The antioxidative and neuroprotective effects of neurosteroids in pilocarpine-induced status epilepticus mouse model

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The antioxidative and neuroprotective effects of neurosteroids in pilocarpine-induced status epilepticus mouse model

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The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the
degree of Master of Medical Science

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June 2014

This certifies that the Master's Thesis of Inja Cho is approved.

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June 2014

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Epilepsy is a neurological disorder associated with complex molecular and biochemical reactions. Oxidative stress resulting from excessive neuronal hyperexcitability, a hallmark of seizures, has been implicated with the initiation and progression of epilepsy. Excessive production of reactive oxygen species (ROS) being coupled with the shortage of antioxidant defense system like superoxide dismutase (SOD) may play a key role in the process of neuronal death and following epileptogenesis.

Neurosteroid, an important native neuromodulator of cerebral metabolism, plays a role in various cerebral physiological processes through its interactions with neurotransmitter-gated ion channels and their receptors, which may also include potent anticonvulsant effects in various animal models.

The purpose of this study is to investigate the neuroprotective role of allopregnanolone, the prototype neurosteroid in brain, in relation to the ROS mechanisms of neuronal injury in a pilocarpine-induced status epilepticus (SE) mouse model.

Adult male C57BL/6 mice were given injections of pilocarpine 30 min after scopolamine treatment. Hippocampal cell death was assessed by cresyl-violet and TUNEL staining. The hippocampal ROS was assessed using *in situ* detection of oxidized hydroethidine (HEt) administered intravenously after SE. SOD level was analyzed by both Western blotting analysis and immunofluorescent staining in subfields of hippocampus, in order to investigate the relationship between the SOD expression and the neuroprotective effect of allopregnanolone.

The number of neurons was severely reduced and TUNEL positive cells were significantly increased in hippocampal CA1and CA3 regions at 3days after SE. In allopregnanolone treated group, the ROS production, TUNEL positive cells and oxidative DNA damages were all significantly decreased compared to the vehicle-injected group after pilocarpine-induced SE, which were similar to that of normal control group. On the other hand, in allopregnanolone-treated group, SOD expression was significantly increased in hippocampus, especially in CA3 region, which has shown the most severe neuronal damage in the vehicle-treated group. In this pilocarpine SE mouse model, the production of ROS and the degree of neuronal death were quite minimal in the dentate gyrus compared to the hippocampal CA1 and CA3, which may suggest the presence of different innate neuroprotective

mechanisms.

In conclusion, excessive ROS production in the pilocarpine-induced SE mouse model is an important molecular mechanism involved with neuronal death in vulnerable subfields of hippocampus. The neuroprotective role of allopregnanolone in this SE-model is mediated through prevention of ROS-induced neurotoxicity, probably by increasing SOD expression in these areas.

Key word : oxidative stress, ROS, SOD, SE, hippocampus, neurosteroid, allopregnanolone

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I. Introduction

Seizures are defined as symptom complexes precipitated by abnormal and excessive neuronal discharges, whereas epilepsy is defined as a condition of enduring predisposition of seizure recurrences. Mechanisms of either seizure occurrence (ictogenesis) or inducing epilepsy (epileptogenesis) are still unknown. However, it has been well documented that a brain insult precipitates acute neuronal death followed by a complex process of brain recovery to establish altered hyperexcitable neuronal networks, which are associated with gliosis, synaptic reorganization, inflammation, altered neurotransmitters and receptors, etc., Status epilepticus (SE) precipitated by either chemicals or electrical stimulation in mice are the most widely used animal models of acquired temporal lobe epilepsy. SE

induces acute cell loss, in which excessive formation of ROS caused by excessive neuronal hyperactivities play a major role.

In normal physiological condition, cells constantly produce ROS but, at the same time, they have proper antioxidant defense system operating in balance to prevent any excessive oxidative stresses. The antioxidant defense system consists of various free radical scavenging enzymes, such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GP), as well as numerous non-enzymatic antioxidants such as glutathione. The balance between ROS and its scavenging systems are disturbed in various pathologic conditions, which may include both acute cerebral injuries and chronic conditions like neurodegenration. In SE-mouse models, production of ROS is rapidly increased by repetitive ictal discharges in the hippocampal region, which may precipitate acute neuronal death in this structure. However, the degree of neuronal injury may be determined by the balance between ROS and its defense mechanism in individual subregions of hippocampus, which may explain the phenomenon of selective vulnerability of hippocampal subregions. In the pilocarpine-induced SE mouse model, neuropathological investigations clearly demonstrated severe neuronal death associated with the increased lipid peroxidation and free radical formation and the decreased glutathione content,[1-3] which was most pronounced in the hippocampus.[4] Especially, pyramidal neurons in CA1 and CA3 regions of hippocampus are highly vulnerable to damage whereas CA2 and DG regions escape from severe neuronal injury.[5,6]

Neurosteroid is a family of steroid being synthesized and metabolized by reductase

in the central nervous system, which has a wide range of potential clinical applications ranging from a sedative drug to the treatment of epilepsy and traumatic brain injury.[7,8] It was found that progesterone and deoxycorticosterone (DOC) carry anticonvulsant effect,[9,10] which is mediated by their metabolites, allopregnanolone and tetrahydro-DOC (THDOC). Neurosteriods including allopregnanolone bind to $GABA_A$ receptor to enhance inhibitory effects on brain activity. Allopregnanolone has powerful anticonvulsive effect shown in various rodent seizure models.[11] They may also exert effects on gene expression by intracellular steroid hormone receptors. However, neuroprotective effect of allopregnanolone in relation with ROS-mediated acute neuronal death has not been investigated yet.

In this study, the neuroprotective effect of allopregnanolone treatment in relationship with the expression of superoxide anion and its scavenger enzyme, superoxide dismutase (SOD) in the hippocampus was investigated in a pilocarpine–induced SE mouse model.

II. Materials and methods

1. Pilocarpine-induced status epilepticus model and assessment of seizure

All procedures were approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Adult male C57BL/6 mice (20 to 25 g, Orientbio, Gyeonggi, Korea) were used in this study. Mice were housed under a 12 hours light/dark cycle with food and water ad libitum. Three to 5 animals were used in each experimental group at each time point. Mice were injected intraperitoneally with methyl scopolamine (1 mg/kg, i.p.; Sigma, St. Louis, MO, USA) to reduce peripheral cholinergic effects. After 30 min, mice were given injections of pilocarpine hydrochloride (325 mg/kg, i.p.; Sigma) or the same volume of saline as a control. All animals treated with pilocarpine displayed motor seizures and showed onset of SE within 1 hour. After pilocarpine administration, only mice exhibiting sustained severe SE with generalized tonic and clonic movements were included to this study. Diazepam (10 mg/kg, i.p.; Samjin, Seoul, Korea) was administered at 2 hours after the onset of SE to stop behavioral seizures.

