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Effects of Valproic acid and Resveratrol on Salicylate Induced Excitotoxicity in Neuronal Cells

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신경세포에서 살리실산으로 유도된 흥분독성에 대한 발프로산과 레스베라트롤의 효과

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이 논문을 이학석사학위 신청 논문으로 제출함

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ABBREVIATIONS

ARC	Activity-regulated cytoskeleton
CMAKII	Ca^{2+}/calmodulin-dependent protein kinase
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
DCF	2'-7'dichlorofluorescin
DCF-DA	2'-7'dichlorofluorescin diacetate
DMEM	Dulbecco's modified eagle's medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
OHC	Out hair cell
PBS	Phosphate-buggered saline
PCR	Polymerase chain reaction
VPA	Valproic acid
RSV	Resveratrol
NR2B	*N*-methyl D-aspartate receptor subtype 2B
ROS	Reactive oxygen species

FBS	Fetal Bovine Serum
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
SDS	Sodium Dodecyl Sulfate
SIRT1	Sirtuin1
TNF	Tumor necrosis factor

ABSTRACT

Effects of Valproic acid and Resveratrol on Salicylate Induced Excitotoxicity in Neuronal Cells

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Tinnitus is a subjective feeling of noise in the ear without any external source. Acute tinnitus can be caused by increasing the activation of *N*-methyl D-aspartate (NMDA) receptor, induce ototoxicity in the cochlea. Previous studies have shown that high doses of salicylate activate NMDA receptors, as a result increasing the intracellular Ca^{2+} influx and induce glutamate excitotoxicity in neurons. Glutamate excitotoxicity is closely associated with failure in the maintenance of calcium homeostasis, mitochondrial dysfunction, and accumulation of reactive oxygen species (ROS). In this study, I have established an in vitro model for salicylate-induced tinnitus in neuronally differentiated SH-SY5Y cells and investigated the effect of valproic acid (VPA) and resveratrol (RSV) on that model.

In the Part I, I examined whether the VPA can modulate glutamate excitotoxicity in salicylate-induced tinnitus model in vitro. VPA has been widely used as a drug for bipolar disorder, epilepsy and migraine headaches. VPA is known to regulate the activation of

NMDA receptor via arachidonic acid in rat. To examine the effect of VPA in salicylate induced glutamate excitotoxicity, differentiated SH-SY5Y cells were pre-treated with VPA and exposed to salicylate. The expression level of *N*-methyl D-aspartate receptor subtype 2B (NR2B), phosphorylated cAMP response element-binding protein (p-CREB), apoptosis markers and ROS level were measured by several biochemical techniques. I observed that the expression level of NR2B, *TNFα* and *ARC* were increased in salicylate-treated cells. Also, salicylate treatment up-regulated intracellular ROS and cleaved caspase-3 in neuronal differentiated SH-SY5Y cells. Furthermore, these effects were reversed in VPA pretreated neuronal cells. These results were also confirmed in the rat cortical neuron. Therefore, VPA pretreatment prevent the activation of NMDA receptor and protect the neuronal cells from the glutamate excitotoxicity.

In the Part II, the protective effects of resveratrol (RSV) were investigated on the salicylate-induced glutamate excitotoxicity in neuronal cells. RSV is a polyphenol compound known as a SIRT1 activator. RSV can minimize intracellular damages and activates the cell repairing system to prevent aging. It can also promote the activity of neurotrophic factors. The expression of NR2B and p-CREB were increased in salicylate-treated cells, whereas it was decreased in RSV-pretreated group. Similarly, production of intracellular ROS and expression level of p53 and cleaved caspase-3 were increased in salicylate-treated cells whereas these changes were reversed in RSV pre-treated. These results suggest that RSV pretreatment protect the cells from excitotoxicity by reducing oxidative stress.

In conclusion, I confirmed that VPA and RSV pretreatment protect the neuronal cells from salicylate-induced excitotoxicity by preventing excess stimulation of NMDA

receptor. Therefore, I suggested that VPA and RSV could be an effective for tinnitus induced by excitotoxicity.

국문초록

신경세포에서 살리실산으로 유도된 흥분독성에 대한 발프로산과 레스베라트롤의 효과

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이명은 근원 없는 소리가 귀에 들리는 주관적인 느낌이다. 급성 이명은 NMDA 수용체의 활성을 증가시킴으로써 발생할 수 있으며 달팽이관에서 이독성을 유발한다. 이전 연구에서는 고용량의 살리실산이 NMDA 수용체를 활성화하여 세포내 Ca^{2+} 유입을 증가시키고 뉴런에서 글루타메이트 흥분독성을 유도하는 것으로 밝혀졌다. 글루타메이트 흥분독성은 칼슘 항상성 유지 실패, 미토콘드리아 기능장애 및 활성 산소종 (ROS) 축적과 밀접한 관련이 있다. 이 연구에서는 나는 신경으로 분화된 SH-SY5Y 세포에서 살리실산로 유도된 이명에 대한 체외 모델을 확립하고 그 모델에 대한 발프로산 (VPA)과 레스베라트롤 (RSV)의 효과를 조사하였다.

