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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

該当なし。

2. 実用新案登録

該当なし。

3. その他

該当なし。

研究成果の刊行に関する一覧表

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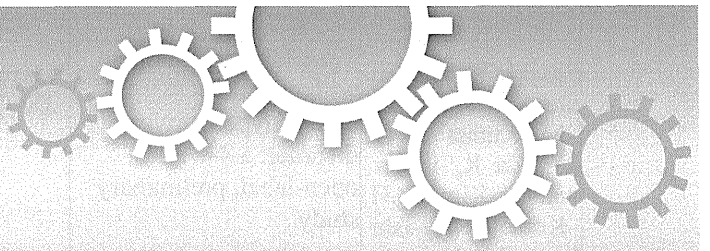

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Minocycline, a microglial inhibitor, reduces 'honey trap' risk in human economic exchange

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Recently, minocycline, a tetracycline antibiotic, has been reported to improve symptoms of psychiatric disorders and to facilitate sober decision-making in healthy human subjects. Here we show that minocycline also reduces the risk of the 'honey trap' during an economic exchange. Males tend to cooperate with physically attractive females without careful evaluation of their trustworthiness, resulting in betrayal by the female. In this experiment, healthy male participants made risky choices (whether or not to trust female partners, identified only by photograph, who had decided in advance to exploit the male participants). The results show that trusting behaviour in male participants significantly increased in relation to the perceived attractiveness of the female partner, but that attractiveness did not impact trusting behaviour in the minocycline group. Animal studies have shown that minocycline inhibits microglial activities. Therefore, this minocycline effect may shed new light on the unknown roles microglia play in human mental activities.

In movies, a female spy often wins the trust of her male target using her physical attractiveness. The male target usually suspects that she is a spy, but because of her attractiveness, he becomes amorously entangled with the female spy despite concerns regarding her trustworthiness. For males, allocating valuable resources to physically attractive females may be evolutionarily adaptive, in that it may increase the probability of producing attractive offspring under natural selection. However, this tendency toward resource allocation to attractive females creates 'noise' that complicates decisions in short-term economic exchanges, leading to the tendency to 'honey trap' males with this behaviour.

In an economic exchange, attractiveness in a female increases sexual arousal in a male that automatically (without careful evaluation of her trustworthiness) facilitates trusting behaviour. While these traits should be adaptive in terms of mate-choice[1], experimental studies have shown that they also affect decisions in social and economic exchange[2,3]. These traits lead to the question of how males can avoid the honey trap.

Recent studies with human subjects show that minocycline, a commonly used tetracycline antibiotic, may facilitate focus on appropriate environmental cues for social decision-making, possibly by reducing noise and other factors (e.g. personality and arousal) that can obstruct decisions. In an economic exchange, one study showed that subjects treated with minocycline make more sober decisions compared to participants treated with placebo[4]. In another study, participants were given dextroamphetamine and those treated with minocycline report less of a 'high' feeling compared to those who did not receive minocycline[4]. Minocycline is also known to improve symptoms associated with psychiatric disorders such as schizophrenia and depression[5–7]. There are past studies examining the effects of physical attractiveness on cooperation in social/economic exchange in different sex pairs, but no study has examined the effects of minocycline on such behaviour in different sex pairs. The hypothesis of this study was that minocycline reduces the risk of the honey trap effect and leads to more appropriate decisions in a short-term economic exchange, through a reduction in the noise triggered by physical attractiveness.

In this experiment, 98 healthy males played a trust game with 8 photographed young females after a 4-day oral treatment course of either minocycline or placebo. Looking at a picture showing a female's face, male players decided how much out of 1300 yen (approximately 13 USD) they would give to each female. Males then evaluated

Table 1 | Mean scores and results of *t*-tests comparing major variables

Item		Conditions		*t*-test	
		Placebo (n = 48)	Minocycline (n = 50)	*t*-value	*p*
Age (years)	Mean	**21.30**	**21.63**	−1.50	0.138
	SD	1.364	1.875		
Offering Rate (0 to 1)	Mean	**0.61**	**0.49**	1.90	0.062
	SD	0.329	0.277		
Mean Attractiveness of All Pictures	Mean	**3.08**	**2.78**	1.37	0.175
(0: Not at all – 10: Perfectly)	SD	1.119	1.076		
Mean Trustworthiness of All Pictures	Mean	**5.52**	**5.37**	0.63	0.528
(0: Not at all – 10: Perfectly)	SD	0.968	1.349		
Mean State Anxiety Score	Mean	**2.11**	**2.28**	−1.50	0.138
(1: Not at all – 10: Very much so)	SD	0.514	0.573		
Mean Trait Anxiety Score	Mean	**2.26**	**2.27**	−0.03	0.979
(1: Not at all – 10: Very much so)	SD	0.514	0.578		

how trustworthy each female was and how physically attractive she was using a 11-point Likert Scale (0: Not at all – 10: Perfectly so). Of note, all of the photographed females had actually decided, in advance, to choose 'betray' against the male players. Therefore, male participants played with untrustworthy female partners, but were unaware of the deception. The impact of attractiveness and trustworthiness on the amount of money given to female partners was analysed. The independent variables were the evaluations/scores of physical attractiveness and trustworthiness given by the male participants.

Results

Table 1 summarizes the mean scores for the major variables and results of a *t*-test used to compare the placebo and minocycline conditions. Consistent with previous reports in which trust games were conducted between healthy male participants[8,9], the offering rate differed marginally between conditions. The State and Trait Anxiety Inventory (STAI)[10] was measured and no significant differences were found for either State or Trait Anxiety scores between conditions.

The primary hypothesis of this study was that the minocycline group would be less affected by the attractiveness of pictured females than the placebo group. To test this hypothesis, an ANOVA was performed with condition (minocycline vs. placebo) and attractiveness (high vs. low) as independent variables and the offering rate of money by participants as the dependent variable. The attractiveness score was not normally distributed ($P = 0.0004$), therefore the score was sub-divided into 2 categories (high vs. low). **Figure 1** shows the mean offer rate by condition and the level of attractiveness. There is a significant interaction effect between condition and attractiveness (F (1,776) = 7.78, $P = 0.005$). Consistent with the primary hypothesis, participants in the placebo group gave larger amounts of money when the partner was more attractive, while participants in the minocycline group did not. According to a simple main effect test, a main effect of attractiveness was detected in the placebo group ($P = 0.0004$), but not in the minocycline group ($P = 0.223$). In addition, Figure 1 shows that, for partners with high attractiveness, the offering rate in the placebo group was significantly higher than in the minocycline group ($P = 0.0004$), but not for less attractive partners ($P = 0.590$).