Allopregnanolone (7mg, 12mg/kg, i.p.; Sigma) dissolved in 40% β-cyclodextrine in distilled water. Allopregnanolone was injected immediately after diazepam treatment.

2. Tissue preparation for histological assessment

Animals were anesthetized and transcardially perfused with heparinized saline. Following perfusion, fresh dorsal hippocampus was dissected and used for western blot analysis and activity assay. For histological analysis brains were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) after the perfusion with heparinized saline and then isolated. They were additionally post fixed in the same fixative overnight at 4 ℃ and then sectioned coronally at 16um using a cryostat. For histological assessment of hippocampal pyramidal damages, cresyl violet staining was performed. Sections were immersed in water and stained in 0.2% cresyl fast violet acetate for five minutes. And then the sections were dipped well in absolute alcohol and rinsed with water, they were cleaned and mounted with mounting solution.[12,13]

3. Detection of superoxide radicals *in situ* hydroethidine

To assess the production of ROS after SE, *in situ* detection of oxidized hydroethidine (HEt) was performed at 12 hours after SE onset. A total of 200 μl of HEt (stock solution 100 mg/mL in dimethyl sulfoxide; Molecular Probes) was administrated intravenously an hour before sacrifice. Brains were obtained by the same method with that of the histologic analysis and prepared samples were observed with a microscope and computerized digital camera system under fluorescent light (excitation 510 to 550 nm and emission 580 nm; BX51; Olympus). Intensity (optical density [OD]) in high-magnification field and expression patterns

of the oxidized HEt were analyzed with computerized analysis system and program (Image j; Molecular Devices).

4. Fluorescent labeling for DNA fragmentation

To identify degenerating neuron, we performed TUNEL staining using a kit (Roche Diagnostics GmbH, Penzberg, Germany). Sections were incubated with TUNEL mixture for an hour at 37 ℃ in a dark chamber. After washing, the sections were counter-stained with Hoechst33258 (2.5×10^{-3} mg/ml; Molecular Probes) and examined under a confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA). The number of TUNEL-positive cells in the subfields of the hippocampus was counted.

5. Detection of oxidative damage 8-hydroxy-2' -deoxyguanosine (8-OHdG)

DNA oxidation was stained with a monoclonal antibody against 8-OHdG (1:100; QED Bioscience, San Diego, CA, USA). For 8-OHdG staining, we followed the manufacturer's protocol MOM kit (Vector Labs, Burlingame, CA, USA). Immunoreactivity of 8-OHdG was Visualized by Vectastain ABC-DAB system (Vector Labs, Burlingame, CA, USA).

6. Immunofluorescence staining for superoxide dismutase (SOD)

The Sections were blocked with PBS containing 5% BSA for an hour at room temperature and incubated with the primary antibody, rabbit anti-SOD2 (1:100, cell

signaling, Darmstadt, USA). As a negative control, the sections were incubated without a primary antibody. The Sections were washed with PBS, and reacted with the FITC-conjugate secondary antibody (1:200, Jackson Immuno Research Laboratories, West Grove, PA) for 1 hour at room temperature and the stained sections were observed under LSM 700 confocal laser scanning microscopy (Carl Zeiss,Thornwood, NY, USA). Intensity (optical density [OD]) in high-magnification field and expression patterns of the expression of SOD2 were analyzed with computerized analysis system and program (Image j; Molecular Devices).

7. Western blot analysis

Dissected hippocampal tissues were homogenized in lysis buffer(20 mM Tris–HCl, pH 7.4, at 4 °C; 137 mM NaCl; 25 mM β-glycerophosphate; 2 mM NaPPi; 1 mM Na3VO4; 1% Triton X-100; 10%glycerol; 2 mM benzamidine; 0.5 mM DTT; 1 mM phenylmethylsulfonyl fluoride). Homogenates were boiled with sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, and 0.002% bromphenol blue, pH 6.9) for 5 min. Proteins were resolved on 8% SDS-poly acrylamide gels and blotted onto polyvinylidinedifluoride membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were washed with TBS-T (50 mM Tris/HCl, 140 mM NaCl, pH7.3 containing 0.1% Tween 20) before blocking non-specific binding with TBS-T plus 5% skim milk for 1 hour. The membranes were incubated with the following polyclonal antibodies: rabbit anti-SOD2 (1:140; Cell signaling, Danvers, MA, USA) for 1 hour. After washing, the blots were incubated with secondary antibodies

conjugated with horseradish peroxidase (1:5000 in TBS-T plus 5% Skim milk) for 1 hour, followed by ECL plus (Amersham Biosciences, Piscataway, NJ, USA) detection.

8. Statistical analysis

Data are expressed as mean ± SE. Statistical comparisons between multiple groups were made using ANOVA followed by Tukey's *post hoc* test, and comparisons between two groups were performed using the unpaired student's t-test (SPSS, version 5.01; SAS Institute Inc, Cary, NC, USA). The level of significance was set at $p^* < 0.05$.

III. Result

1. Reduced neuronal damage in the allopregnanolone treated group after SE

Cresyl violet staining was performed to detect neuronal damage in hippocampus (Figure 1). In normal control group, neuronal structures were well preserved in the pyramidal layer of CA1 and CA3 regions. In the vehicle-injected group, pyramidal neurons in CA1 and CA3 regions were damaged and decreased in numbers at one day after SE (data not shown) and more significantly decreased at 3days after SE. In the allopregnanolone-treated group, neuronal cells were well preserved in CA1 and CA3 regions after SE. In the DG region, granule cells were well preserved in both vehicle-injected and allopregnanolone-treated groups after SE.