제1부에서는 발프로산이 체외에서 살리실산 유도 이명 모델에서 글루타메이트 흥분독성을 조절할 수 있는지 조사했다. 발프로산은 양극성 장애, 간질

및 편두통 치료제로 널리 이용되어 왔다. 발프로산은 쥐에서 아라키돈산을 통해 NMDA 수용체의 활성화를 조절하는 것으로 알려져 있다. 살리실산으로 유도된 글루타메이트 흥분독성에서 발프로산의 효과를 조사하기 위해 분화된 SH-SY5Y 세포를 발프로산 전처리 후 살리실산에 노출시켰다. NMDA 수용체 서브 유닛인 NR2B, 인산화된 cAMP 반응 요소 결합 단백질 (p-CREB), 아포토시스 마커들 및 ROS의 발현 수준이 여러 생화학적 기술로 측정했다. 살리실산이 처리된 세포에서 NR2B 및 관련 유전자 *TNFα*, *ARC*의 발현이 증가하는 것을 관찰했다. 또한, 살리실산 처리는 신경으로 분화된 SH-SY5Y에서 세포 내 ROS 및 절단된 카스파제-3를 증가시켰다. 추가적으로 이러한 효과는 쥐의 피질 뉴런에서도 확인되었다. 따라서 발프로산 전처리는 NMDA 수용체의 활성화를 방지하고 글루타메이트 흥분독성으로부터 신경세포를 보호한다.

제2부에서는 레스베라트롤(RSV)의 보호효과가 신경세포에서 살리실산 유도 글루타메이트 흥분독성에 대해 조사했다. 레스베라트롤은 SIRT1 활성화제로 알려진 풀리페놀 화합물이다. 레스베라트롤은 세포 내 손상을 최소화하고 세포 복구 시스템을 활성화하여 노화를 방지한다. 또한 신경 영양 인자의 활성을 촉진할 수 있다. NR2B 및 p-CREB의 발현은 살리실산이 처리된 세포에서 증가한 반면, 레스베라트롤이 처리된 그룹에서 감소하였다. 유사하게, 세포 내 ROS의 생성과 p53 및 절단된 카스파제-3의 발현 수준은 살리실산이 처리된 세포에서 증가된 반면, 레스베라트롤이 전처리된 세포에서 역전되었다. 이러한 결과는 레스베라트롤 전처리가 산화적 스트레스를 감소시킴으로써 흥분독성으로부터 세포를 보호함을 시사한다.

결론적으로, 나는 발프로산과 레스베라트롤 전처리가 NMDA 수용체의 과도한 활성화를 방지함으로써 살리실산으로 유발된 흥분독성으로부터 신경세포를 보호한다는 것을 관찰했다. 따라서 발프로산과 레스베라트롤이 흥분독성으로 인한 이명에 효과적일 것이라고 제안한다.

I. INTRODUCTION

Patients suffering from tinnitus hear a phantom noise without any external sound source, and gradually associated with hearing loss. Not all tinnitus patients need a treatment, and most often get used to the phantom sound (Tang, et al. 2019). However, if the symptoms get worse, it can induce anxiety, emotional disturbances, sleep disturbances, work disorders, and many other symptoms (Langguth, et al. 2013). Tinnitus is caused by damages in cochlea, auditory nerve, or central auditory pathway at all levels (Yi, et al. 2016). The causes of damage to the auditory nerve include shock, inflammation, aging, drug abuse and external loud noise (Wan, et al. 2015).

Drug abuse, such as antibiotics, anticancer, and salicylate, are deadly causes of tinnitus. It has been reported that antibiotics like aminoglycoside give oxidative damage to the hair cells by increasing the reactive oxygen species (ROS) (Chang, et al. 2016). Another antibiotics quinine is known to cause tinnitus by interfering the K^+ channel in outer hair cells (OHC) (Alvan, et al. 2017).