Discussion

This study demonstrated that minocycline is the first drug shown to reduce the honey trap effect on young males. A previous report using a trust game with an anonymous male partner showed that

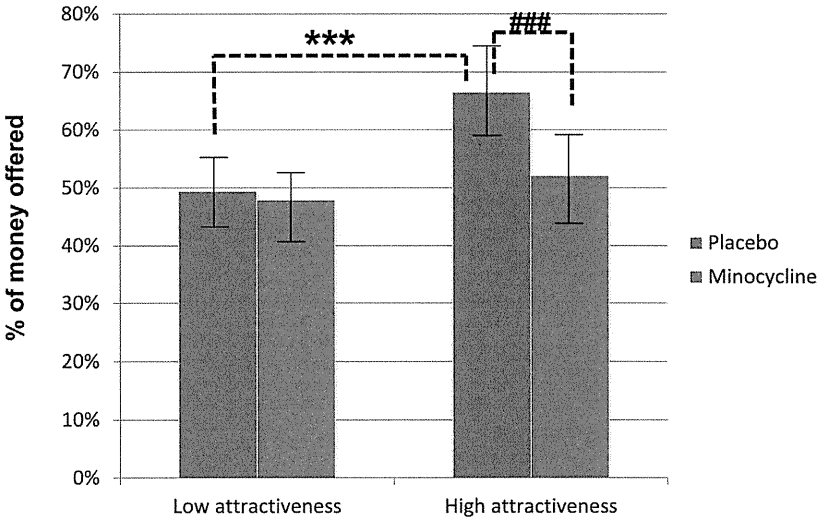

Figure 1 | Mean Offering Rate (percentage of money offered) by the Male Participants to Less- and More-Attractive Female Partners. Error bars represent the standard deviation for each condition. *** For the placebo group, the offering rate to highly attractive female partners is higher than that to partners with low attractiveness ($P = 0.0004$). ### The offering rate to highly attractive partners in the placebo group is higher than that in the minocycline group ($P = 0.0004$).

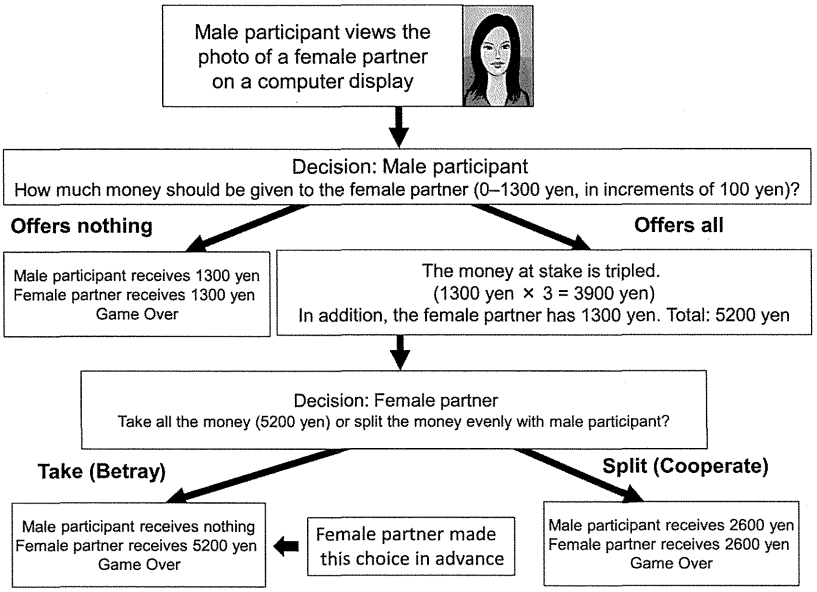

Figure 2 | Trust Game Structure with the Most Extreme Cases.

minocycline reduces decision-making based on personality and trait[9]. Rather, minocycline facilitated decision-making based on situational factors such as game structure and evaluation of others' trustworthiness[8,9]. Consistent with evidence that minocycline attenuates the subjective high feeling associated with dextroamphetamine treatment[4], the current results indicate that minocycline may reduce the effect of arousal and lead to sober decision-making. Recent clinical trials suggest that minocycline improves symptoms in patients with schizophrenia and depression[5–7]. In the current experiment, anxiety was measured and no difference was identified between the minocycline and placebo conditions. Future studies should clarify the effects of treatment with minocycline on psychological processes including mood, impulsivity, and cognitive performance in both healthy volunteers and patients with psychiatric disorders.

In rodent models, minocycline is the most commonly used drug for suppressing microglial activity in the brain[11–13]. In addition, a clinical trial with human subjects has shown that a long-term minocycline treatment (200 mg/d) suppresses microglial activation in various areas of the brain including the putamen, thalamus, and frontal cortex[14]. Microglia are glial cells with immunologic/inflammatory functions that contribute to various brain pathologies, including neurodegenerative diseases[15–17] and psychiatric disorders (e.g. schizophrenia[18–20] and autism[21,22]). Recent animal-model studies have shown that stress increases microglial activation[23–25] and causes anxiety-like behaviours[26]. This behavioural change can be modulated with minocycline treatment[26]. In addition, recent evidence from rodent studies showed that in normal brains, microglia make direct contact with synapses[27–31], suggesting that in this study minocycline may change synaptic reactions by suppressing microglial activity. The amygdala, one of the brain regions most affected by minocycline[32], is activated during judgments of trustworthiness in human faces[33,34]. However, no studies have investigated how microglial activation directly contributes to human social decision-making and how these effects are modulated by minocycline. Taken together, these results suggest that microglial activity in the amygdala may modulate cognitive and emotional processes involving physical attractiveness and evaluation of trustworthiness.