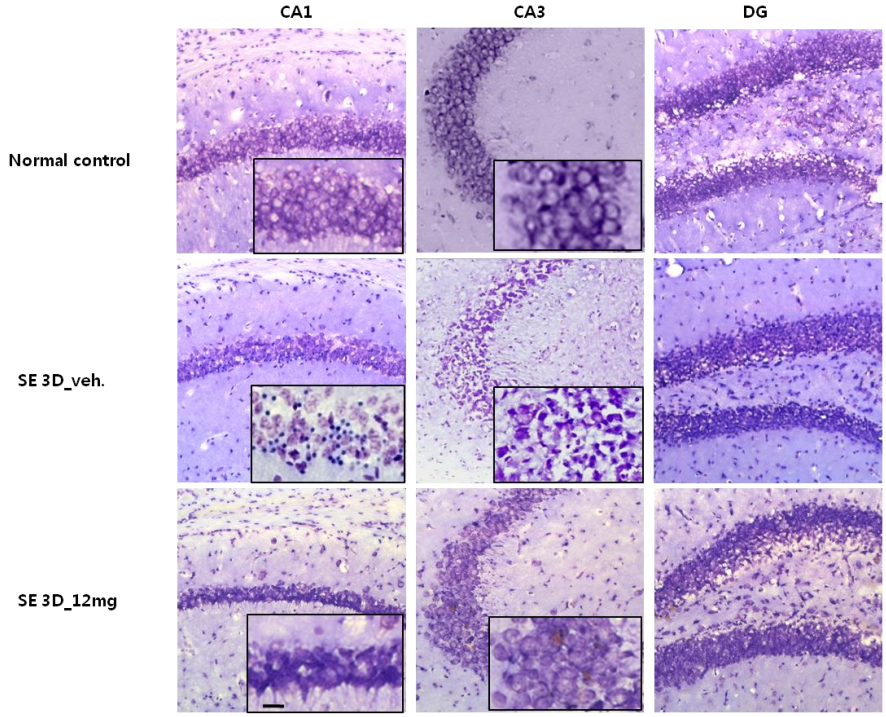
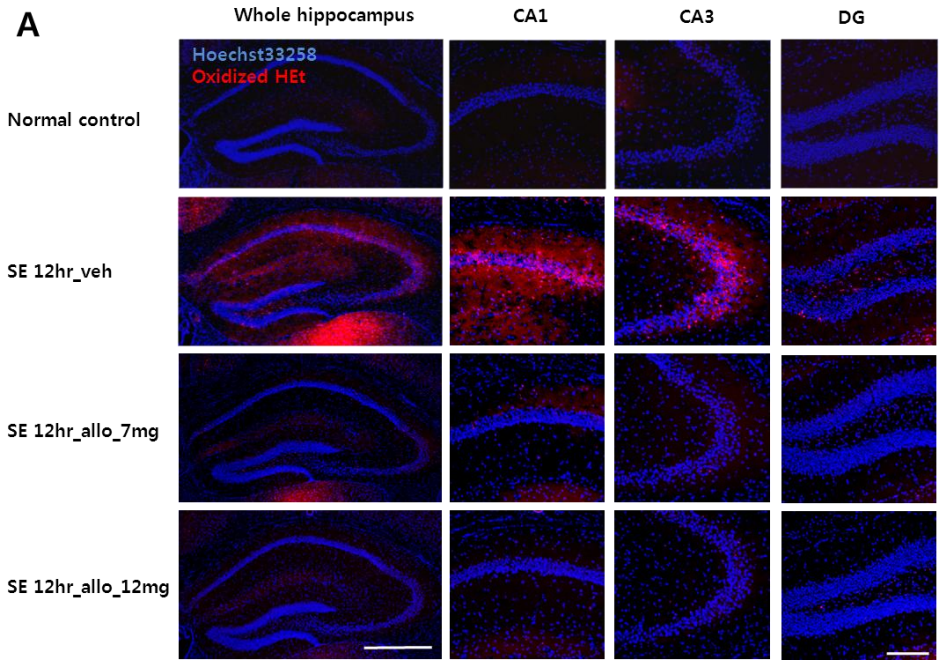


Figure 1. Cresyl violet staining in hippocampus after pilocarpine-induced status epilepticus

In the pyramidal layers of CA1 and CA3, neuronal cell death was prominent at 3 days after SE in the pilocarpine-induced SE group, while they were well preserved in the allopregnanolone-treated group after SE. Scale bar=20 μm.

2. Decreased production of SE – induced ROS by allopregnanolone

To measure the production of ROS after SE, HEt oxidation was observed with a microscope and computerized digital camera system under fluorescent light. In the pilocarpine–induced SE group, oxidized HEt (red) was significantly increased in both CA1 and CA3 regions, while they were much less marked in DG. Allopregnanolone treatment effectively prevented ROS production at those regions, thus the intensity of oxidized HEt was comparable to that of the control group (Figure 2A). The intensity of oxidized HEt was quantitated by using Image J software. In the vehicle–injected group after SE, the intensity of oxidized HEt was significantly higher in both CA1 and CA3 regions compared to that of the control group (Figure 2B), (p<0.001). Only a mild increase of oxidized HEt was detected in the hilus of DG region. In the allopregnanolone-treated group, the intensity of oxidized HEt was significantly decreased compared to that of the vehicle–treated group.


A
Whole hippocampus
CA1
CA3
DG
Hoechst33258
Oxidized HEt
Normal control
SE 12hr_veh
SE 12hr_allo_7mg
SE 12hr_allo_12mg

