikeshima-Kataoka et al., 2015; Griffiths et al., 2020). Thus, our results also indicate that 2ccPA contributes to neuroprotection via astrocytes in TBI as well as the anti-inflammation via astrocyte secreted factor, TN-C.

We suggested the repair mechanism of 2ccPA in stab wounds, in the acute phase, 2ccPA promotes the secretion of TN-C from astrocytes and suppresses the extravasation from the BBB as well as the apoptosis of neurons in the vicinity of the stab wound region. The suppression of extravasation and apoptosis by 2ccPA seems to contribute to the attenuation of inflammation after day 3. This notion is supported by our previous results that 2ccPA suppresses the expression of pro-inflammatory cytokine mRNA levels and regulates polarization towards the M2 neuroprotective phenotype (Hashimoto et al., 2018). It is suggested that this suppression of inflammation by 2ccPA results in the suppression of neuro-apoptosis, reduction in the number of activated astrocytes, and downregulation of TN-C expression. In addition, TN-C has been reported to promote the proliferation of neural stem cells and damage repair in rat model of TBI (Dai et al., 2019). This finding provides a potential future therapeutic target, TN-C, for injury repair after TBI.

In conclusion, the present study found that 2ccPA contributes to neuroprotection via astrocytes after stab wound injury and that 2ccPA is a promising therapeutic agent for TBI. Moreover, TN-C secreted from astrocytes suppressed neuronal cell death in primary cortical neurons. Therefore, 2ccPA contributes to neuroprotection via astrocytes in TBI as well as prevents inflammation via astrocyte secreted factor, TN-C.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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