Other possible effects of minocycline should be taken into account. Apart from inhibiting microglial activation, minocycline has also been reported to interact with brain glutamate and dopamine neurotransmission[35] and to have direct effects on neuronal cells[36]. Some reports suggest positive links between microglia and glutamate and dopamine interaction[37,38]. Further research should be performed to clarify the effects of this potential interaction. The dose of minocycline (200 mg/d) used in this experiment was based on previous reports with human subjects[4,14] and different doses may have different effects. Therefore, further trials should be conducted to investigate the effect of minocycline dosing.

To date, the biological mechanisms that underlie the honey trap effect remain poorly understood and no drug has been conclusively proven to attenuate honey trap effects during human social decision-making. The results of the present study suggest that minocycline is the first drug to have a novel pharmacologic function in humans—inhibition of honey trap effects. The current findings may shed new light on the mechanism underlying microglial effects on human mental activities and represent a novel psychopharmacologic approach for modulation of microglia.

Methods

This double-blind randomised trial, one of a series of trust game studies with human male subjects[9], was approved by the Kyushu University Ethical Committee under the administration of the UMIN Clinical Trials Center (**UMIN000004803**). After a complete description of the study, all participants provided written informed consent. Either minocycline or placebo was administered to participants for 4 days, after which they participated in a trust game[39].

Subjects. Participants were recruited using on-campus advertisements. Therefore, all participants were undergraduate or graduate students at Kyushu University. Healthy adult males (age range, 20–30 years) who were capable of providing informed consent were included. Participants were excluded if they met any of the following 4 criteria: (1) any history of experiencing side effects associated with antibiotics, including minocycline; (2) any history of severe heart, liver, or kidney disease; (3) a history of allergic syndromes; and (4) any history of psychiatric disorders. Their mental and physical health was confirmed via interview with a psychiatrist (TAK). After this screening process, 101 healthy adult males were enrolled in the study.

Drug administration. Participants received a hand-out describing their detailed dosing schedule. They were asked to record the exact time each dose was taken, and to keep and submit all capsule packaging, as evidence of medication administration. Participants began the medication (either minocycline or placebo) on the evening of Day 1 and continued taking the medication twice daily (morning and evening) for 3 additional days. The game experiment was conducted on Day 5. Participants were instructed to take the last capsule 3 h prior to their scheduled appointment time, ensuring that all participants had similar drug levels during the actual experiment.

Each capsule contained either 100 mg minocycline (in the treatment group) or 100 mg lactose (in the placebo group). This minocycline dose (200 mg/d) is within the typical range for daily dosing used to treat infections[40] and has also been used in recent clinical trials[4,6,14]. Using a double-blind procedure in advance, participants were randomly assigned to either the treatment group or the placebo group.

Procedure. After 4 days of drug administration, participants were interviewed by a physician regarding drug side effects, other medications, and adherence to the drug administration protocol. Participants then took part in the following trust game.

Trust Game with photographed female partners. In this 2-player game[39], each player was initially given 1300 JPY. The first player (the male participant) then decided how much of the 1300 JPY to give to the second player (the female partner). The amount of money given to the female partner was tripled and the female partner then decided whether to split her money equally with the male participant (namely, cooperate) or to take the entire amount of money (namely, betray). The trust game structure illustrating the most extreme cases is shown in **Figure 2**. All of the female partners were photographed and had decided in advance to take the entire amount of money. However, the male participants were not aware of this decision.

The male participant's decision regarding how much money to give to the female partner is thought to reflect the level of trust the male participant places in his partner. The amount of money given was expected to function as a behavioural measure of the trust the male participant has in the female partner. In this experiment, male participants had no information about the female partner except for a photograph. Therefore, it is likely that male participants based their decisions regarding how much to trust each female partner, on impressions formed on the basis of the photos. After the experiment, each participant was paid an amount of money corresponding to the result of a randomly selected game from all 8 games.

Photo materials. Prior to the experiment, 61 young females were recruited using on-campus advertisements (mean age, 20.08 years; SD, 1.31 years). Each female participant was asked how they would behave in the role of the female partner in the trust game described above, especially in the case of an anonymous male participant that had chosen to give them the entire amount of money. Eleven participants answered 'take the entire amount' rather than 'split equally'. Eight female participants gave permission to use their photos in the experiment (mean age, 19.88 years; SD, 0.93 years). The photographs included the head and shoulders, with a neutral facial expression. During the experiment, each participant was asked if they knew each of the female partners shown in the photographs, in order to avoid confounding effects associated with previous acquaintance. However, there were no acquaintances identified among the participant pairs.

Statistical analyses. Ninety-eight Japanese males, out of 101 initially enrolled, completed all experiments (mean age, 21.49 years; SD, 1.65 years). Of the participants, 3 (1 in the minocycline condition and 2 in the placebo condition) failed to complete the experimental procedure, so the analyses were performed with data from the 98 participants. All data analyses were performed with SPSS (Version 19, IBM Corp., Armonk, NY USA).

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Author contributions

Conceived and designed the experiments: T.A.K., M.W. Performed the experiments: T.A.K., M.W., S.T., K.I. Analysed the data: M.W. Contributed reagents/materials/analysis tools: T.A.K., M.W., K.H., A.M., H.U., S.K. Wrote the paper: M.W., T.A.K.