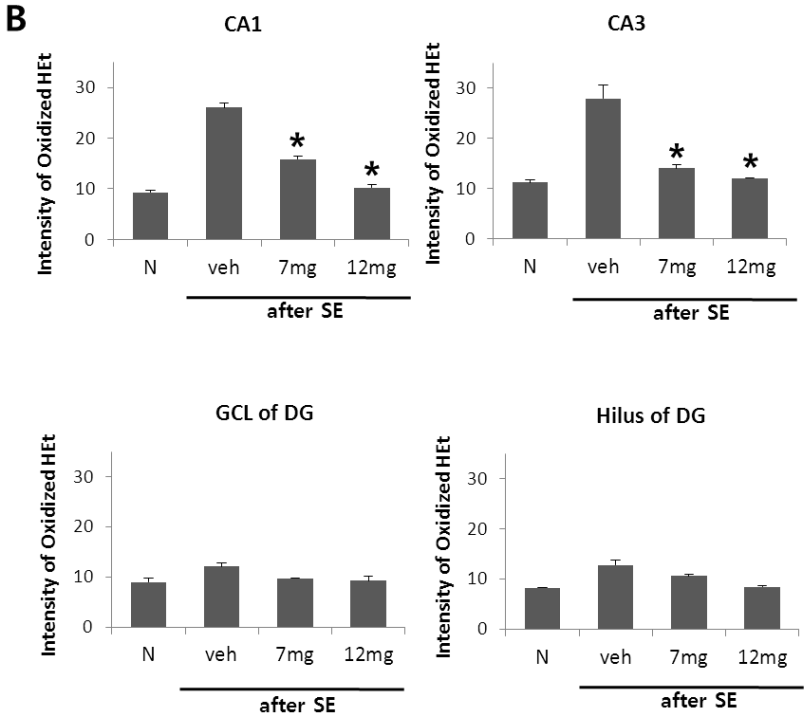
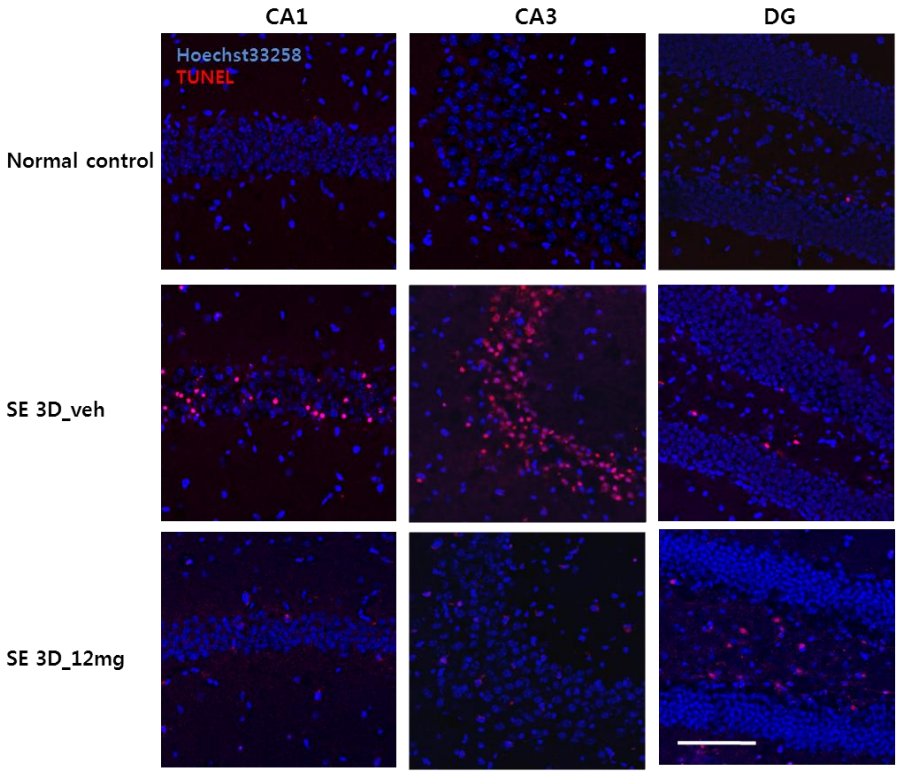

B
CA1
CA3
GCL of DG
Hilus of DG
Intensity of Oxidized HEt
0
10
20
30
N
veh
7mg
12mg
after SE
*

Figure 2. ROS production in hippocampus measured by oxidized HEt at 12 hours after pilocarpine-induced status epilepticus

(A) ROS production of hippocampus. (B) Intensity of ROS production in the hippocampal CA1, CA3 and DG regions at 12 hours after SE. In CA1 and CA3 pyramidal cell layers, ROS production was markedly increased in the vehicle-injected group at 12 hours after SE compared to the control group but not in the DG region. Allopregnanolone-treated group manifested significantly decreased ROS production compare with vehicle-injected group in CA1 and CA3 regions. N, normal control; Veh, vehicle injected after pilocarpine- induced SE; 7 mg and 12 mg, allopregnanolone- treated after SE; GCL, granule cell layer. Scale bar= 500 and 200 μm. *p<0.001 vs. vehicle group; (ANOVA with Tukey′s *post hoc* test).

3. Decreased DNA fragmentation in the allopregnanolone-treated group

Hippocampal neuronal damage after SE was observed by TUNEL staining for DNA fragmentation (Figure 3). For the semi-quantitative measures of DNA damage, the number of TUNEL positive cells was counted. In the control group, TUNEL positive cells (red) were not detected. At 3days after SE, TUNEL positive cells were significantly increased in the CA1 and the CA3 regions, but only a few in the hilus of DG. The number of TUNEL positive cells were markedly decreased and rarely detected in CA1 and CA3 regions at 3 days after SE by treatment with allopregnanolone.


CA1
CA3
DG
Hoechst33258
TUNEL
Normal control
SE 3D_veh
SE 3D_12mg

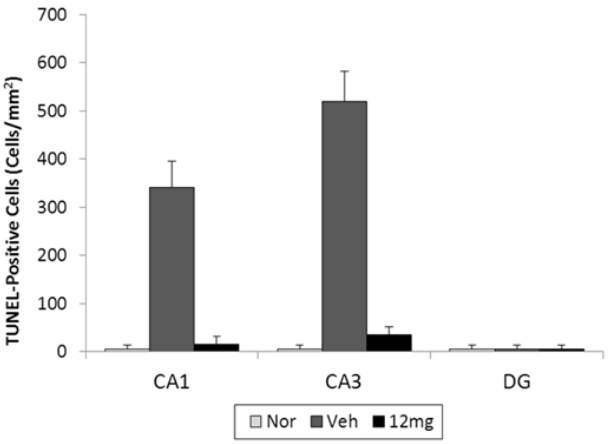
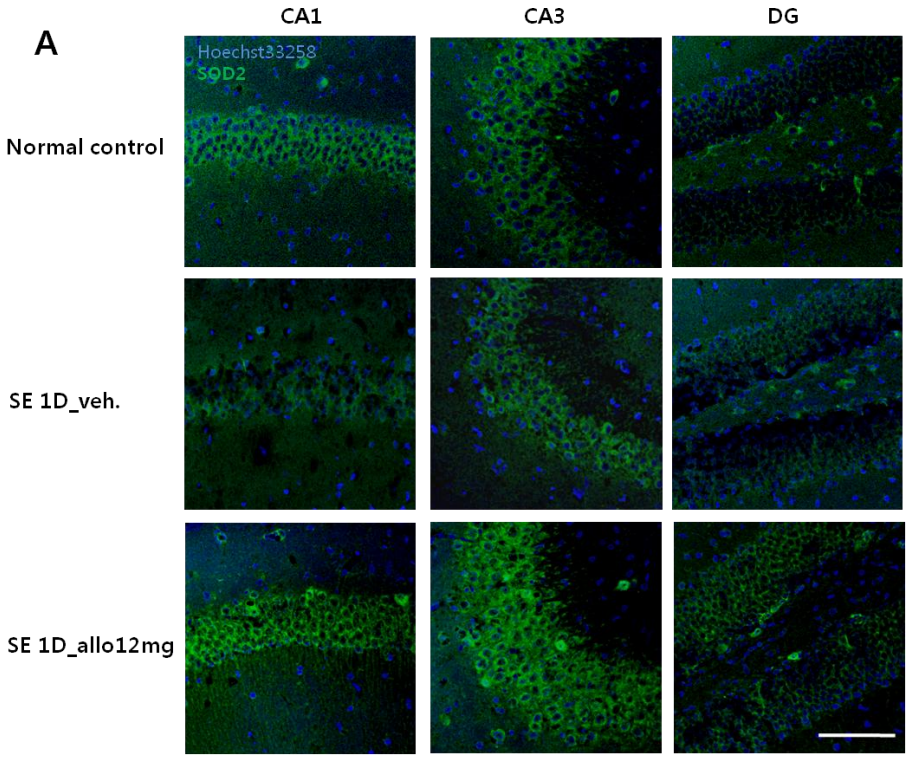