Salicylate is commonly used as a drug for mild pain, anti-inflammation and fever in therapeutic doses (Ratchford, et al. 2017). It is also used mainly for rheumatoid arthritis (Zhang, et al. 2018; Iacono, et al. 2019). However, it has been known to cause temporary hearing loss and tinnitus when high doses of salicylate were administered (Sheppard, et al. 2014). Salicylate as a therapeutic agent inhibits cyclooxygenase-2 (COX-2), which involves in the production of prostaglandins that cause pain, fever and inflammation (Poorani, et al. 2016). Meanwhile, sustained inhibition of COX-2 accumulates arachidonic acid and it causes activation of *N*-methyl D-aspartate (NMDA) receptor (Ruel, et al. 2008). The arachidonic acid in plasma membrane causes excessive activation of

NMDA receptor by bending the synaptic membrane. High dosage of salicylate stimulates NMDA receptors and increases intracellular Ca^{2+} influx, which causes excitotoxicity within the cell (Qin, et al. 2019). Previous studies have suggested that overactivation of NMDA receptor causes not only glutamate excitotoxicity but also ototoxicity in the cochlea (Hong, et al. 2018). Ca^{2+} inflow by the activated NMDA receptor caused mitochondrial dysfunction and increased intracellular ROS level. One of the damaging results of excess calcium in the cytosol is initiating apoptosis through cleaved caspase processing (Dutta and Trapp 2011). NMDA receptor mediate excitotoxicity and apoptosis in neuron lead to tinnitus (Fig. 1).

Valproic acid (VPA) is a well-known drug used for bipolar disorder, epilepsy and migraine headaches (Nanau and Neuman 2013). It has been reported that VPA regulates the conversion of arachidonic acid through COX in the rat brain (Bosetti, et al. 2003). VPA also modulates the activity of NMDA receptor by down-regulating the expression of two NMDA receptor-interacting proteins, postsynaptic density protein PSD-95 and CaMKII (Bosetti, et al. 2005; Basselin, et al. 2008). Thus, VPA gently relieves the spontaneous excitatory postsynaptic potentials by controlling the NMDA receptors. Also, it can protect the cells by reducing oxidative stress caused by glutamate induced excitotoxicity (Terzioğlu Bebitoğlu, et al. 2020).

Resveratrol (RSV) is a polyphenol substance contained in most berries. It is known to have various consequences such as antioxidant, anti-aging and anti-inflammatory effects (Xia, et al. 2017). Prior studies have shown that RSV can induce neuroprotective effects by reducing oxidative damage and chronic inflammation. It also has the ability to promote neurotrophic factors (Zhang, et al. 2015). RSV regulates the unnecessary activation of glutamate and choline receptors by controlling SIRT1 expression in vivo, which plays a

role in neuroprotective effect (Karthick, et al. 2016).

In this study, I investigated that salicylate induces glutamate excitotoxicity in neuronal cells. I observed that salicylate treatment increased the neuronal cell death by activating NMDA receptor and apoptosis signaling. However, VPA and RSV pretreatments were effectively prevented neuronal cells against salicylate-induced excitotoxicity.