Additional information

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Pretreatment of aripiprazole and minocycline, but not haloperidol, suppresses oligodendrocyte damage from interferon-γ-stimulated microglia in co-culture model

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ABSTRACT

Recent imaging studies have indicated that the pathophysiology of schizophrenia is closely related to white matter abnormalities and microglial activation. Additionally, recent clinical trials have suggested that atypical antipsychotics may have brain protective properties and that minocycline, an antibiotic with inhibitory effects on microglial activation, improves symptoms of schizophrenia. We have reported that not only atypical antipsychotics with dopamine D2 receptor (D2R) antagonism but also aripiprazole, a unique antipsychotic drug with D2R partial agonism, inhibit microglial activation in vitro. Thus, atypical antipsychotics may exert a beneficial influence on both microglia and oligodendrocytes, while the underlying mechanisms have not been clarified. Here, we investigated whether antipsychotics suppress oligodendrocyte damage by inhibiting microglial activation utilizing a co-culture model with microglia and oligodendrocytes. Pretreatment of aripiprazole and minocycline suppressed apoptosis of oligodendrocytes in the co-culture model with interferon-γ (IFN-γ)-activated microglia, while haloperidol, a traditional antipsychotic drug, did not. Aripiprazole and minocycline inhibited the production of tumor necrosis factor-alpha (TNF-α) from IFN-γ-activated microglia. Moreover, aripiprazole and minocycline attenuated the phosphorylation of signal transducer and activator of transcription 1 (STAT1) in microglia. Overall, our results suggest that aripiprazole and minocycline may have antipsychotic effects through reducing oligodendrocyte damage caused by microglial activation. These results put forward a novel therapeutic hypothesis in schizophrenia research. Future in vivo studies to confirm the present results should be performed.

1. Introduction

Multiple theories have been put forth regarding the pathogenesis of schizophrenia, while the underlying mechanisms remain to be identified (Fatemi and Folsom, 2009; Jaaro-Peled et al., 2009). Recent imaging studies have shown that first-episode schizophrenia patients have a significant volume reduction in white matter with abnormal brain connectivity (Price et al., 2006; Schlosser et al., 2007). Deviant myelination of schizophrenia patients has been evident in postmortem studies (Uranova et al., 2004; Uranova et al., 2007; Bernstein et al., 2009) and imaging studies (Miyata et al., 2009; Kubota et al., 2011; Kubota et al., 2013). Combined with evidence of dysregulation of myelination-related genes, a disruption of oligodendrocyte function in schizophrenia has been strongly implicated (McCullumsmith et al., 2007).

Mittelbronn et al. demonstrated that local distribution of microglia in the normal adult human brain differs by up to one order of magnitude and that there is significantly more microglia in white matter than in gray matter (Mittelbronn et al., 2001). Therefore, microglial activation plays an important role especially in white matter disorders (Schnieder and Dwork, 2011). Microglial cytotoxicity of oligodendrocytes is mediated through free radical-related molecules (nitric oxide (NO) and/or peroxynitrite) and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (Buntinx et al., 2004; Steelman and Li, 2011). TNF-α is known to compromise the growth of oligodendrocytes and the expression of mRNA for myelin basic protein (MBP) in cultures (Cammer and Zhang, 1999). Furthermore, TNF-α is reported to inhibit the survival and proliferation of oligodendrocyte progenitors and their subsequent differentiation into mature myelinating phenotypes (Feldhaus et al., 2004). Recent postmortem and imaging studies have suggested the microglial activation (Radewicz et al., 2000; Steiner et al., 2006, 2008c; van Berckel et al., 2008; Doorduin et al., 2009; Takano

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et al., 2010) and oligodendrocyte dysfunction (Uranova et al., 2004; Uranova et al., 2007; Bernstein et al., 2009) in schizophrenia patients. Based on the above evidence, microglial activation may be involved in the pathological process of schizophrenia by damaging oligodendrocytes.

Various antipsychotics, which had classically been regarded to modulate solely neurons and synaptic networks, have recently been revealed to have direct anti-inflammatory properties on activated microglia from a series of in vitro studies (Kato et al., 2007, 2011a, 2013). We have reported that not only antipsychotics with dopamine D2 receptor (D2R) antagonism but also aripiprazole with D2R partial agonism inhibit microglial activation in vitro (Kato et al., 2008, 2011b). In spite of a different pharmacological profile, aripiprazole is effective against the positive and negative symptoms of patients with schizophrenia like other antipsychotics with lower side effects (Kasper et al., 2003; Potkin et al., 2003; Tandon et al., 2006). In addition, aripiprazole has been proved to be effective not only for schizophrenia but also for other psychiatric disorders such as major depressive disorders and bipolar disorders (Keck et al., 2006; Berman et al., 2007). On the other hand, minocycline, a semi-synthetic tetracycline antibiotic, is known to be one of the most well-known inhibitors of microglial activation (Yrjanheikki et al., 1998; Du et al., 2001). Minocycline, which can easily cross the blood–brain barrier, has been reported to provide neuroprotection via suppressing microglial activation in a number of neuronal disorders including amyotrophic lateral sclerosis (ALS), Parkinson's disease, Huntington's disease and Alzheimer's disease (Kriz et al., 2002; Van Den Bosch et al., 2002; Plane et al., 2010). Recent studies using animal models of schizophrenia have suggested that minocycline can be beneficial for the treatment of schizophrenia (Zhang et al., 2007; Fujita et al., 2008; Mizoguchi et al., 2008; Levkovitz et al., 2010). Furthermore, therapeutic improvement in psychotic symptoms has been demonstrated by minocycline in patients with schizophrenia (Ahuja and Carroll, 2007; Miyaoka et al., 2007; Miyaoka, 2008; Chaves et al., 2010; Levkovitz et al., 2010; Kelly et al., 2011; Chaudhry et al., 2012).

To the best of our knowledge, the protective effects of antipsychotics and minocycline on oligodendrocytes via the modulation of microglial inflammatory responses have never been reported. Our previous investigations have suggested that aripiprazole is the most effective antipsychotic, which directly and significantly inhibits microglial activation in vitro (Kato et al., 2008, 2011b). In the present study, we thus investigate the underlying mechanism of how aripiprazole, haloperidol and minocycline affect the degenerative process of oligodendrocytes via the modulation of microglial activation utilizing a co-culture model with microglia and oligodendrocytes.

2. Materials and methods

All experimental procedures were conducted in accordance with the Standard Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

2.1. Chemicals and reagents

Aripiprazole was generously provided from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Recombinant interferon-gamma (IFN-γ) was purchased from R&D systems (Minneapolis, MN, USA). Minocycline hydrochloride, haloperidol and other chemical reagents were purchased from Sigma Chemicals (St. Louis, MO, USA). Minocycline was dissolved with distilled water. Aripiprazole and haloperidol were dissolved initially into 20 mM with dimethyl sulfoxide (DMSO) and then were diluted into the final concentration for each experiment.