TUNEL-Positive Cells (Cells/mm²)
700
600
500
400
300
200
100
0
CA1
CA3
DG
Nor
Veh
12mg

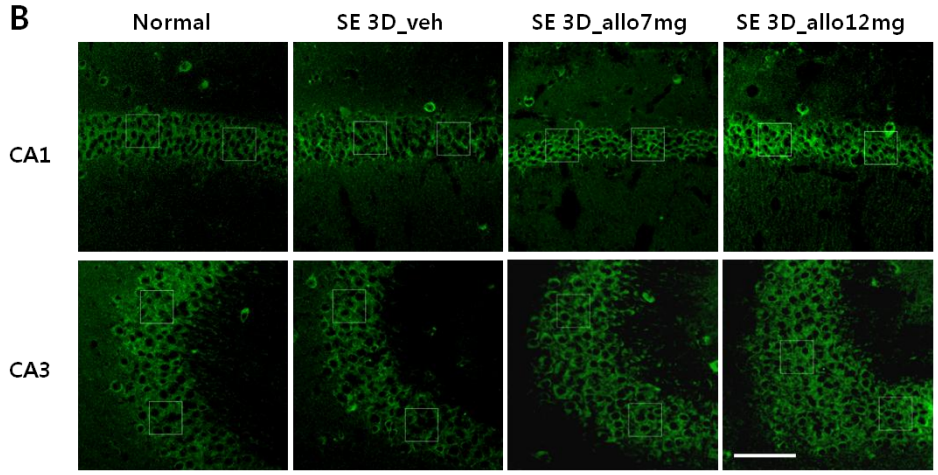
Figure 3. TUNEL staining (cell death marker) of hippocampal subfields in allopregnanolone-treated group after SE

At 3 days after SE, TUNEL positive-cells (red) were markedly increased in the CA1 and the CA3 hippocampal regions compared with the control group. The allopregnanolone-treated group showed significant reduction of TUNEL-positive cells compared to the vehicle–injected group. Blue, Hoechst; Red, TUNEL; Scale bar=50 μm; Nor, normal control; Veh, vehicle injected after pilocarpine-induced SE; 12 mg, allopregnanolone-treated after SE.

4. Increased Expression of SOD2 in the allopregnanolone-treated group by immunofluoresence staining

Immunohistostaining was performed to evaluate the expression of SOD2, an important free radical scavenger enzyme in brain. SOD2 is expressed in the mitochondrial inner space, which is observed as a cytosolic pattern (Figure 4A). In the DG region, SOD2 expression did not show any differences among the control, the vehicle-treated, and the allopregnanolone-treated groups. However, SOD2 positive cell were increased significantly in the allopregnanolone-treated group in a dose dependent manner. The fluorescent intensity of SOD2 expression was measured by using image J software (Figure 4B), which has shown markedly increased fluorescent intensity of SOD2 expression in both CA1 and CA3 regions in the allopregnanolone 12mg -treated group compared with the vehicle-treated group (Figure 4C), which was statistically significant (p=0.007 and p=0.028, respectively).


A
CA1
CA3
DG
Hoechst33258
SOD2
Normal control
SE 1D_veh.
SE 1D_allo12mg


B
Normal
SE 3D_veh
SE 3D_allo7mg
SE 3D_allo12mg
CA1
CA3

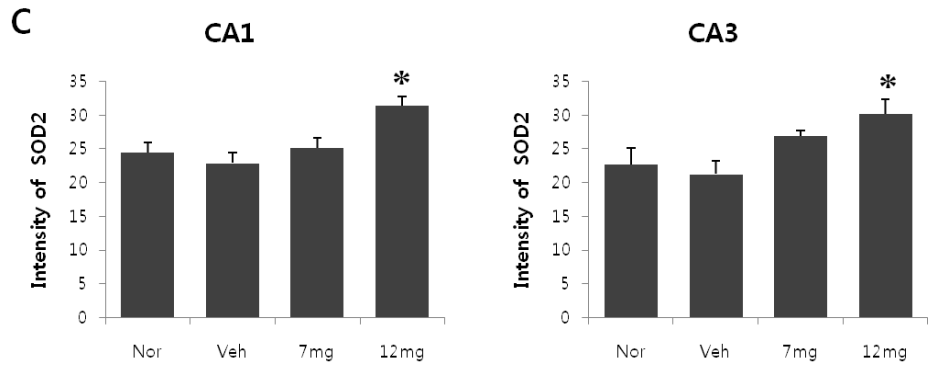


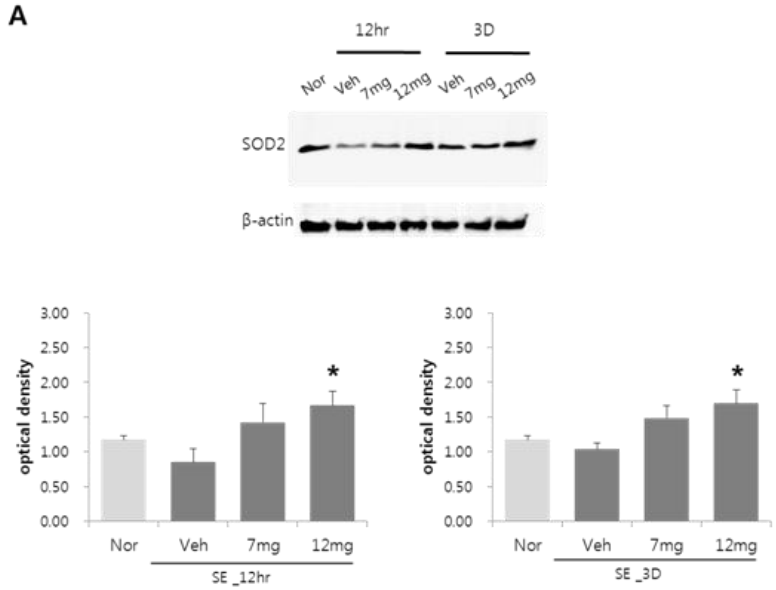
Figure 4. Immunohistochemical staining of superoxide dismutase2 (Mn SOD) in the hippocampal subfields

(A) the double- staining for SOD2 expression (green) and nucleus (blue) in the hippocampal subfields, CA1, CA3 and DG, at 1 day after SE. (B) The SOD2 expression of hippocampal neurons in the CA1 and the CA3 regions at 3 days after SE. (C) The intensity of SOD2 expression in the CA1 and the CA3 regions at 3 days after SE. Allopregnanolone-treated group shows increased SOD2 expression in a dose dependent manner at the hippocampal CA1 and CA3.

