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Microglia and astroglia prevent oxidative stress-induced neuronal cell death: Implications for aceruloplasminemia

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Abstract

We partially characterized the transferrin-independent iron uptake (Tf-IU) of neuronal and glial cells in the previous report. In the present study, we further examined a mechanism of which glial cells protect neuronal cells against iron stress using neuron–microglia (N–MG) and neuron–astrocyte (N–AS) co-cultures. When each solely purified cell was treated with iron citrate, cell death occurred in N and MG. However, AS proliferated under the same condition. Both N–MG and N–AS co-cultures were effective in resistance to excessive iron. The total and specific Tf-IU activities of N–MG co-cultures similar to those of N did not increase in a density-dependent manner. Contrarily, the total activity of AS was extremely high and the specific activity was extremely low as a result of proliferation. Regarding of effect of co-cultures on H_2O_2-induced cell death, N–MG co-cultures were less effective, but N–AS co-cultures were more effective in protecting N from the oxidative stress. These results suggest that N–MG co-cultures suppress the Tf-IU and N–AS co-cultures stimulate AS proliferation to protect neuronal cells. Brain cells from aceruloplasminemia with mutations in the ceruloplasmin gene take up iron by Tf-IU. Therefore, the different mechanisms of neuronal cell protection by MG and AS may explain the pathophysiological observations in the brains of patient with aceruloplasminemia.
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Keywords: Microglial; Astrocyte; Neuronal cell death; Oxidative stress; Neurodegenerative disorders; Aceruloplasminemia

Several neurodegenerative diseases, including Alzheimer's diseases (AD), Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, Hallervorden–Spatz disease, and aceruloplasminemia are associated with altered iron metabolism and free radical injury [7]. Iron deposition has been observed in the cortex and amyloid plaques of patients with AD [4,36], substantia nigra in Parkinson's disease [15,47,48], the spinal cord in amyotrophic lateral sclerosis [33,19], pallidal triad in Hallervorden–Spatz disease [10], and basal ganglia and dentate nucleus in aceruloplasminemia [13,25–27,53]. Especially, in aceruloplasminemia, it is apparent that iron deposited in the glial cells of the basal ganglia and thalamus [25]. The iron deposition is more prominent in astrocytes [16,17] and also in the microglial cells [14] than in the neurons in the same region. The neuropathological hallmark of this disease is intracellular iron overload, which is thought to lead to neuronal cell death through increased oxidative stress. To understand mechanism of altered iron metabolism in brain cells in this disorder, in vitro experiments are essential to explain in these phenomena observed in aceruloplasminemia.

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Oxidative stresses are implicated in various brain diseases [1,3,11]. The major reactive oxidants in cells are superoxide (O_2^-), and hydroxyl radicals which are more reactive, the latter being derived in partly from H_2O_2 [12]. Recently, we found that reactive oxygen species levels in iron-loaded neuronal cells are twice that of iron-loaded glial cells [34]. Amyloid β proteins (Aβ), which are associated with plaques in the brains of Alzheimer patients increase the H_2O_2 level in primary central nervous system cultures. Aβ-induced increases in H_2O_2 may reflect increased O_2^- production and conversion to H_2O_2 by superoxide dismutase [1]. Based on these reports, in the present study, using neuron–microglia (N–MG) or neuron–astrocyte (N–AS) co-cultures treated with iron or H_2O_2 to produce oxidative stress, we examined how MG and AS protect neurons from oxidative stress. When co-cultured with MG or AS, neurons were remarkably resistant to the oxidative stresses. Not only were MG sensitized to iron- or H_2O_2-induced oxidative stress but AS resistant to the oxidative stresses could also give neuron resistance to the stressful stimuli by co-cultures. Especially, we further found that MG take up more iron than neuron at lower level than AS and that AS take up a higher level of iron than neuron and MG. In this way, we hoped to reproduce in vitro the iron deposition in microglia and astroglia observed in aceruloplasminemia. This is the first report on the relation between effects of co-cultures of neuron with microglia or astrocytes on stress-induced cell death and non-transferrin iron uptake.

1. Materials and methods

1.1. Primary cultures of neurons, microglial cells and astroglial cells

1.1.1. Preparation of neurons

Neurons were prepared from 17-day-old embryonic rat brain essentially according to the method of Shimojyo et al. [46]. The neonatal rat brains were cleaned by removing the meninges, minced, and dissociated with 0.25% trypsin. The cells were seeded onto poly-L-lysine-coated dishes at a density of 5.3×10^5 cells/cm^2 with Eagle's minimum essential medium (MEM) containing 2% fetal calf serum (FCS) and were further cultured for 2 days. The purity of this neuronal culture was estimated to be 96–97% by staining with antibody to microtubule-associated protein 2 (MAP2).

1.1.2. Preparation of microglia and astrocytes from primary cultures of newborn rat brain

The primary culture of rat brain for obtaining microglia (MG) and astrocytes was carried out essentially according to the method described previously [30–32,46]. The cerebral hemispheres from newborn rats were cleaned by removing the meninges and blood vessels, minced, and trypsinized in phosphate-buffered saline (PBS) pH 7.4, containing 0.25% trypsin at 37 °C for 10 min. After the reaction was stopped by the addition of FCS, the dissociated cells were filtered through a 100-mesh screen and washed twice with PBS by centrifugation. Cells were seeded on a 75-cm^2 culture flask at a density of $(1.5–2.0) \times 10^7$ cells per flask with phenol-red-free Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. The cultured cells were maintained in the same medium for the first 9 days, with the medium being changed every 3 days, and thereafter in MEM containing 2% FCS. We prepared microglia and astrocytes from the above primary culture.

Microglia were obtained from primary cultures during days 10–15 of culture. Cells weakly attached on the primary cell layer of the mother culture were detached by gentle shaking of the flasks, and the resultant microglial suspension