2.2. Cell cultures

Primary oligodendrocyte cultures were prepared as described (Chen et al., 2007) with the following modifications. Using a dissecting microscope, the subventricular zone was removed from the 3-day-old Sprague–Dawley rats (n = 6). The tissues were mechanically dissociated into single cells on 100-mm pore nylon mesh cell strainers (BD Falcon, Franklin Lakes, NJ, USA), and collected in PBS. Cells were then filtered through 40-mm nylon mesh cell strainers (BD Falcon). The cell suspension was centrifuged for 5 min at 800 g, and the cell pellet was resuspended in cold neurobasal medium (NB; Gibco, Carlsbad, CA, USA)/B27/L-Glu/PSA/EGF/bFGF consisting of NB supplemented with B27 (Gibco), L-glutamine (L-Glu; Gibco), penicillin/streptomycin/amphotericin B (Gibco), 20 ng/mL EGF (Sigma-Aldrich) and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma-Aldrich). The cell suspension was plated in poly-L-lysine (PLL) coated 12 well plates at 1.5×10^5 cells per well. Around day 7, T3 (Sigma-Aldrich) and T4 (Sigma-Aldrich) were added into the cultured-media (T3: 30 μg/mL, T4: 40 μg/mL). 14 days after T3 and T4 addition, differentiated oligodendrocytes were subjected to further experiments.

Primary microglia are known to be a mixture of heterogeneous microglia. To avoid contamination of heterogeneous microglia population, immortalized microglia are frequently used especially in co-culture experiments (Bi et al., 2011; Cui et al., 2012; Dentesano et al., 2012; Gresa-Arribas et al., 2012). We thus decided to use a single microglial cell line in our co-culture system. The immortalized rat microglial cell line HAPI (Cheepsunthorn et al., 2001; Zhou et al., 2008) was kindly provided by Drs. Morales NP. and Hyodo F. (Kyushu University). The cells were cultured in DMEM (low glucose; Invitrogen), 5% FBS (Hyclone), 4 mM glutamine (Invitrogen), 100,000 U/L penicillin G, 100 mg/L streptomycin (Mediatech), and maintained in 5% CO_2 at 37 °C.

2.3. Co-culture experiment with microglial cell line and primary oligodendrocyte

The HAPI microglial cells were plated on tissue culture inserts for 12-well plates (Greiner Bio One GmbH, Frickenhausen, Germany) at a density of 5.0×10^5 cells. The microglial cells were incubated for 12 h in the presence or absence of aripiprazole (20 μM), haloperidol (20 μM) or minocycline (10 μM). In order to minimize oligodendrocyte damage due to pretreatment of IFN-γ challenge, we have chosen to treat the target drugs (aripiprazole, haloperidol and minocycline) before IFN-γ challenge. This experimental design is represented in Fig. 1B. Each tissue culture insert was placed on the primary oligodendrocytes in 12-well plates (Fig. 1A). 100 U/mL IFN-γ (R&D) was added immediately to the cultured medium of HAPI cells and primary oligodendrocytes. 24 h after the incubation, the tissue culture inserts were removed from the 12-well plates. Morphological changes in the primary oligodendrocytes were observed under a phase-contrast microscope. This experiment was independently conducted three times.

2.4. Immunofluorescence

Co-cultured primary oligodendrocytes were rinsed twice in 0.1 mol/L HEPES/KOH and were fixed with 4% paraformaldehyde for 10 min and then rinsed with 0.1 mol/L HEPES/KOH for 10 min. Indirect immunofluorescence was performed using the following antibodies: rabbit anti-cleaved caspase-3 polyclonal antibody (1:200 dilution, Cell Signaling, Danvers, MA) and mouse anti-PLP monoclonal antibody (1:100 dilution, Abcam, Cambridge, MA). Cells were incubated in primary antibodies diluted in 0.1% Triton-X 100 in PBS containing 5% normal goat serum at 4 °C overnight. After rinsing twice with PBS for 5 min, FITC- or Texas red-conjugated secondary antibodies (Southern Biotech, Birmingham, AL, USA) were used for detection. Fluorescent images were captured with a fluorescence microscope (OLYMPUS BX50; Olympus Co. Ltd, Tokyo, Japan).

For phosphorylated STAT1 (pSTAT1) immunofluorescence, the HAPI cells were plated on 24-well tissue culture plates (Greiner Bio One GmbH) at 2×10^4 cells per 500 μL medium. The cells were pre-incubated in the presence or absence of minocycline (10 μM) or aripiprazole (10 μM) for 12 h followed by the addition of 100 U/mL