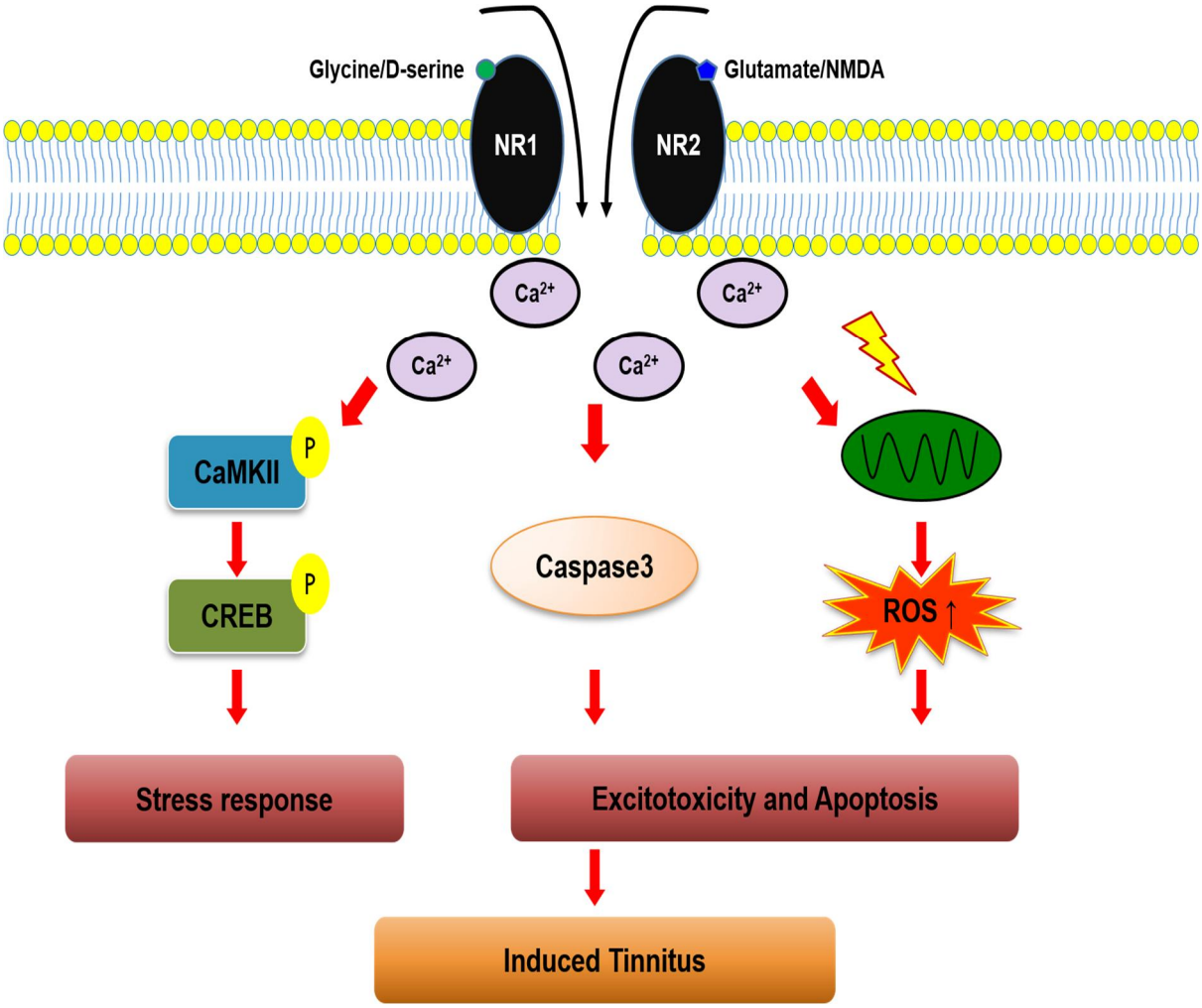


Fig. 1. Molecular pathway of tinnitus induced by glutamate excitotoxicity.

Excessive activation of the NMDA receptor increases intracellular calcium influx. Increased calcium influx causes intracellular damage. In response to these stresses, CaMKII/CREB signaling is phosphorylated. In addition, caspase3 activation and up-regulated ROS level induce apoptosis and excitotoxicity in neuronal cells. Finally, excessive excitotoxicity of neuronal cells leads to tinnitus.

II. MATERIALS AND METHODS

II-1. Cell culture and treatment

SH-SH5Y cells were cultured in DMEM/F12 growth media (with 10% fetal bovine serum) at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Cells were detached with 0.25% trypsin-EDTA solution, a density of 1.5 × 10^6 cells of DMEM containing 10% FBS in 100 mm dish. For neuronal differentiation, the growth medium was replaced with differentiation medium, which contained 0.1% FBS, 1% Pen Strep, and 1 μM retinoic acid (Sigma, St. Louis, MO, USA), and the cells were then further incubated for 2 days.

At embryonic day 18, cerebral cortex was dissected from Sprague Dawley rat pups and prepared for cultivation. Tissues were dissociated using a dissociation buffer containing 10 units/mL of papain (Worthington Biochemical, Lakewood, NJ, USA) and 100 units/mL of DNase I (Roche, Basel, Switzerland) for 30 min at 37°C. The dissociated tissue was further broken into single-cell suspension by using Neurobasal A medium (Invitrogen, Grand Island, NY, USA) and cells were seeded on poly-D-lysine hydrobromide (150 μg/mL; Sigma-Aldrich)-coated 12 well plates at a density of 0.5 × 10^6 cells/well. An hour after plating, the seeding medium was replaced by a growth medium composed of Neurobasal A, 1 × B27 supplement (Invitrogen), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.5 mM glutamine (Invitrogen). Cells were then incubated at 37°C in 5% CO_2.

Both SH-SY5Y cells (0.1% serum media) and rat cortical neurons were treated with

either VPA 200 μg/ml or RSV 2 μM in serum media for 12 h and subsequently treated with salicylate 40 μg/ml for 8 h.

II-2. Real-time PCR (Quantitative PCR)

Total RNA was isolated from SH-SY5Y using RNAiso reagent (TAKARA, Japan) following the manufacturer's instructions. Primescript II 1st strand cDNA synthesis kit (TAKARA, Japan) was exploited to reverse transcribe 2 μg total RNA with 5 μM Random primers, 1 mM of each dNTP, and the supplied buffer. cDNA was amplified using Power SYBR Green PCR master mix (Applied Biosystems Inc.; U.S.A.) with gene-specific primers for human *NR2B, ARC, TNFα*. The real-time PCR (StepOnePlus™ Real-Time PCR System) cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, and 1min in at 60°C. The primers are summarized in Table 1. These were produced by GenoTech (GenoTech Corp., Daejeon, South Korea) and IDT (Integrated DNA Technologies Inc.; Coralville, IA, U.S.A.)