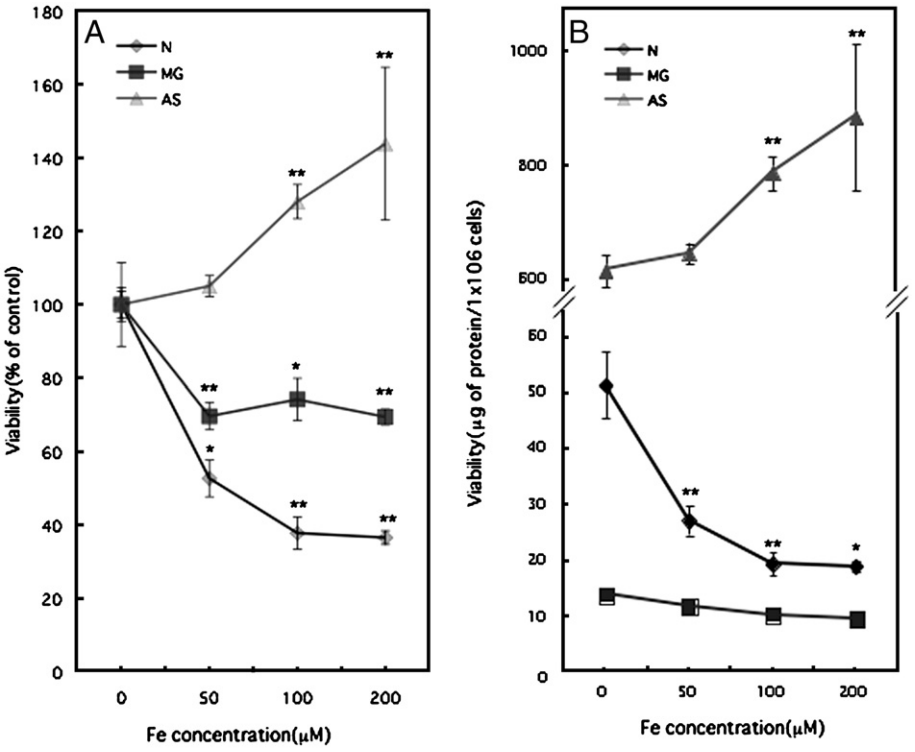


Fig. 1. Effect of Fe loading to neurons (N), microglia (MG) and astrocytes (AS) on cell viability. N, MG and AS of 5×10^5 cells were treated with 50, 100 and 200 μM ferric citrate for 1 h at 37 °C. After treatment, the cells were washed three times with PBS and cultured in the medium for N for 24 h at 37 °C. After incubation, each cell viability was assessed as described in Materials and methods. A shows the cell viability expressed as a percentage of viable cells with treatment to control cell without iron treatment. And B shows the amount of protein of viable cells with or without Fe treatment, respectively. The data were obtained from two separate experiments with three cultures. Asterisk, $P<0.05$, two asterisks, $P<0.001$ versus iron untreated cells.

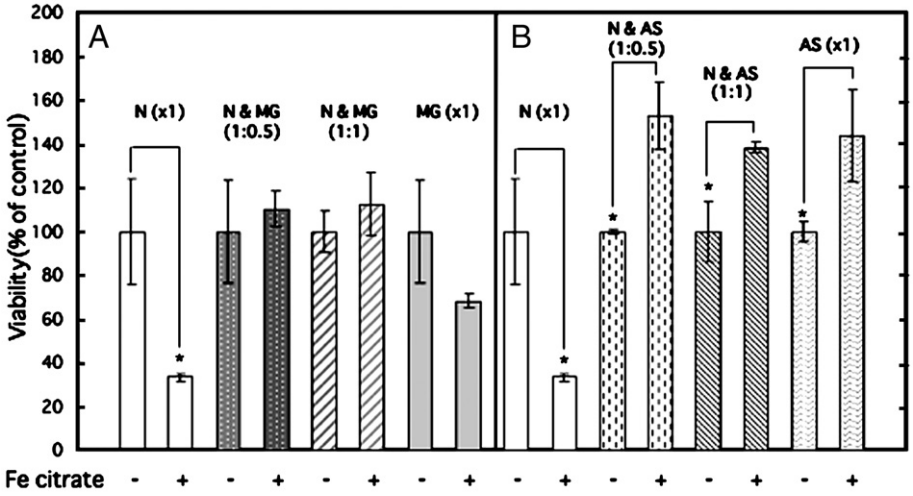


Fig. 2. Effect of co-culture of neurons (N) with microglia (MG) or astrocytes (AS) on cell viability. N, MG, AS, N–MG co-cultures (A) and N, AS, N–AS co-cultures (B) were treated with 200 μM ferric citrate for 1 h at 37 °C. After treatment, the cells were washed three times with PBS and cultured in the medium for N for 24 h at 37 °C. After the incubation, cell viability was assessed as described in Materials and methods. The data were obtained from two separate experiments with each three cultures. Asterisk, $P<0.05$, two asterisks, $P<0.001$ versus iron untreated cells.

was transferred to culture dishes at a density of 0.5×10^5 cells per dish (in the case of 6-cm dishes) or 1.7×10^5 cells per dish (in the case of 9-cm dishes). The purity of the microglia cultures was estimated to be over 99.9% as judged by staining with antibody to ionized calcium binding adaptor molecule 1 (Iba1).

Astrocytes were prepared as follows. Primary cultures that had been maintained in MEM containing 2% FCS for 5–12 days were shaken vigorously with a rotary shaker to remove non-astrocytic cells. The resultant astrocytic monolayer was detached and dissociated by trypsin treatment (0.05% trypsin/PBS) and subsequent filtration with a 100-mesh screen, and the cell suspension was seeded onto the culture dishes at a density of 0.5×10^5 cells/cm^2. The purity of the astrocytes was estimated to be 97–98% by staining with antibody to glial fibrillary acidic protein.

1.2. Co-cultures of cortical neurons with microglia or astrocytes

N–MG and N–AS co-cultures were carried out as follows. The desired cell number of MG or AS was seeded onto the dishes on which neuron had been previously plated 1 day before. The single-seeded and the co-cultured cells were further maintained for 2 days, and were used for the determination of neuroprotection against oxidative stress. In the co-culture experiments, neurons were seeded at a density of 1.4×10^5 cells/cm^2 in 6-well plates and cultured for 3–4 days. Microglia or astrocytes were seeded onto the neuronal culture at a density of 0.7×10^5 and 1.4×10^5 cells/cm^2 and then cultured in the same medium as the neuronal cells.

The experiments using animals were conducted in accordance with the Guide for Animal Experimentation at Soka University.

1.3. Treatment with ferric citrate and hydrogen peroxide

Neurons, MG, AS, N–MG and N–AS co-cultures were treated with 50, 100, and 200 μM ferric citrate or 50, 100 and 500 μM H_2O_2 for 1 h at 37 °C. After treatment, the cells were washed three times with PBS and cultured in the same medium as the neurons for 24 h at 37 °C. After the incubation, sensitivities of these cell were estimated by counting viable cells. The iron concentration used here was estimated from the data obtained in AD brain [8]. This concentration equal to 0.5 to 3.0 mM was used in the present study.