Blue, Hoechst; Green, SOD2; scale bar=20 μm; Nor, normal control; Veh, vehicle injected after pilocarpine-induced SE; 7 mg and 12 mg, allopregnanolone-treated after SE. *P<0.05 vs. vehicle group; (ANOVA with Tukey′s *post hoc* test).

5. Measurement of SOD2 expression in the allopregnanolone-treated group by western blot analysis

For quantitative assessment of SOD2 expression, western blot analysis was performed for hippocampal proteins. There was no significant change of SOD2 expression in both whole fraction and subfield fractions after SE, which was comparable to that of the control group. However, in the allopregnanolone-treated group, SOD2 expression was increased in the whole fraction at 1 and 3 days after SE (Figure 5A), which has reached to the significant level in the group subjected to 12mg of allopregnanolone (p=0.032 and p=0.012). Measurement of SOD2 expression in each subfields of hippocampus revealed significant increases in both CA1 and CA3 regions, however, its relationship with the dose of allopregnanolone was present only in the CA3 region (Figure 5B), (p=0.025). In CA1, the SOD2 expression was increased after the administration of allopregnanolone that reached to the statistical significance only at 7mg of allopregnanolone (p=0.044).


A
12hr
3D
Nor
Veh
7mg
12mg
Veh
7mg
12mg
SOD2
β-actin
optical density
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
Nor
Veh
7mg
12mg
SE _12hr
optical density
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
Nor
Veh
7mg
12mg
SE _3D

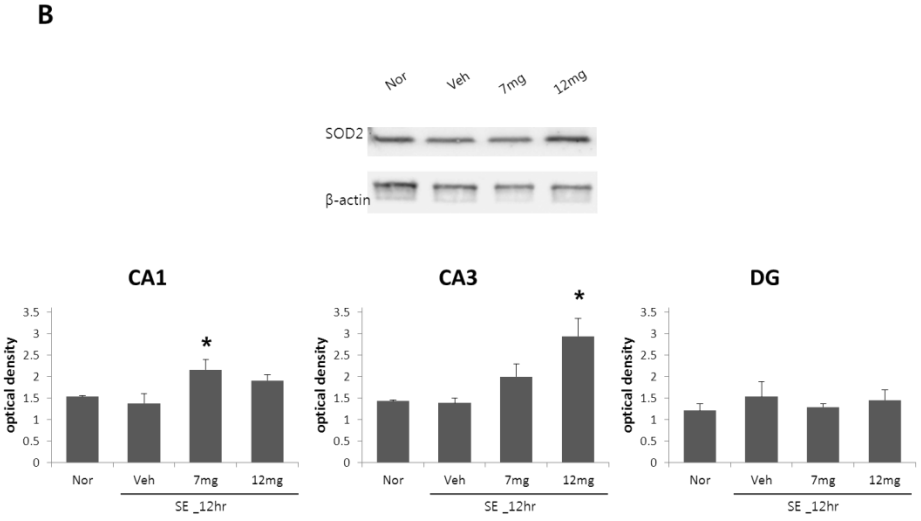

B
Nor
Veh
7mg
12mg
SOD2
β-actin
CA1
optical density
3.5
3
2.5
2
1.5
1
0.5
0
*
Nor
Veh
7mg
12mg
SE _12hr
CA3
optical density
3.5
3
2.5
2
1.5
1
0.5
0
*
Nor
Veh
7mg
12mg
SE _12hr
DG
optical density
3.5
3
2.5
2
1.5
1
0.5
0
Nor
Veh
7mg
12mg
SE _12hr

Figure 5. The hippocampal SOD2 expression in normal, pilocarpine- induced SE and treatment of allopregnanolone-treated group after SE

Western blot analysis showed increased SOD2 expression in allopregnanolone-treated group after SE. (A) The expression of SOD2 in whole hippocampal tissue. (B) The expression of SOD2 in hippocampal subfields at 12 hours after SE.

Nor, normal control; Veh, pilocarpine- induced SE; 7mg and 12mg, allopregnanolone-treated group after SE. *p<0.05 vs. vehicle group; (ANOVA with Tukey′s *post hoc* test).

6. Decrease of SE-induced oxidative DNA damage by allopregnanolone

To confirm the oxidative DNA damage, we examined the immunohistostaining of 8-OHdG, an oxidative DNA marker, in hippocampus CA1 and CA3 regions. In normal control, 8-OHdG positive cells were not detected. In the vehicle-treated SE group, 8-OHdG positive neurons were increased at 1 day after SE and further increased at day 3 (Figure 6). In the allopregnanolone-treated group after SE, 8-OHdG positive neurons were present but much less abundant than the vehicle-treated group in the CA1 and CA3 regions. 8-OHdG staining in DG did not show any significant differences from the control group after SE.

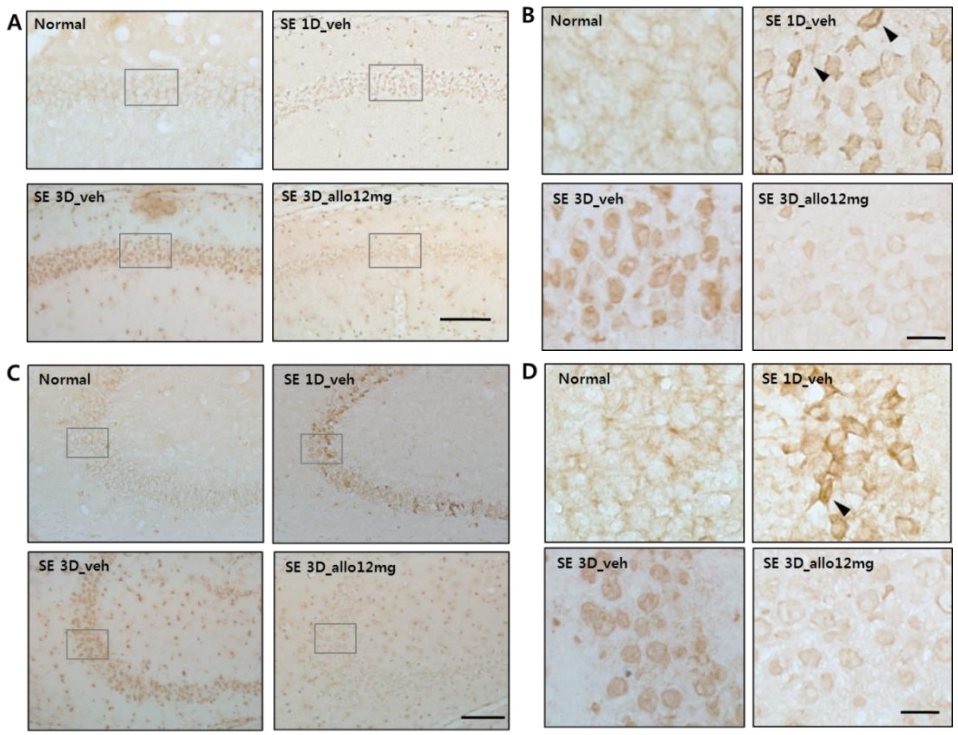


Figure 6. Detection of oxidative DNA damage using immunohistostaining of 8-OHdG in hippocampus CA1 and CA3 regions