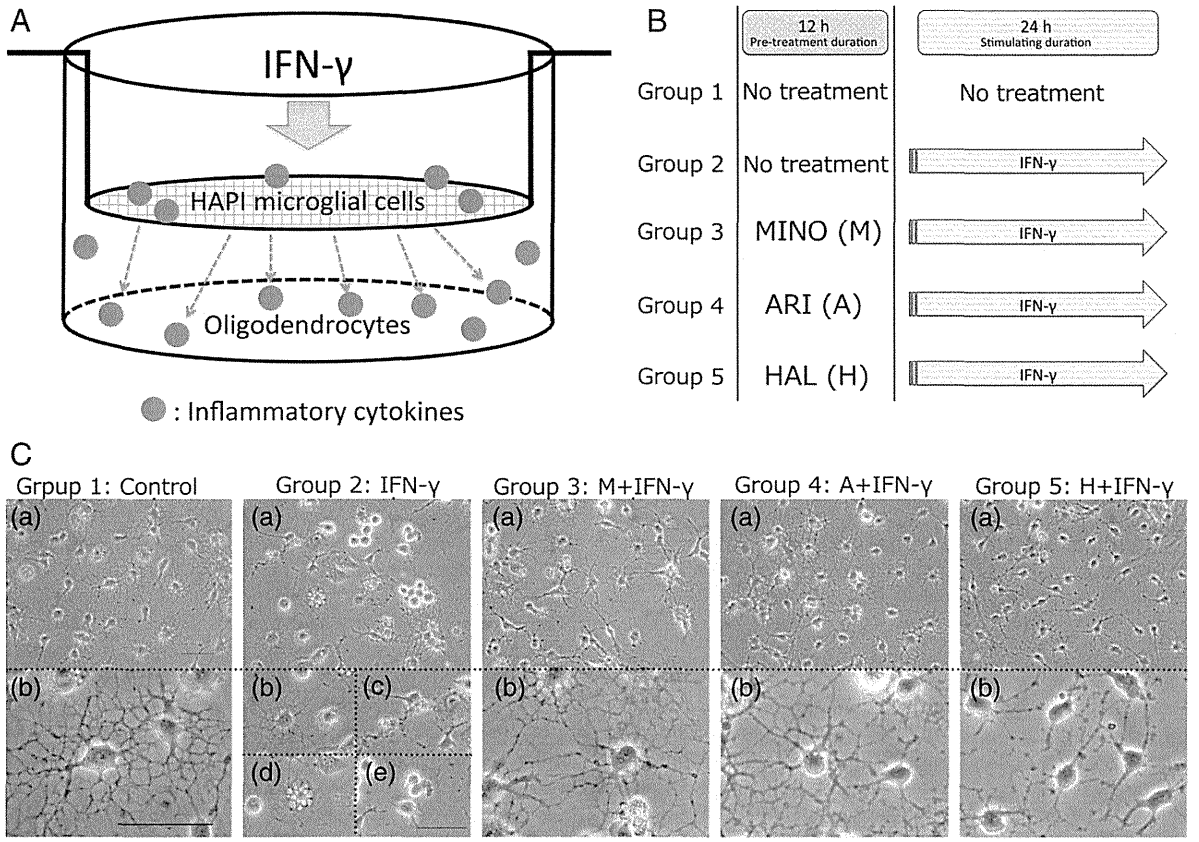

Fig. 1. Experimental design of co-culture experiment and the morphological changes of primary oligodendrocytes. (A) The co-culture system of primary oligodendrocytes/HAPI microglial cells. HAPI cells are pre-incubated with haloperidol, aripiprazole or minocycline for 12 h followed by a co-culture with primary oligodendrocytes in IFN-γ-containing medium for 24 h. (B) The time course of applying each drug and grouping into five as follows: Group 1 (no treatment HAPI cells + primary oligodendrocyte), Group 2 (IFN-γ-alone stimulated HAPI cells + primary oligodendrocyte), Group 3 (minocycline/IFN-γ stimulated HAPI cells + primary oligodendrocyte), Group 4 (aripiprazole/IFN-γ stimulated HAPI cells + primary oligodendrocyte) and Group 5 (haloperidol/IFN-γ stimulated HAPI cells + primary oligodendrocyte). Abbreviations in B: M, minocycline; A, aripiprazole; H, haloperidol. (C) The morphological changes of oligodendrocytes after co-culture experiment. Low (a) and high (b–e) magnification of each condition is shown. In Group 2, although some normal shapes were observed (b), degenerative changes such as retraction of their processes, (c) swollen cytoplasm and large vacuoles (d), and roundish morphology (e) were mainly observed. Degenerative changes were not evident in Group 3 and Group 4, but fragmented shapes were observed in Group 5. Abbreviations in B: MINO, minocycline; ARI, aripiprazole; HAL, haloperidol.

IFN-γ to cultured medium for 30 min ($n = 4$, respectively). Then, the cells were rinsed twice in 0.1 mol/L HEPES/KOH and were fixed with 4% paraformaldehyde for 10 min and rinsed with 0.1 mol/L HEPES/KOH for 10 min. They were blocked with 1.0% bovine serum albumin in PBS containing 0.3% Triton X-100 and 0.05% sodium azide for 1 h at 20 °C. After blocking, they were incubated in rabbit phospho-STAT1 antibody (Ser 727) (1:1000; Cell Signaling Technology, Danvers, MA) for 4 days at 20 °C. After rinsing three times with PBS for 5 min, a mixture of Cy3-conjugated secondary antibody (1:300; Jackson ImmunoResearch Laboratories) and YOYO-1 (1:10,000; Invitrogen, Carlsbad, CA) was used for detection. Fluorescent images were captured with a fluorescence microscope (Axio Scope A1, Carl Zeiss, Oberkochen, Germany) and an optical sectioning system (Apotome.2, Carl Zeiss).

2.5. Digital image analysis

Four cultures were selected from each condition, and two specimens per culture were processed for pSTAT1 and YOYO-1 staining. Eight-bit black and white images were captured with a digital camera (Retiga EX, Roper Scientific, San Diego, CA) using a dry objective lens (×20, NA). The same capturing conditions of digital camera were used for all specimens.

The intensity of pSTAT1 was measured using ImageJ 1.42 (NIMH). The outlines of nucleus were traced manually, and then the mean score of pSTAT1 intensity was measured for each. To avoid double counting, we used the Measure and Label plugin. All data were presented as the mean ± SEM and were analyzed by a one-way ANOVA, followed by Tukey's HSD test. Statistical significance was established at a level of $p < 0.05$.

For preparing illustrations, selected images were processed using Adobe Photoshop CS4 (Adobe Systems, Mountain View, CA). Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way.

2.6. Real time-polymerase chain reaction (real time-PCR)

The HAPI microglial cells were incubated for 12 h in the presence or absence of minocycline (1 μM). 100 U/mL IFN-γ (R&D) was then added to the cultured medium of HAPI microglial cells. Total RNA was isolated 6 h after the incubation by the RNeasy Mini Kit (Qiagen). The reverse transcription (RT) reaction was performed by the superscript II (Invitrogen, Carlsbad, CA). The expression of iNOS and TNF-α was quantified using SYBR Green master mix (TOYOBO, Osaka, Japan). The gene-specific primers are used as follows: iNOS (forward: 5′-TGTCTTGGTTCTATGGAATCACTC-3′ and reverse: 5′-CGTTCACAGACC TAATCTAAACCT-3′), TNF-α (5′-ATGATCCGAGATGTGGAACTGGCA-3′ and reverse: 5′-AATGAGAAGAGGCTGAGGCACAGA-3′), β-actin (forward: 5′-TGTCTTGGTTCTATGGAATCACTC-3′ and reverse: 5′-CG TTCACAGACCTAATCTAAACCT-3′). The linearity of the amplifications as a function of cycle number was tested in preliminary experiments, and the mRNA expression levels were normalized to the expression levels of the house keeping gene β-actin.