1.4. Uptake of ^{55}Fe-citrate by neuron, glial cells and their co-cultured cells

^{55}Fe-citrate was used to measure the transferrin-independent iron uptake (Tf-IU) activity [18,35,49]. ^{55}Fe-citrate was prepared from $^{55}FeCl_3$ (Dupon-New England Nuclear, USA) by adding excess ferric citrate (Sigma-Aldrich, USA). We identified ferric citrate as a soluble polymer form with a molecular weight lower than transferrin in PBS using gel filtration [35]. To measure the Tf-IU or non-Tf iron activity of the single-seeded cells and co-cultures, these cells were washed three times to remove Tf derived from fetal calf serum in the medium. The endogenous Tf in the cells was removed and depleted by pre-incubating for 1 h in serum-free medium. Then, the single-cultured cells and the co-cultures of N–MG or N–AS were incubated in DMEM at 37 °C for 1 h with 200 μM^{55}Fe-citrate. The cells were washed three times in cold PBS. The cell pellet was solubilized in detergent (Solvable, NEN, USA), mixed in scintillator (Econoflour, NEN), and measured for radioactivity.

1.5. Cell viability

Trypan blue exclusion assay was used to assess cell viability as follows: 5 μl of trypan blue (0.5% in PBS) was added to 40 μl of resuspended cells. Trypan blue excluding and including cells were counted as viable and non-viable cells in a hemocytometer in three randomly selected fields. MG and AS protect neurons from stressful stimuli-induced cell death. Using an antibody against microtubule-associated protein 2 (MAP2), a specific marker of neuron, immunoblot analysis is used to estimate neuronal survival. However, the MAP2 expression is promoted by co-cultures with MG or AS [51,52]. This means that the amount of MAP2 does not reflect the number of viable neurons after stressful stimuli. Therefore, the number of viable cells of co-cultures with MG or AS after the treatment was decided by this method to estimate cell viability.

In a single culture of MG or AS, the cell viability was expressed as a percentage of viable cells with treatment to control cell without iron treatment. In each co-cultures, the cell viability means percent of total viable cells with treatment to the total N–MG or N–AS co-cultures without the treatment.

Table 1
Specific and total activities of transferrin-independent iron uptake in neuron, microglia and astrocyte

	Neuron	Microglia	Astrocyte
Specific activity (dpm/μg cell protein)	82±4	1202±107	9±1
Total activity (dpm)	7701±419	6849±305	15,733±323

The total and specific activities of Tf-IU of each cell was decided as described in Materials and method. Briefly, after pre-incubation for 1 h in the serum-free medium, these cells were washed, incubated with 200 μM ^{55}Fe-citrate at 37 °C for 1 h. After washing three times with PBS, ^{55}Fe-radioactivity was measured to estimate the Tf-IU activity. Results are means±S.D. of three experiments.

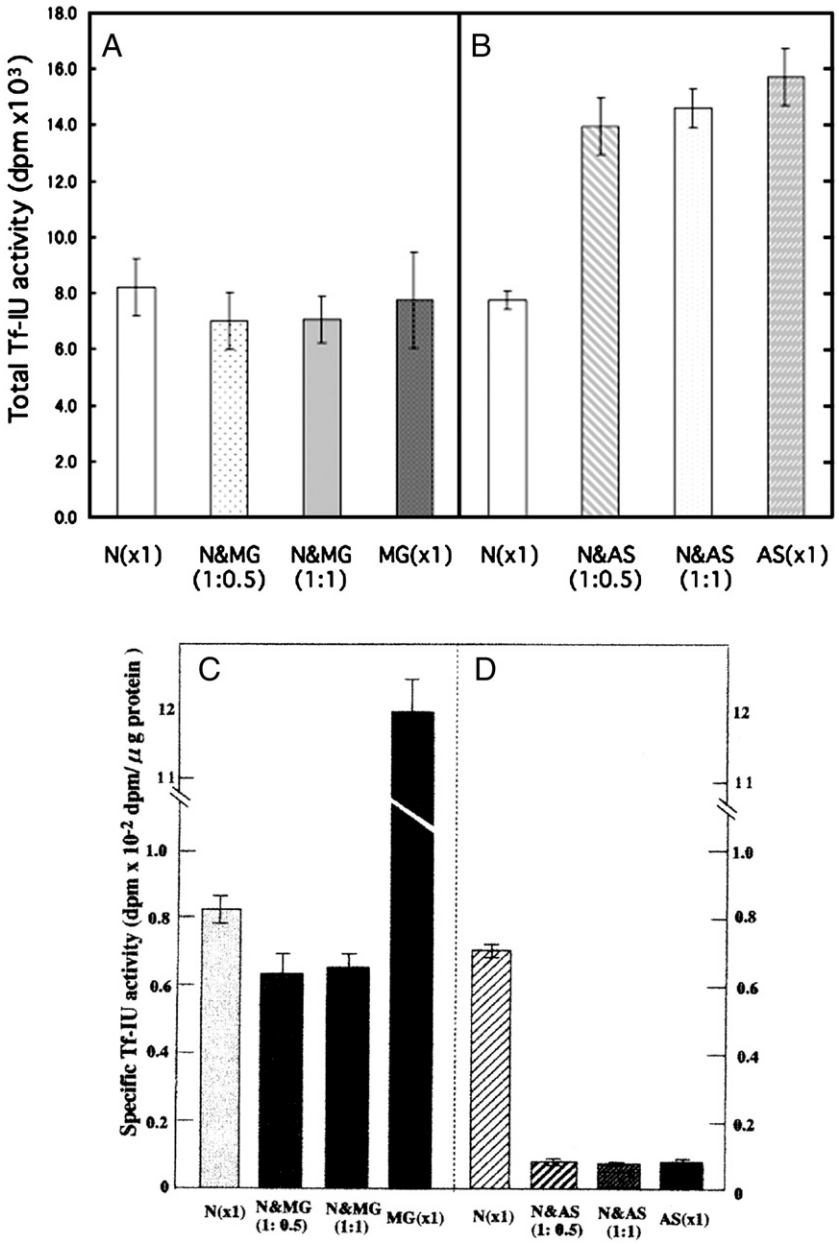


Fig. 3. Effect of f co-culture of neurons (N) with microglia (MG) or astrocytes (AS) on non-transferrin iron uptake. ^{55}Fe-citrate was used to measure transferrin-independent iron uptake (Tf-IU) [35]. As described in Materials and methods, after the pretreatment in DMEM without FCS for 1 h at 37 °C, the single-seeded cells and the co-cultures were incubated in the medium at 37 °C for 1 h with 200 μM ^{55}Fe-citrate. The cells were washed three times in cold PBS and measured as described in Materials and methods. Results are means ± S.D. of three cultures from the typical experiment. (A) Total Tf-IU activity of N, MG and N–MG co-cultures shows the total amount of ^{55}Fe-radioactivity by these cells in DMEM at 37 °C for 1 h after incubation with 200 μM ^{55}Fe-citrate. (B) Total Tf-IU activity of N, AS and N–AS co-cultures shows the total amount of ^{55}Fe-radioactivity by these cells in DMEM at 37 °C for 1 h after incubation with 200 μM ^{55}Fe-citrate. (C) Specific Tf-IU activity of N, MG and N–MG co-cultures shows the amount of ^{55}Fe-radioactivity taken up by cells per mg protein. (D) Specific Tf-IU activity of N, AS and N–AS co-cultures shows the amount of ^{55}Fe-radioactivity taken up by cells per mg protein.