(A) 8-OHdG staining of hippocampal CA1 region. (B) The magnification of box in (A). (C) 8-OHdG staining of hippocampal CA3 region. (D) The magnification of box in (C). The 8-OHdG was very faint in normal control group, which was markedly increased at 3 days after SE in the vehicle-treated group. The allopregnanolone-treated group showed much less 8-OHdG -positive cells than the vehicle-treated group at day 3 after SE. Scale bar=100 μm and 20 μm.

IV. Discussion

Pilocarpine-induced SE precipitated an acute neuronal damage in the CA1 and CA3 regions of hippocampus, which were apparent at day 1 with further progression at day 3 after SE. However, in this SE model, the DG escaped from any significant neuronal damage, which might suggest the involvement of different protective mechanisms in different subfields of hippocampus. Previous investigations demonstrated that the excessive production of ROS was responsible for the acute neuronal death in various cerebral insult models including SE models.[14,15] ROS represents agents indicating oxidative stresses, such as superoxide, hydroxyl radical, nitric oxide, nitrite, nitrate and H_2O_2. The relationship between SE and ROS has been well established in SE-models by previous studies.[14,16] Excessive epileptiform discharges cause excessive ROS productions, which is, in turn, responsible for the subsequent neuronal death and following epileptogenesis. Hippocampus is one of the most vulnerable cerebral regions to oxidative stresses.[4] Especially, CA1 and CA3 subfields of hippocampus appear particularly vulnerable, whereas dentate gyrus (DG) granule cells are resistant to seizure-induced cell loss.[5,6] ROS may also affect excitatory neurotransmission system by increasing glutamate release initiating excitotoxicity consisting of denaturation of a variety of lipids and proteins and DNA-damage leading to neuronal death.[17]

In this study, we found increased ROS production in the areas of severe neuronal damage (CA1 and CA3) but not in the DG, which was a nice correlation to support the hypothesis of ROS being the primary mechanism of neuronal damage in the

pilocarpine-induced SE model. The elevation of ROS production preceded the neuronal death as it was significantly elevated at 12 hours after SE, when gross neuronal damages were still not apparent. Elevation of ROS production also nicely correlated with the degree of neuronal damage as well as the severity of DNA damage by ROS, thus supporting its pivotal role in neuronal injury mechanisms precipitated by pilocarpine-induced SE.

We found that allopregnanolone, a representative neurosteroid in mammals, carries a significant neuroprotective effect in this SE-model. The acute neuronal damage by SE in CA1 and CA3 was successfully prevented by the administration of allopregnanolone at the completion of SE, thus its protective effect might be mediated through a modulation of neuronal death mechanisms rather than its direct effect on SE. Intravenous administration of allopregnanolone was associated with a significant reduction of ROS expression compared to the vehicle-treated animal, which was visualized by Het oxidation. The number of TUNEL-positive cells, an early cell death marker, was significantly increased in CA1 and CA3 subfields, but not in the region of DG at 3 days after SE. These selective vulnerability of CA1 and CA3 subfields compared to the DG of hippocampus had been reported in other studies.[12,18] To further evaluate the oxidative neuronal damages precipitated by excessive ROS production, we performed 8-OHdG staining, a marker of oxidative DNA damage leading to oxidative stress-induced neuronal death. As expected, 8-OHdG positive neurons were markedly increased in both CA1 and CA3 subfields but not in the DG, which was again effectively prevented by the allopregnanolone

treatment. These excellent correlations among ROS overproduction, TUNEL-positive cells and 8-OHdG-positive cells in their spatial distribution as well as their effective prevention by allopregnanolone treatment has provided a strong support for the link between the allopregnanolone-mediated neuronal protection and the ROS-mediated neuronal damage.

To further identify the impact of allopregnanolone on ROS system, we investigated the expression of SOD2 expression in these subfields, an important scavenging enzyme of ROS in brain. SOD is an important antioxidant defense system consisting of three types of isoenzymes being encoded by three different genes. The copper/zinc SOD (cytoplasmic SOD or SOD1) is found in the cytosol, whereas manganese SOD (mitochondrial SOD or SOD2) is located in the mitochondrial matrix. The other type of SOD, being called extracellular SOD (SOD3), is expressed at low level in plasma and extracellular fluids.[19] These three forms of SOD catalyze the dismutation of superoxide anion to hydrogen peroxide, thereby reducing the risk of hydroxyl radical formation.[20] Previous studies clearly demonstrated the protective effect of SOD in various models of acute brain insults. Mice with overexpressed SOD2 were resistant to the kainite–induced hippocampal damage[21] and SOD1 played a significant protective role against focal and global cerebral ischemia. [22,23] In this study, SE did not alter the SOD expression in various subfields of hippocampus, which was similar to that found in the control group. However, SOD2 expression was found significantly increased by allopregnanolone-treatment in the CA1 and CA3 subfields, but not in the DG. In Western blot analysis,

SOD2 in hippocampus was increased at both 12 hours and 3 days after SE, which has reached to the significant level in allopregnanolone 12mg–treated group. In the quantitative analysis of SOD expression in each subfields of hippocampus, its elevation reached to the significant level in both CA1 and CA3 subregions, but not different from the control group in the DG subfield. The increase of SOD2 expression related to the dose of allopregnanolone was found for the measurement of whole hippocampus and the CA3 region, but not for the CA1 region, which requires further confirmation in future investigations. The assessment of dose-dependent increase of SOD2 expression in hippocampus may require a dose-ranging study using more variable doses of allopregnanolone in a wide range.