II-3. Immunoblot analysis

Total proteins were isolated in 50 μl RIPA buffer (Santa Cruz Biotechnology, CA, USA) containing inhibitor cocktail, PMSF for 30 min on ice and then centrifuged at 16,000 rpm for 20 min. Protein concentration were measured by BSA assay kit (Thermo Fisher Scientific; Waltham, MA, U.S.A.). Protein was then subjected to immunoblotting using antibodies against NMDAε2, p53, CREB, p-CREB, GAPDH (Santa Cruz Biotechnology, CA, USA) and anti-caspase 3, active (cleaved) form (Merck Millipore, CA, USA) 4°C

overnight. The appropriate horseradish peroxidase-conjugated anti-goat, anti-rabbit and anti-mouse were used as secondary antibodies (Santa Cruz Biotechnology, CA, USA). Band detection was performed using enhanced chemiluminescence (ECL) (GE Healthcare, Buckinghamshire, UK) detection system and exposed to X-ray films.

II-4. Immunocytochemical staining

SH-SY5Y cells were cultured on poly-D-lysine coated coverslips (Thermo Fisher Scientific, Waltham, MA, USA) and differentiated into neuronal cells. After the treatments the cells were fixed by 4% paraformaldehyde and then permeabilized with cold methanol. Fixed cells were incubated with primary antibodies for NR2B (Santa Cruz Biotechnology, CA, USA) and p-CREB (Santa Cruz Biotechnology) for 1 h 30 min at room temperature (RT). After three times washing with PBS, the cells were incubated with Secondary antibodies, Alexa 555-conjugated donkey anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA) and Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes Inc.) together with Hoechst 33342 (Molecular Probes Inc.) in PBS for 1h 30min at RT respectively. Cells were mounted (ProLong Gold anti-fade reagent; Molecular Probes Inc., USA). The stained cells were visualized under a Nikon Eclipse Ti2 fluorescence microscope (Nikon, Tokyo, Japan). Images were taken by a DS-Ri2 digital camera (Nikon, Japan).

II-5. Detective of intracellular ROS

Intracellular ROS levels in treated SH-SY5Y cells were measured by 2',7'-dichlorofluorescin diacetate (DCFH-DA), which is converted to fluorescent product, 2',7'-dichlorodihydrofluorescin (DCF). Cells were seeded on 24-well plate and incubated with 20 μM DCFH-DA diluted in serum media at 37°C for 20min. After PBS washing, Cell were observed under a Nikon Eclipse Ti2 fluorescence microscope (Nikon, Tokyo, Japan) and captured by a DS-Ri2 digital camera (Nikon, Japan).

II-6. Statistical analysis

Data are represented as the means ± standard deviation (SD) of the indicated number of experiments and significance was estimated using a Student's t-test for unpaired observations. Statistical comparisons between groups were made using an independent *t*-test. p-value of < 0.05 was considered statistically significant.

Table 1. Primers used in real time PCR

Gene	Forward primer (5'→3')	Reverse primer (3'→5')	Gene accession
Human *β-actin*	ATC CGC AAA GAC CTG TAC GC	TCT TCA TTG TGC TGG GTG CC	NM_001101
Human *NR2B*	GGAGAGGTGGTCATGAAGAG	CATTGCTGCGTGACACCATG	NM_000834.4
Human *TNFα*	GTTGTAGCAAACCCTCAAGCTG	CCAGCTGGTTATCTCTCAGCTC	NM_000594.3
Human *ARC*	ACAACAGGTCTCAAGGTTCCC	AGCCGACTCCTCTCTGTAGC	NM_015193.4
Rat *GAPDH*	CTGCCACTCAGAAGACTGTGG	TTCAGCTCTGGGATGACCTTG	NM_017008.4
Rat *NR2B*	GGAGATGGAAGAACTGGAAGCTC	GACACCTGCCATATTGTCGATG	NM_012574.1
Rat *TNFα*	CCACCACGCTCTTCTGTCTAC	GATGATCTGAGTGTGAGGGTCTG	NM_012675.3
Rat *ARC*	GTCTGCTGCATAGAAGGAACCAG	AGGGTGCCCACCACATACTGA	NM_019361.1

Table 2. Primary antibodies used in western blotting

1st antibody	2nd antibody	Titer	Company (Cat.NO.)
NR2B	Mouse	1:200	Santa Cruz (#sc-365597)
p-CREB	Goat	1:500	Santa Cruz (#sc-7978)
CREB	Rabbit	1:100	Santa Cruz (#sc-186)
p53	Rabbit	1:500	Santa Cruz (#sc-6243)
Cleaved caspase-3	Rabbit	1:150	Merck (#AB3623)
GAPDH	Goat	1:1000	Santa Cruz (#sc-48167)

III. RESULTS

Part Ⅰ. Valproic acid prevents salicylate-induced neuronal cell death

III-1. VPA pretreatment significantly block the NMDA receptor in excitotoxicity neuronal cell line SH-SY5Y.