1.6. Evaluation of purity of neuronal culture from primary culture

To evaluate neuronal cell death and survival, immunoblot analysis was used as described by Lim et al. [21,37]. The N–MG and N–AS co-cultures were solubilized in Laemmli's sample solution [2,20] containing 2% sodium dodecyl sulfate. After electrophoresis, the electrophoretic bands were transferred to nitrocellulose sheets and immunoblotted with a monoclonal antibody against MAP2.

1.7. Statistical test

The significance of any differences from the control series was tested with *t*-test.

2. Results

2.1. Sensitivities of neurons, microglia and astrocyte to iron stress stimuli

To estimate the sensitivities of the cells to iron stressful stimuli, the viability of highly purified neuron (N), microglia (MG) and astrocyte (AS) cultures was examined at 24 h after treatment with 50, 100 and 200 μM ferric citrate for 1 h at 37 °C (Fig. 1). When the cell number of N and MG without the treatment was compared

to those of cells after treatment, their cell number significantly decreased in a dose-dependent manner. At concentration of 200 μM ferric citrate, the viability of N and MG was approximately 70 and 40% of these cells without treatment (Fig. 1A). However, the viable cell number of AS extremely increased in a manner of the dose-dependence. When the protein concentration of AS were compared with those of N and MG, the amount of AS was more than 10-fold and 100-fold of N and MG, respectively (Fig. 1B). These results suggest that iron functions as a stressor for both N and MG but functions as a growth factor.

2.2. Effect of neuron–microglia and neuron–astrocyte co-cultures on iron stressful stimuli

We previously reported that glial-predominant cells protect neurons against iron toxicity [34]. To examine how MG or AS contributes to this neuroprotection, we investigated the effect of N–MG and N–AS co-cultures on iron stress stimuli. As shown in Fig. 2A, individually purified N, MG and N–MG co-cultures were treated with 200 μM ferric citrate. After 24 h, not only viable cell number of individually purified N or MG but total viable cell number of N–MG co-cultures were counted to examine the effect of the co-cultures. As a result, 65% and 35% (right bar of N × 1 in Fig. 2A and right bar of MG × 1 in Fig. 2A) of the neuronal and microglial control cells without iron stress (left bar of N × 1 in Fig. 2A and left bar of MG × 1 in Fig. 2A) occurred cell death, respectively. When the viable cell number of N–MG co-cultures without treatment was compared with that of the co-cultures with treatment at density of the ratio of both 1:0.5 and 1:1, no change was observed in the total number of viable cells. These results suggest that MG protect neuron from iron stress-induced cell death. The MAP2-immunoblot analysis supported that neurons co-cultured with MG were saved from iron-induced cell death (data not shown).

In the same way, solely purified N, AS and N–AS co-cultures were treated with ferric citrate under the same condition. Not only viable cell number of individually purified N or AS but total viable cell number of N–AS co-cultures was counted to examine the effect of the co-cultures. 65% and 5% (right bar of N × 1 in Fig. 2B and right bar of AS × 1 in Fig. 2B) of the neuronal or astroglial control cells without iron stress (right bar of N × 1 in Fig. 2B and right bar of AS × 1 in Fig. 2B) occurred the cell death, respectively. As a result of co-culture with neurons and AS, approximately 50% of the viable cell number of N–AS without treatment increased at two kinds of ratios (1:0.5 and 1:1). No significant difference between N–AS co-cultures at two kinds of ratios (1:0.5, 1:1) and single-cultured AS. These results suggest that AS also protect neurons from iron stress-induced cell death (Fig. 2B). The MAP2-immunoblot analysis also supported that neurons co-cultured with astrocytes were saved from iron-induced cell death (data not shown).

2.3. Iron uptake by purified neurons, microglia and astrocytes

It is thought that non-transferrin (Tf)-bound iron increases in serum of aceruloplasminemia because ceruloplasmin does not function as a ferroxidase to bind iron to apo-Tf [13,14]. We previously reported kinetics of free iron or transferrin (Tf)-bound iron uptake in cultured human fibroblasts [35]. Free iron accumulates in mammalian cells because non-Tf bound iron uptake has a large Vmax for iron uptake. Therefore, we measured non-Tf bound iron uptake in each cell. As shown in Table 1, there was no significant difference in total activity between neuron and MG. In contrast, AS had the largest Tf-independent iron activity and took up non-Tf iron more than twice as much non-Tf iron as did neurons and microglia. Concerning the specific radioactivity of the uptake, remarkable difference was noted among them. The specific radioactivity of neurons was 9-fold that of astrocytes. The specific radioactivity of microglia was 15-fold that of neurons and 130-fold that of astrocytes. Microglia had the greatest specific activity. These results suggest that MG concentrate non-Tf iron and AS take up large amount of free iron though both glial cells internalize iron out of cells.