2.7. Nitrite production assessment

The accumulation of NO_2^-, a stable end-product, extensively used as an indicator of NO production by cultured cells, was assayed using the Griess reaction. The HAPI cells were plated on 96-well tissue culture plates (Greiner Bio One GmbH) at 1×10^5 cells per 200 μL medium. The cells were pre-incubated in the presence or absence of minocycline for 12 h followed by the addition of 100 U/mL IFN-γ to the cultured medium. After 24 h of incubation, the cell-free supernatants were assayed for NO accumulation using a Griess kit (Dojindo Molecular Technologies, Kumamoto, Japan) and a plate reader (Labsystems Multiscan MS, Frankfurt, Germany).

2.8. Enzyme-linked immunosorbent assay

TNF-α concentrations in the conditioned media from HAPI cells that were pre-incubated with minocycline (1 μM) and subsequently stimulated with 100 U/mL IFN-γ were measured by enzyme-linked immunosorbent assay (ELISA) with Quantikine kits for Rat TNF-α (Invitrogen) according to the manufacturer's instructions. Optical densities were determined by measurement of indicator color shift at 450 nm on a microplate reader (Multiscan MS, Labsystems, Finland).

2.9. Cell viability

Cell viability of the present experimental conditions was determined by colorimetric measurements of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT). The original medium was removed from the 96-well plates, and the cells were incubated for 2 h at 37 °C in the presence of phenol red-free minimum essential medium (Invitrogen) containing 0.5 mg/mL MTT. A 100 μL MTT lysis buffer (5% sodium dodecyl sulfate (SDS) and 5 mM HCl) was then added to each well, and the plates were incubated at 37 °C overnight to dissolve the formazan that had formed in the wells. MTT is reduced to formazan in the mitochondria of living cells. Reduced MTT was measured by means of a plate reader (Labsystems Multiscan MS, Frankfurt, Germany) at a wavelength of 570 nm.

2.10. Quantitative analyses and statistics

To determine the expression ratio of cleaved caspase-3 in oligodendrocytes, the number of DAPI positive nuclei and the cleaved caspase-3 positive oligodendrocytes was counted from 4 optic fields for each condition. Next, the intensity of pSTAT1 in HAPI cells was measured for each condition. All data are represented as the means ± standard error of means and they were analyzed by a one-way analysis of variance (ANOVA). The Tukey method was used for post hoc comparisons. Significance was established at a level of $p < 0.05$. All statistical analyses were performed with SPSS version 13.0 for Windows.

3. Results

3.1. Cell viability

In the present experiments, the cell viability of HAPI microglial cells was not affected by aripiprazole (20 μM), haloperidol (20 μM) and minocycline (10 μM) (data not shown). Therefore, we used these drugs under this concentration in the present study.

3.2. Protective effects of minocycline and antipsychotics on oligodendrocyte damage

IFN-γ is a typical Th1 cytokine and a major immuno-activator released by infiltrating T cells as well as activated microglia in the CNS (Kawanokuchi et al., 2006). Elevated mRNA levels of IFN-γ have been reported in the brain of schizophrenia patients (Freudenreich et al., 2010), and we thus applied IFN-γ as a stimuli for microglial cells. To investigate direct interactions between microglial activation and the degeneration of oligodendrocytes, a co-culture experiment with HAPI microglial cells and primary oligodendrocytes was performed utilizing the tissue culture inserts (Fig. 1A). In order to evaluate the protective effects of aripiprazole, haloperidol and minocycline, we compared five different groups with various drugs. In order to minimize oligodendrocyte damage due to pretreatment of IFN-γ challenge, we have chosen to treat the target drugs (aripiprazole, haloperidol and minocycline) before IFN-γ challenge. Our experimental design is represented as Fig. 1B. Group 1 [Negative Control] showed a differentiated oligodendrocyte and exhibited the morphology of multipolar mature oligodendrocytes rather than bipolar early precursor cells (Woodruff et al., 2001; Jakovcevski and Zecevic, 2005) (Fig. 1C; Group 1; a and b). Although Group 2 [Positive Control; INF-γ] showed a typical morphology of oligodendrocyte (Fig. 1C; Group 2; a and b) partly, degenerative changes of the oligodendrocyte were frequently observed such as retraction of their processes (Fig. 1C; Group 2; c) swollen cytoplasm/large vacuoles (Fig. 1C; Group 2; d) and roundish morphology (Fig. 1C; Group 2; e) compared to Group 1. In contrast, Group 3 [INF-γ + minocycline] (Fig. 1C; Group 3; a and b) and Group 4 [INF-γ + aripiprazole] (Fig. 1C; Group 4; a and b) displayed no obvious degenerative changes of oligodendrocytes. Group 5 [INF-γ + haloperidol] showed the fragmentation of oligodendrocytes processes (Fig. 1C; Group 5; a and b), indicative of cytoskeletal breakdown.

A striking degeneration of oligodendrocytes was observed in the treatment of IFN-γ (Fig. 1C; Group 2). We then performed immunocytochemistry for proteolipid protein (PLP), a mature oligodendrocyte marker, and cleaved caspase-3 to confirm whether the apoptosis was caused by microglial activation. PLP positive cells in Group 2 were markedly immunoreactive for cleaved caspase-3 (Fig. 2A; Group 2). The expression of cleaved caspase-3 was inhibited by aripiprazole and minocycline (Fig. 2A; Group 3 and Group 4). In the haloperidol pretreatment group the immunoreactivity of cleaved caspase-3 was observed slightly (Fig. 2A; Group 5). A quantitative analysis showed that the expression ratio of the cleaved caspase-3 was significantly lower in Group 3 and Group 4 (Fig. 2B) than in Group 2. Group 5 tended to be lower than Group 2 (Fig. 2B). These results suggest that aripiprazole and minocycline are equally efficacious drugs in the inhibition of oligodendrocyte damage.