In the previous study, high dose of salicylate induced excitotoxicity by increasing the activation of the NMDA receptor in vivo tinnitus model. Therefore, I confirmed that salicylate can induce excitotoxicity by excessive activity of the NMDA receptor subunit (NR2B) in neuronal differentiated SH-SY5Y. The mRNA and protein of NR2B were measured by qPCR, immunocytochemical staining and immune blot. The mRNA expression of NR2B increased in the salicylate-treated group and significantly decreased in the VPA pretreatment group (Fig. 2A). Similarly, the NR2B protein level increased when excitability was induced with salicylate, and the increase could be suppressed through VPA pretreatment (Fig. 2B and 2C). Immunoblot analysis with NR2B were repeated and data were quantified (Fig. 2D). Salicylate treatment is known that not only increases the NMDA receptor but also increases the pro inflammation cytokine and immediate early gene. The expression of inflammatory cytokine *TNFα* and immediate early gene *ARC* were also recovered to reduce when VPA was treated to salicylate-induced SH-SY5Y cells (Fig. 2E and 2F). It can be seen that the NMDA receptor, which

is excessively increased due to salicylate, can be suppressed by VPA pretreatment, and the increased genes are also reduced.

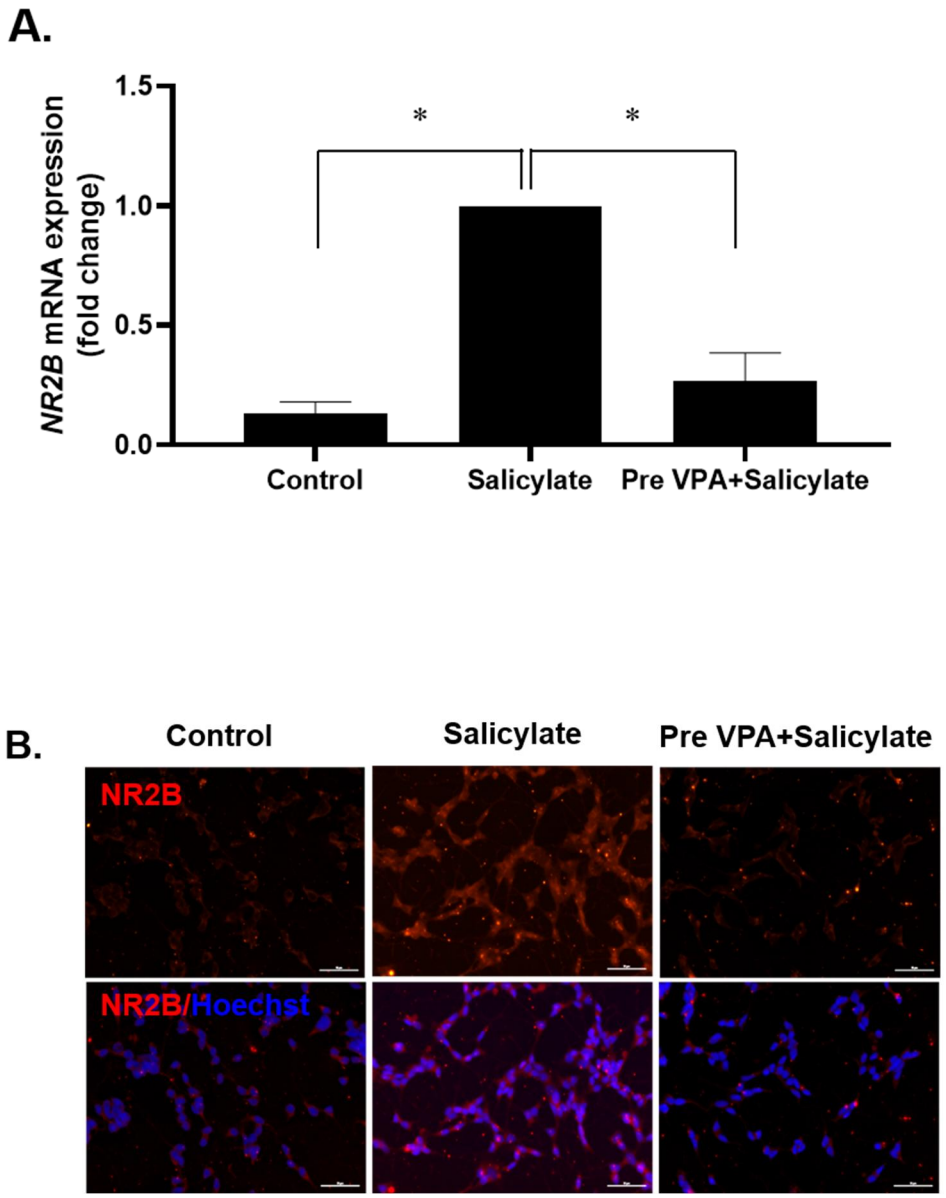
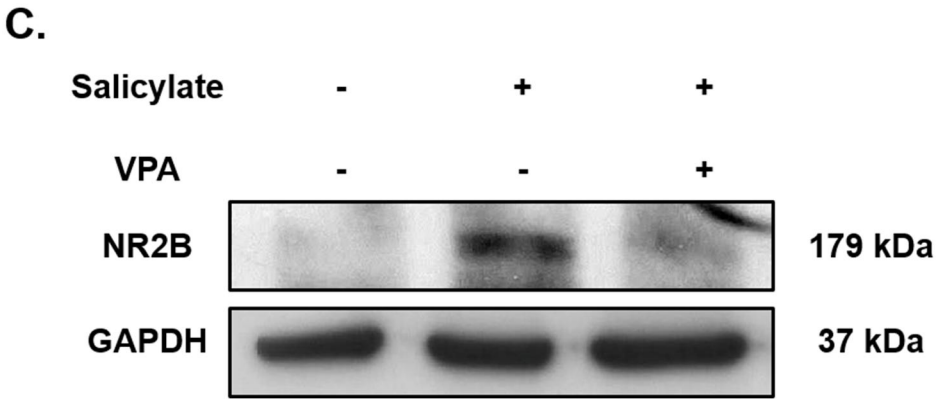
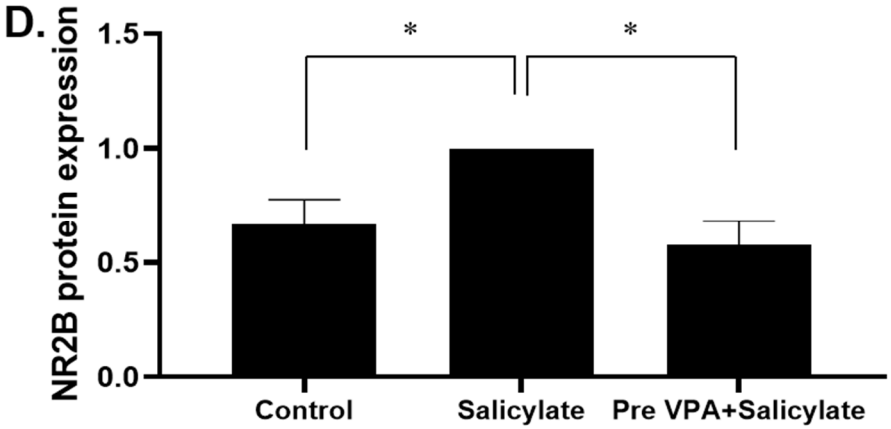