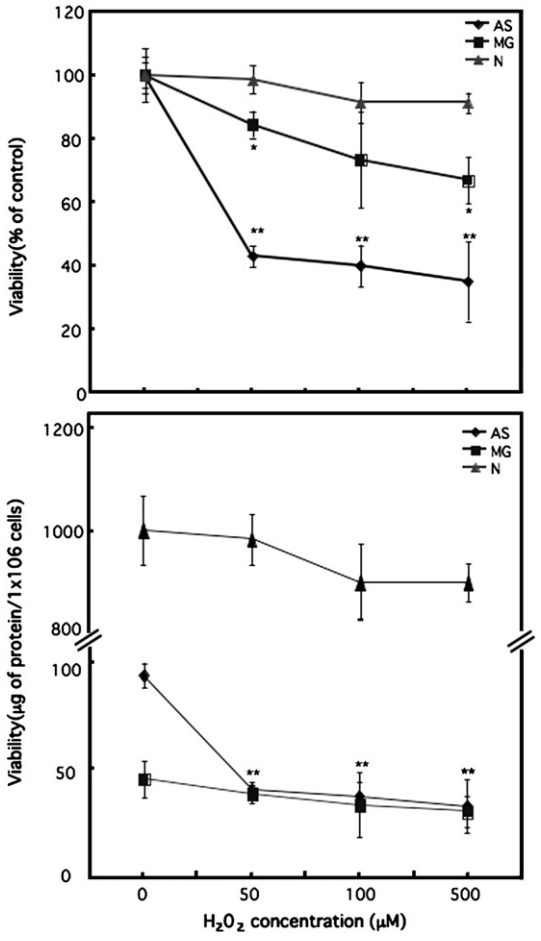


Fig. 4. Effect of oxidative stress to neurons (N), microglia (MG), and astrocytes (AS) on cell viability. After N, MG and AS of 1×10^6 cells were treated with 50, 100 and 500 μM H_2O_2 for 1 h at 37 °C, the cells were washed three times with PBS and cultured in the medium for N for 24 h at 37 °C. After the treatment, cell viability was assessed as described in Materials and methods. The data were obtained from two separate experiments with three cultures and showed the typical results obtained by conducting three cultures from one experiment of the two independent experiments. Results are means ± S.D. of three cultures from the typical experiment.

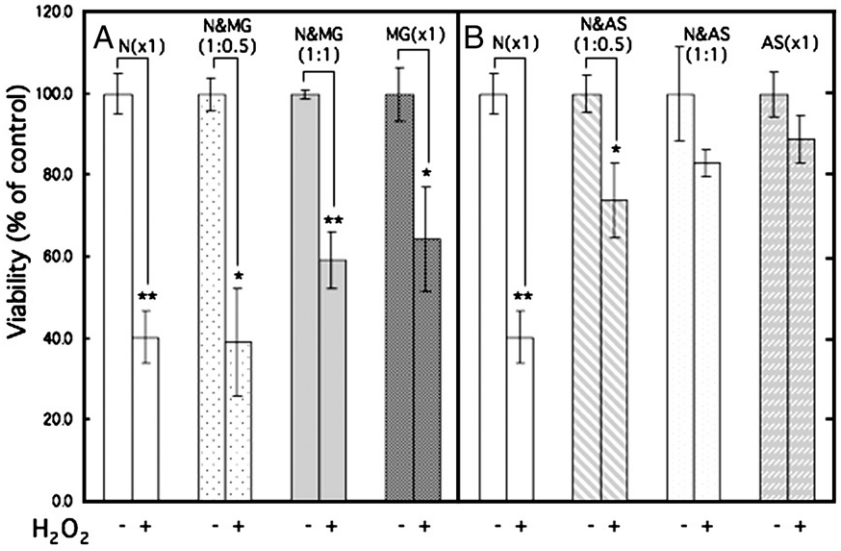


Fig. 5. Effect of co-culture of neurons (N) with microglia (MG) or astrocytes (AS) on H_2O_2-induced cell death. N, MG, AS, N–MG, and N–AS co-cultures were treated with 500 μM H_2O_2 for 1 h at 37 °C. After incubation, the cells were washed three times with PBS and cultured in the medium for N for 1 h at 37 °C. After the treatment, each cell viability was assessed as described in Materials and methods. The data were obtained from two separate experiments each with three cultures. Asterisk, $P<0.05$, two asterisks, $P<0.001$ versus iron untreated cells.

2.4. Effect of neuron–microglia and neuron–astrocyte co-cultures on non-transferrin iron uptake

The total Tf-IU activities of neuron, MG, AS, N–MG and N–AS co-cultures were measured using non-Tf iron, ^{55}Fe-citrate under the condition without endogenous Tf as described in Materials and methods. When the total Tf-IU activities of these cells were compared with each other, no difference between the total activities of single cell cultures and co-cultures though MG were seeded onto the neuronal culture at densities of 1:0.5 and 1:1, Tf-independent iron uptake of N–MG co-cultures did not increase in proportion to the cell number of the co-culture. In other words, although the cell number increased, non-Tf uptake did not increase with cell density (Fig. 3A). Next, when the total Tf-IU activities of N, AS and their co-cultures were compared with them, no difference between the total activities of AS and N–AS co-cultures though the total Tf-IU activity of AS was greater than that of neurons (Fig. 3B).

For the specific Tf-IU activities, MG have extremely high specific activity, which is 14- and 19-fold greater than those of neurons and N–MG co-cultures (Fig. 3C). In contrast, as shown in Fig. 3D, AS have extremely low specific activity, which is 7-fold smaller than those of neurons though no difference between N–MG co-cultures. These results suggest that MG suppress the Tf-IU by co-culture and that AS relatively decrease the Tf-IU not by co-culture but by proliferation.

2.5. Sensitivities of neurons, microglia and astrocytes to oxidative stress stimuli

The viability of solely purified neurons, MG and AS was examined after treatment with 0 to 500 μM H_2O_2 for 1 h to estimate the sensitivity of these cells to H_2O_2 stress stimuli. As shown in Fig. 4A, neurons were the most sensitive to oxidative stress. MG were more resistant than neurons. AS were the most resistant cell to oxidative stress. Surprisingly, although 70% of control neurons without the oxidative stress occurred cell death after treatment with 500 μM H_2O_2, more than 90% of AS were viable at the same H_2O_2 concentration (Fig. 4B).

2.6. Effect of co-cultures with neurons and microglia or with neurons and astrocytes on oxidative stress stimuli

As shown in Fig. 5, after purified neurons, MG, AS, N–MG and N–AS co-cultures were treated with 500 μM H_2O_2 for 1 h, the viability of each cultured and co-cultured cells was estimated by counting the total viable cell number of single-cultured cells or co-cultured cells to compare with those of iron stressful stimuli. The level of the viability of co-cultures with neurons and MG at the ratio 1:0.5 was similar to neurons, but the viable level of the co-cultures with the ratio 1:1 was higher than that of neurons, which was similar to that of MG (Fig. 5A).

The level of the viability of co-cultures with neurons and AS at the ratio 1:0.5 was higher than neurons, and the viable levels of the co-cultures with the ratio 1:1 were similar to that of AS (Fig. 5B). From these results, it may be suggested that co-cultures with N and MG or with N and AS were effective in cell protection of the couple for H_2O_2-induced cell death.