3.3. Minocycline inhibits TNF-α released by IFN-γ-activated microglia, but not nitric oxide

The above results strongly indicate that IFN-γ-activated microglial cells secrete inflammatory molecules at high enough levels to be degenerative to primary oligodendrocytes in their local environment. Microglia are reported to be a major source of locally produced NO and TNF-α (Li et al., 2005, 2008). In addition, previous studies have demonstrated that oligodendrocyte cell death is dependent on TNF-α production (Li et al., 2008). Therefore, we hypothesize that the protective effect of aripiprazole and minocycline on oligodendrocyte damage may be induced by inhibiting TNF-α from IFN-γ-activated microglial cells. Previously, we have shown that aripiprazole significantly inhibits TNF-α generation from IFN-γ-activated microglial cells (Kato et al., 2008), however the effect of minocycline has not been reported. To investigate whether the detrimental effects of minocycline were attributable to TNF-α production on IFN-γ-activated microglial cells, we performed quantitative PCR for two targets of iNOS and TNF-α. These results showed that the iNOS mRNA level in minocycline-pretreated microglial cells was not altered compared to that of IFN-γ alone-treated microglial cells (Fig. 3A). In contrast, the expression of TNF-α mRNA was significantly reduced in minocycline-pretreated microglial cells (Fig. 3B). Therefore, we then measured the NO and TNF-α releases. The released NO was unaltered in minocycline pretreated microglial cells (Fig. 3C). In contrast, the secreted TNF-α was significantly inhibited by minocycline

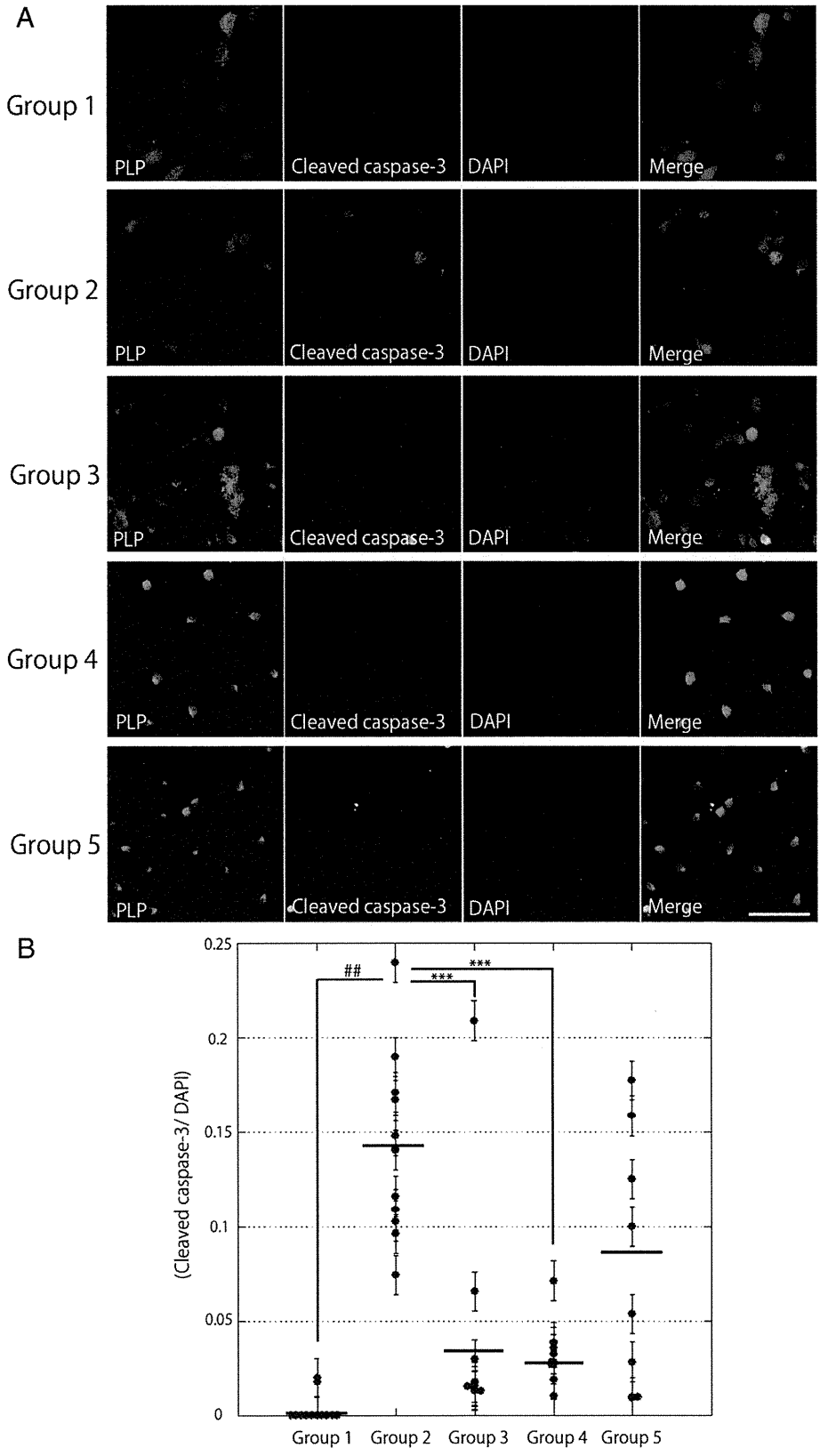

Fig. 2. Double immunofluorescence for PLP and cleaved caspase-3. (A) Numerous cleaved caspase-3-positive cells were observed in Group 2 compared to Group 1. In contrast, in Group 3 and Group 4, the cleaved caspase-3 expression was inhibited. In Group 5, the expression of cleaved caspase-3 was slightly observed, but there is no significance compared to Group 2. (B) The results based on three independent experiments were expressed as the ratio of the values obtained in cleaved caspase-3 positive nuclei or DAPI positive nuclei. Each result was shown as the mean ± SEM (##$p < 0.001$ in comparison to the control group. ***$p < 0.001$ in comparison to the IFN-γ stimulated group). Scale bar = 50 μm.