Fig. 2. (continued)





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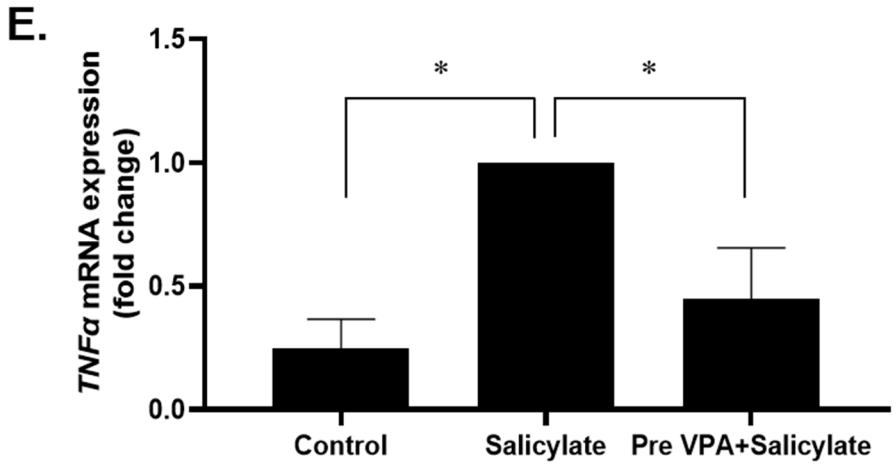