3. Discussion

We previously reported that glial cells are more resistant to oxidative stress than neuronal cells and that iron more markedly accumulates in glial cells than in neuronal cells. We hypothesized that glial cells contribute to the metal accumulation resulting in contribution to neuroprotection from oxidative stress caused by iron [34]. In the previous study, because we used glial-predominant cells, we could not investigate which of

the glial cells, microglia or astrocyte contributes to neuroprotection. Therefore, individually and highly purified neuronal and glial cells were needed for further investigation. In the present study, to test which and how glial cells accumulate iron, we measured the specific and total activities of transferrin-independent iron uptake in neurons, MG and AS (Table 1). AS had a total activity twice as high as that of MG or neurons. AS also had the largest capacity for iron accumulation. Furthermore, we observed that the transferrin-independent iron uptake (Tf-IU) in glial-predominant cells was 3-times as much as that in control glial-predominant cells [34]. Surprisingly, iron functioned not as an oxidative stressful stimulus but as a growth factor for astrocytes. On the other hand, iron citrate did as an oxidative stressor, but not as a growth factor (Fig. 1). In contrast, microglia had an extremely high specific activity, compared with AS. Therefore, we concluded that both MG and AS contribute to iron accumulation. Iron was concentrated in MG, which have a small storage capacity, and accumulated in AS, which have a large storage capacity.

Takeda et al. [50] reported that MG take up more non-transferrin iron than do AS or oligodendrocyte in hypotransferrinemic (Hp) mice, which have a point mutation or small deletion in the Tf gene. Because excess non-transferrin iron increases in the plasma of Hp mice, most of plasma iron is non-transferrin iron. Non-transferrin iron is deposited in the brain, liver and pancreas in Hp mice as was seen in patients with aceruloplasminemia. Aceruloplasminemia is an autosomal recessive disorder of iron metabolism caused by mutations in the ceruloplasmin (CP) gene [13,25,26,53]. CP functions as a copper transporter, an amine oxidase, an antioxidant and a ferroxidase, which oxidizes ferrous iron for transfer into transferrin. CP is essential to the normal movement of iron from cell to plasma [44] and functions to promote iron efflux from brain cells via ferroxidase activity. In patients with aceruloplasminemia, iron would be unable to move from brain cells into the brain extracellular fluid and cerebrospinal fluid for eventual excretion, resulting in iron accumulation in related tissue [43]. Iron accumulation in the brain, liver and pancreas is seen in patients with aceruloplasminemia. In the brain, iron deposition occurs in glial cells of the basal ganglia and thalamus. Abundant iron is evident within MG [25,16,17,14] and AS [16,17] of patients with this disease. In our previous in vitro study, we observed that both glial-predominant cells including AS and MG take up iron [34]. In the present in vitro study, not only AS with large capacity but MG with poor capacity could also concentrate large amount of iron (Table 1). The present model system may be applicable as an in vitro model for a mechanism of iron accumulation in patients with aceruloplasminemia.

Many studies of neuroprotection against oxidative stress have been reported. Most of them have examined neuroprotective factors and intracellular signaling pathways. Only a few investigators have reported neuroprotection against nitric oxide using MG–N co-culture [52] and reactive oxygen and nitrogen species using AS–N co-culture [51]. Therefore, we used co-cultures of neurons with MG or co-cultures of neurons with AS in sensitivity or resistance to ferric citrate and H_2O_2 as stressful stimuli under the same condition to further examine how MG and AS protect neurons from iron accumulation and oxidative stress caused by iron.

To investigate the effect of these co-cultures on iron-induced cell death, the total and specific activities of Tf-IU of individually cultured cells and their co-cultures were compared. N–MG co-cultured cells had specific and total activities similar to those of neurons. The specific and total activities of N–AS co-cultures were roughly comparable with those of AS. AS have extremely low specific activity, which is 7-fold smaller than those of neurons. These results suggest that the suppression of Tf-IU by N–MG co-cultures results in neuroprotection from iron stress-induced neuronal cell death and that the Tf-IU of N–AS co-cultured cells is suppressed by astrocyte proliferation (Fig. 1) resulting in the neuroprotection from the stress-induced neuronal cell death. A report by Makar et al. on the important role of astrocytes in antioxidant processes in the brain may support our evidence [22].

The effect of co-cultures on H_2O_2-induced neuronal cell death has not yet been investigated in in vitro experiments, although there were studies on the effect of reactive oxygen species, including NO, couples of NO and O_2, and hydroxy radical ($OH^{\cdot}$), on the cell death. To study the effect of N–MG or N–AS co-culture on reactive oxygen species-induced cell death [51,52], we used H_2O_2 because Aβ, which is associated with plaques in the brains of patients with AD, increases the H_2O_2 level in primary central nervous system cultures. In the present experiment, we examined the effect of co-cultures of N/MG or N/AS on oxidative stress. As shown in Fig. 5, resistance to H_2O_2 of co-cultures of N–MG and N–AS has the same level as that of the individually cultured microglia and astrocyte.

In our previous study, we found that reactive oxygen species were generated in both iron-loaded neuronal and glial-predominant cells and showed the generation of O_2^- in both control cells [34]. Behl et al. detected H_2O_2 using 2′,7′-dichlorofluorescein diacetate in Aβ-treated B12 cells derived from the peripheral nervous system. This detection could reflect O_2^- production and conversion to H_2O_2 by superoxide dismutase in nerve cells [1]. The selective increase in lipid peroxidation in the inferior temporal cortex of AD brains [36] also supports our data. Moreover, in our previous study, the neuronal cell death might have been because of the cell toxicity of reactive oxygen species. Comparing the sensitivities of the cells to oxidative stress, glial-predominant cells were more resistant to Al and Fe toxicities than neuron-predominant cells. Neurons are more vulnerable than glia to insults such as ischemia, hypoxia and brain trauma [37,38]. It was also reported that the antioxidant capacity of neurons in culture is lower than that of glial cells in culture [22]. Our findings are compatible with these reports. The relationship between the amount of reactive oxygen species in both cells and the viabilities of these cells may reflect the difference between the antioxidant defense systems of neurons, microglia and astrocyte (Fig. 1).

There have been several reports on neuroprotection by glial cells. Microglia prevent nitric oxide-induced neuronal apoptosis by secreting heat-labile and heat-stable neuroprotective factors in vitro [52], whereas microglia cause neuronal cell death by secreting neurotoxic substances including reactive oxygen

species [39,40]. In this experiment, we presented another mechanism that both MG and AS can protect neurons from both iron and oxidative stress by suppressing Tf-IU and by proliferating.

Mammalian cells take up iron by Tf-dependent and Tf-independent in ron uptake in the brain [28]. Quite recently, several iron transporters that might be candidates of Tf-IU have been cloned [9,5]. The Nramp1 gene encoding the natural resistance-associated macrophage protein 1 (Nramp1) is expressed on neurons as well as microglia [6]. Divalent metal transporter 1 (DMT1, an isoform of Nramp1) is distributed in neurons [45]. DMT1 were expressed on neurons, microglia and astrocytes [29]. It has been reported that DMT1 with iron responsive element (+IRE) and without the IRE (−IRE) were expressed in N, MG and AS. It was found that there is a relationship between the increased iron influx and DMT1 expression [50].