Neurosteroids rapidly alter neuronal excitability through their direct interactions with $GABA_A$ receptors[24-28], which are the major inhibitory neurotransmitter system in brain. Allopregnanolone (3α-hydroxy-5α-pregnan-20-one) is metabolized from progesterone by the enzyme called reductase[29] and has been found to exert powerful and broad-spectrum anticonvulsant effects being useful in clinical practice, especially for patients with catamenial epilepsy. Previous studies reported that allopregnanolone and related neurosteriods bind to $GABA_A$ receptors to potentiate its inhibitory functions in brain.[11] In this study, we did not investigate the relationship between $GABA_A$ receptors and ROS overproduction in a systemic way, however all animals were given diazepam, a potent $GABA_A$ receptor agonist, to terminate on-going SE, which did not seem to prevent the ROS overproduction. In addition, it has been found that a prolonged SE causes $GABA_A$ receptor trafficking

to decrease their presence in the synaptic membrane to make any significant impact of $GABA_A$-receptor mediated neuroprotective mechanisms less likely. However, it may be worthwhile to consider potential links between $GABA_A$ receptor potentiation and ROS production in the pilocarpine-induced SE model in future investigations.

In conclusion, this study identified that ROS overproduction plays an important role for the extensive neuronal death in CA1 and CA3 hippocampal subfields after pilocarpine-induced SE, which was effectively prevented by the administration of allopregnanolone at the time of SE-termination. Prevention of ROS overexpression in the CA1 and CA3 by allopregnanolone was linked to the overexpression of SOD2, a potent ROS scavenging enzyme, in these subfields showing selective vulnerability to SE.

V. Conclusion

This study demonstrated that excessive ROS production is the key player for the hippocampal neuronal damages found in the pilocarpine-induced SE mouse model. Reduction of neurons, increase of oxidative DNA-damage and DNA fragmentation by TUNEL staining were obvious in CA1 and CA3 at 3 days after SE, but not in DG, a well-known phenomenon of selective vulnerability of hippocampal subregions. The ROS related neuronal damages in CA1 and CA3 were successfully prevented by the administration of allopregnanolone, which was associated with marked increase of SOD2 expression in these regions, which were not found in the vehicle-treated group.

In the allopregnanolone-treated group, production of ROS, oxidative DNA damage and TUNEL positive cells were comparable to that of the normal control group, but were significantly decreased compared to that of the vehicle-treated SE group. The successful prevention of ROS -related neuronal damages in CA1 and CA3 by the administration of allopregnanolone was associated with markedly increased expression of SOD2 in these regions, which were not found in the vehicle-treated group. The increased SOD2 expression was most pronounced in CA3, which has reached to the significant level with a pattern of dose-dependent increase in a quantitative measurement by using western blot and immunohistostaining. Our results suggested that the neuroprotective effect of allopregnanolone in a pilocarpine-induced SE model is primarily related to the restoration of a balance in the ROS system in hippocampal subregions of selective neuronal vulnerability, the

compensation of ROS overproduction from SE by an increase of SOD2 expression after treatment of allopregnanolone. Although our study did not provide any clues for the mechanism of SOD2 expression and its modulation by allopregnanolone, it has provided evidence for the link between ROS system and neurosteroid in the mechanism of acute neural damage in a pilocarpine-induced SE model

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ABSTRACT (IN KOREAN)

pilocarpine으로 유도된 SE쥐에서, neurosteroids 투여 후 나타나는 항산화 효과 및 세포보호 효과

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조인자

간질 중첩증(status epilepticus: SE)은 여러 원인에 의해 간질발작이 정상 억제기전을 벗어나 지속적으로 간질 발작이 일어나는 상태를 말한다. 이로 인해 흥분독성, 활성산소종(ROS), 미토콘드리아 장애, 염증 등 여러 기전을 통해 뇌세포 사멸이 일어나게 되고, 과도하게 발생한 활성산소종은 간질발생기전에 있어서 뇌세포 손상 및 사멸에 중요한 기전 중 하나로 인식되고 있다. 이러한 ROS등에 의한 산화적 스트레스에 대응해 신체는 Superoxide dismutase(SOD), glutathione(GSH)와 같은 방어기전을 통해 세포를 보호하게 된다. 연구에 따르면 해마 내 소구역 CA3,

CA1는 뇌세포사멸이 두드러짐에 반해 CA2나 DG는 상대적으로 손상에 대한 저항이 강하다. 이러한 기전에 대한 원인은 여전히 많이 알려져 있지 않다.

Neurosteroids는 뇌에서 생성되어 신경조직에서 활성을 갖는 스테로이드 호르몬이다. 또한 발작감수성을 조절하며 최근 항경련 효과(anticonvulsant effects)도 보고 되고 있으나, 간질병소생성(epileptogenesis) 조절에서 neurosteroid의 정확한 역할은 아직 알려진 바가 없다. Allopregnanolone은 Neurosteroids 중 하나로 이에 대한 강력한 항경련효과가 보고되고 있다. 이는 $GABA_A$ 수용체에 결합 함으로써 항경련효과를 나타내는 것으로 보여지며, 이러한 $GABA_A$ 조절을 통한 메커니즘 외에는 아직 알려진 바가 많지 않다.

본 연구에서는 수컷 C57BL/6 에 pilocarpine을 투여함으로써 SE를 유도시킨 쥐 모델을 이용하였으며, 해마의 구역에 따라 활성산소종의 측정과, 8-OHdG 면역 염색을 이용, 산화적 DNA 손상을 통해 일어나는 세포사멸을 관찰했다. 또한 활성산소종의 방어물질 중 하나인 SOD의 발현양을 정성적, 정량적으로 관찰 하였다. 또한 neurosteroids의 대사물질 중 하나인 allopregnanolone의 투여로 산화적 스트레스가 감소하고 이에 따른 세포사멸이 감소되는지 확인 하였다. 또한 SE와의 SOD 발현양의 차이를 관찰 하였다.

본 연구를 통해, SE 모델에서 활성산소종의 과도한 발생을 확인 할 수

있었으며,

이에 따른 산화적 DNA 손상을 통한 세포사멸이 일어나는 것을 관찰 할 수 있었다. 또한 allopregnanolone에 의해 SE에 의해 과도하게 발생 된 활성 산소가 감소하는 것을 확인 할 수 있었으며, 그에 따라 신경세포보호 효과를 관찰하였다. 또한 allopregnanolone의 투여 후에 SOD의 발현 증가를 확인 함으로써, allopregnanolone이 항산화 보호체계의 증가를 유도시킴으로써 신경세포보호 효과를 나타냄을 확인 하였다.

결론적으로 이번 연구를 통해 allopregnanolone이 항산화 보호체계의 조절로 인해 신경세포보호효과를 가지는 것을 확인 하였다.

핵심되는 말: 산화스트레스, 활성산소종, SOD, 간질중첩증, neurosteroid, allopregnanolone