To understand how N–MG co-cultures suppress transferrin-independent iron uptake, we focused on the relationship between iron metabolism and neuronal cell death. It is known that heme oxygenase 1 (HO1), a heme-degrading enzyme, is involved in iron metabolism and prevents cell death by regulating cellular iron [41,42]. HO1 is a microsomal enzyme committed to heme degradation and considered an antioxidant and anti-inflammatory enzyme because of its ability to reduce the intracellular levels of the pro-oxidant heme and contribute to the formation of bilirubin [23,24]. Studies of the effect on HO1 expression of co-culture of cortical neurons with microglia or astrocytes are in progress.

References

[1] C. Behl, J.B. Davis, R. Lesley, D. Schubert, Hydrogen peroxide mediates amyloidβprotein toxicity, Cell 77 (1994) 817–827.

[2] Y. Cai, C. Zhang, T. Nawa, T. Aso, M. Tanaka, S. Oshiro, H. Ichijyo, S. Kitajima, Homocysteine-responsive ATF3 gene expression in human vascular endothelial cells: activation of c-Jun NH(2)-terminal kinase and promoter response element, Blood 96 (2000) 2140–2148.

[3] J.T. Coyle, P. Puttfarcken, Oxidative stress, glutamate, and neurodegenerative disorders, Science 262 (1993) 689–695.

[4] J.R. Connor, B.S. Snyder, J.L. Beard, R.E. Fine, E.J. Mufson, Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease, J. Neurosci. Res. 31 (1992) 327–335.

[5] A. Donovan, A. Brownlie, Y. Zhou, J. Shepard, S.J. Pratt, J. Movnihan, B.H. Paw, A. Drejer, B. Barut, A. Zapata, T.C. Law, C. Bruqnara, S.E. Lux, G.S. Pinkus, J.L. Pinkus, P.D. Kingslev, J. Palis, M.D. Fleming, N.C. Andrew, L.I. Zon, Positional cloning of zebrafish ferroportin 1 identifies a conserved vertebrate iron exporter, Nature 403 (2000) 776–781.

[6] C.A.W. Evans, M.S. Harbuz, T. Ostenfeld, A. Norrish, J.M. Blackwell, Nramp1 is expressed in neurons and is associated with behavioural and immune response to stress, Neurogenet. 27 (2001) 69–78.

[7] M. Gerlach, D.B. Shachar, P. Riederer, M.B. Youdim, Altered brain metabolism of iron as a cause of neurodegenerative diseases? J. Neurochem. 63 (1994) 793–807 (Review).

[8] P.F. Good, D.P. Perl, L.M. Bierer, J. Schmeider, Selective accumulation and iron in the neurofibrillary tangles of Alzheimer's disease: a laser microprobe (LAMMA) study, Ann. Neurol. 31 (1992) 286–292.

[9] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Borton, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, Nature 388 (1997) 482–488.

[10] W. Halliday, The nosology of Hallervorden–Spatz disease, J. Neurol. Sci. 134 (1995) 84–91 (suppl.).

[11] B. Halliwell, Reactive oxygen species and central nervous system, J. Neurochem. 59 (1992) 1609–1623.

[12] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, Oxford, 1989, pp. 188–266.

[13] Z.L. Harris, Y. Takahashi, H. Miyajima, M. Serizawa, R.T. MacGillivray, J.D. Gitlin, Aceruloplasminemia: molecular characterization of this disorder of iron metabolism, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 2539–2543.

[14] Z.L. Harris, L.W.J. Klomp, J.D. Gitlin, Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis, Am. J. Clin. Nut. 67 (1998) 972s–977s (suppl).

[15] E.C. Hirsch, J.P. Brandel, P. Galle, F. Javoy-Agid, Y. Agid, Iron and aluminum increase in substantia nigra of patients with Parkinson's disease: an X-ray microanalysis, J.Neurochem. 56 (1991) 446–451.

[16] K. Kaneko, K. Yoshida, K. Arima, S. Ohara, H. Miyajima, T. Kato, M. Ohta, S. Ikeda, Astrocytic deformity and globular structures are characteristic of the brains of patients with aceruloplasminemia, J. Neuropathol. Exp. Neurol. 61 (2002) 1069–1077.

[17] K. Kaneko, A. Nakamura, K. Yoshida, F. Kametani, K. Higuchi, S. Ikeda, Glial fibrillary acidic protein is greatly modified by oxidative stress in aceruloplasminemia brain, Free Radic Res. 36 (2002) 303–306.

[18] J. Kaplan, I. Jordan, A. Sturrock, Regulation of the transferrin-independent iron transport system in cultured cells, J. Biol. Chem. 266 (1992) 2997–3004.

[19] E.J. Kasarskis, L. Tandon, M.A. Lovell, W.D. Ehmann, Aluminum, calcium, and iron in the spinal cord of patients with sporadic amyotrophic lateral sclerosis using laser microprobe mass spectroscopy: a preliminary study, J. Neurol. Sci. 130 (1995) 203–208.

[20] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[21] J.H. Lim, T.C. Wen, S. Matsuda, J. Tanaka, N. Meda, H. Peng, J. Aburaya, K. Ishihara, M. Sakanaka, Protection of ischemic hippocampal neurons by ginsenoside Rb1, a main ingredient of ginseng root, Neurosci. Res. 28 (1997) 191–200.

[22] T.K. Makar, M. Nedergaad, A. Preuss, A.S. Gelbard, A.S. Perumal, A.J.L. Cooper, Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain, J. Neurochem. 62 (1994) 45–53.

[23] M.D. Maines, The heme oxygenase system: a regulator of second messenger gases, Annu. Rev. Pharmacol. Toxicol. 37 (1997) 517–554.

[24] C. Mancuso, M. Perluigi, C. Cini, C.D. Marco, A. Maria, G. Stella, V. Calabrese, Heme oxygenase and cyclooxygenase in the central nervous system: a functional interplay, J. Neurosci. Res. 84 (2006) 1385–1391.

[25] H. Miyajima, Y. Nishimura, K. Mizoguchi, M. Sakamoto, T. Shimizu, N. Honda, Familial apoceruloplasmin deficiency associated with blepharospasm and retinal degeneration, Neurology 37 (1987) 761–767.

[26] H. Miyajima, S. Kono, Y. Takahashi, M. Sugimoto, M. Sakamoto, N. Sakai, Cerebellar ataxia associated with heteroallelic ceruloplasmin gene mutation, Neurology 57 (2001) 2205–2210.

[27] H. Morita, S. Ikeda, K. Yamamoto, S. Morita, K. Yoshida, S. Nomoto, M. Kato, N. Yanagisawa, Hereditary ceruloplasmin deficiency with hemosiderosis: a clinicopathological study of a Japanese family, Ann. Neurol. 37 (1995) 646–656.

[28] T. Moos, Brain iron homeostasis, Dan. Med. Bull. 49 (2002) 279–301.

[29] T. Moos, E.H. Morgan, The significance of the mutated divalent metal transporter (DMT1) on iron transport into the Belgrade rat brain, J. Neurochem. 88 (2004) 233–245.

[30] K. Nakajima, M. Nakajima, M. Hamanoue, M. Shimojo, N. Takei, S. Kohsaka, Characterization of microglia isolated from a primary culture of embryonic rat brain by a simplified method, Biomed. Res. 10 (1989) 411–423.

[31] K. Nakajima, M. Shimojo, M. Hamanoue, S. Ishiura, H. Sugita, S. Kohsaka, Identification of elastase as a secretory protease from cultured rat microglia, J. Neurochem. 58 (1992) 1401–1408.

[32] K. Nakajima, S. Honda, Y. Tohyama, Y. Imai, S. Kohsaka, T. Kurihara, Neurotrophin secretion from cultured microglia, J. Neurosci. Res. 65 (2001);

K. Nakajima, Y. Tohyama, S. Kohsaka, T. Kurihara, Ability of rat microglia to uptake extracellular glutamate, Neurosci. Lett. 307 (2001) 171–174.

[33] H. Oba, T. Araki, K. Ohtomo, S. Monzawa, G. Uchiyama, K. Koizumi, Y. Nogata, K. Kachi, Z. Shiozawa, M. Kobayashi, Amyotrophic lateral sclerosis: T2 shortening in motor cortex at MR imaging, Radiology 189 (1993) 843–846.

[34] S. Oshiro, M. Kawahara, Y. Kuroda, C. Zhang, Y. Cai, S. Kitajima, M. Shirao, Glial cells contribute more to iron and aluminum accumulation but are more resistant to oxidative stress than neuronal cells, Biochim. Biophys. Acta 1502 (2000) 405–414.

[35] S. Oshiro, H. Nakajima, T. Markello, D. Krasnewich, I. Bernardini, W. Gahl, Redox, transferrin-independent and receptor-mediated endocytosis iron uptake system in cultured human fibroblasts, J. Biol. Chem. 268 (1993) 21586–21591.

[36] A.M. Palmer, M.A. Burns, Selective increase in lipid peroxidation in the inferior temporal cortex in Alzheimer's disease, Brain Res. 645 (1994) 338–342.

[37] H. Peng, T.C. Wen, J. Tanaka, S. Matsuda, J. Desaki, S. Sudo, B. Zhang, M. Sakanaka, Epidermal growth factor protects neuronal cells in vivo and in vitro against transient forebrain ischemia- and free radical-induced injuries, J. Cereb. Blood Flow Metab. 18 (1998) 349–360.

[38] D.P. Perl, P.F. Good, Comparative techniques for determining cellular iron distribution in brain tissues, Ann. Neurol. 32 (1992) S76–S81 (Suppl).

[39] D. Piani, M. Spranger, K. Frei, A. Schaffner, A. Fontana, Macrophage-induced cytotoxicity of *N*-methyl-D-aspartate receptor positive neurons involves excitatory amino acids rather than reactive oxygen intermediates and cytokines, Eur. J. Immunol. 22 (1992) 2429–2436.

[40] E. Polazzi, T. Gianni, A. Contestabile, Microglial cells protect cerebellar granule neurons from apoptosis: evidence for reciprocal signaling, Glia 36 (2001) 271–280.

[41] K.D. Poss, S. Tonegawa, Heme oxygenase 1 is required for mammalian iron reutilization, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10919–10924.

[42] K.D. Poss, S. Tonegawa, Reduced stress defense in heme oxygenase 1-deficient cells, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10925–10930.

[43] Z.M. Qian, Q. Wang, Expression of iron transport proteins and excessive iron accumulation in the brain in neurodegenerative disorders, Brain Res. Rev. 27 (1998) 257–267.

[44] H.P. Roeser, G.R. Lee, S. Nacht, G.E. Cartwright, The role of ceruloplasmin in iron metabolism, J. Clin. Invest. 49 (1970) 2408–2417.

[45] J.A. Roth, C. Horbinski, L. Feng, K.G. Dolan, D. Higgins, M.D. Garrick, Differential localization of divalent metal transporter 1 with and without iron response element in rat PC12 and sympathetic neuronal cells, J. Neurosci. 20 (2000) 7595–7601.

[46] M. Shimojo, Y. Imai, K. Nakajima, S. Mizushima, A. Uemura, S. Kohsaka, Interleukin-2 enhances the viability of primary cultured rat neocortical neurons, Neurosci. Lett. 151 (1993) 170–173.

[47] E. Sofic, W. Paulus, K. Jellinger, P.M. Riederer, M.B.H. Yodium, Selective increase of iron in substantia nigra zona compacta of Parkinsonian brains, J. Neurochem. 56 (1991) 978–982.

[48] N. Song, H. Jiang, J.X. Xie, Divalent metal transporter 1 upregulation is involved in the 6-hydroxydopamine-induced ferrous iron influx, J. Neurosci. Res. (2007) Jul 30 [Electronic publication ahead of print].

[49] A. Sturrock, J. Alexander, J. Lamb, Characterization of a transferrin-independent uptake system for iron in HeLa cells, J. Biol. Chem 265 (1990) 3139–3145.

[50] A. Takeda, A. Devenyi, J.R. Connor, Evidence for non-transferrin-mediated uptake and release of iron and manganese in glial cell cultures from hypotransferrinemic mice, J. Neurosci. Res. 51 (1998) 454–462.

[51] J. Tanaka, K. Toku, B. Zahng, K. Ishihara, M. Sakanaka, N. Maeda, Astrocytes prevent neuronal death induced by reactive oxygen and nitrogen species, Glia 28 (1999) 85–96.

[52] K. Toku, J. Tanaka, H. Yano, J. Desaki, B. Zhang, L. Yang, K. Ishihara, M. Sakanaka, N. Maeda, Microglial cells prevent nitric oxide-induced neuronal apoptosis in vitro, J. Neurosci. Res. 53 (1998) 415–425.

[53] K. Yoshida, K. Furihata, S. Takeda, A. Nakamura, K. Yamamoto, H. Morita, S. Hiyamuta, S. Ikeda, N. Shimizu, N. Yanagisawa, A mutation in the ceruloplasmin gene is associated with systemic hemosiderosis in humans, Nat. Genet. 9 (1995) 267–272.