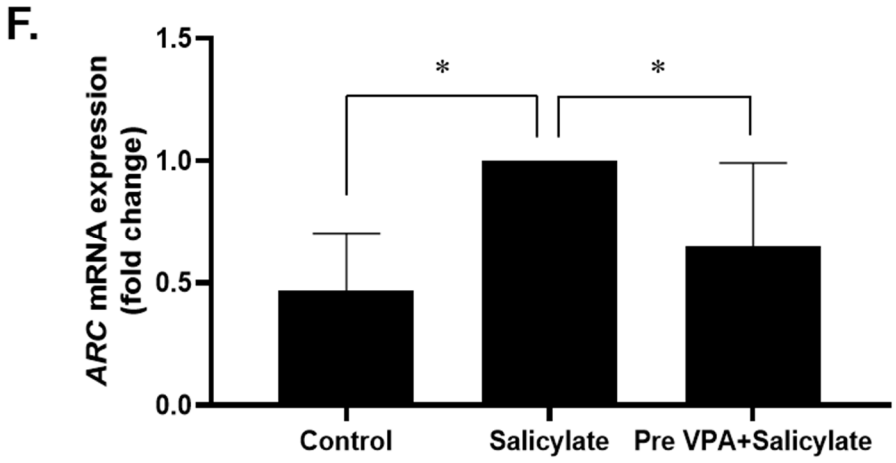


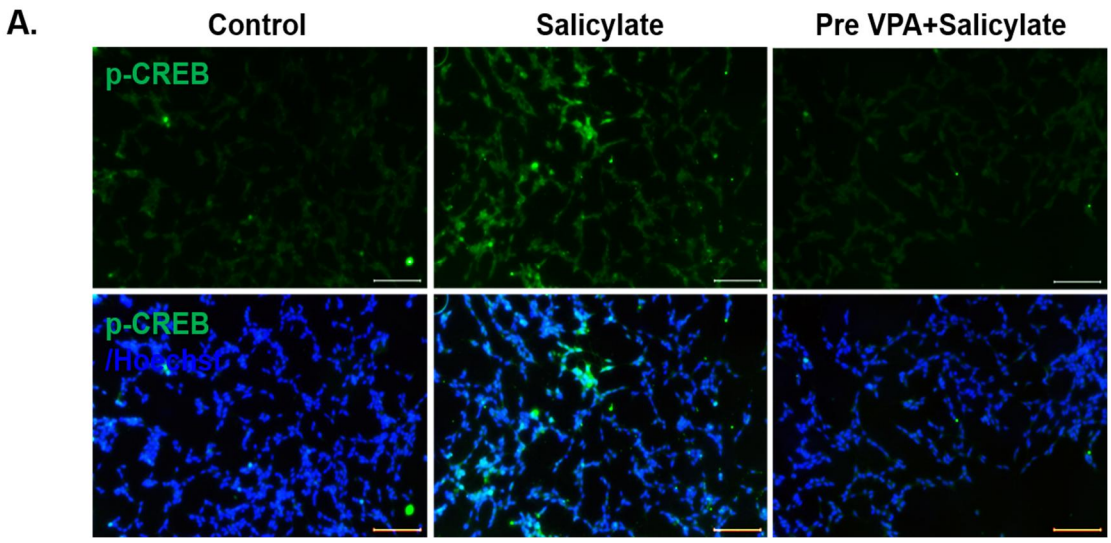
Fig. 2. (continued)

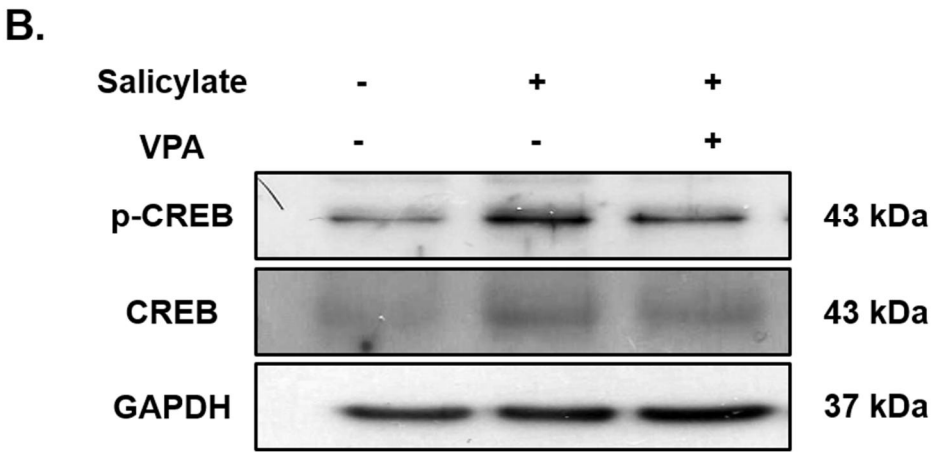
Fig. 2. Expression ofNR2B at mRNA and protein levels, inflammation cytokine and immediate early gene in neuronal differentiated SH-SY5Y

The mRNA expression of NR2B was measured in neuronal differentiated SH-SY5Y cells by quantitative PCR (A; *t*-test, * $p < 0.05$, mean ± SD, $n = 6$). Neuronal differentiated SH-SY5Y were fixed and stained with NR2B (red) antibody and honest 33342 (blue), and visualized under fluorescent microscopy (B). Consistent results were obtained from immunoblot analysis. Bands were quantified using the Image J software and normalized by GAPDH. (C and D; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$). The quantitative PCR results with inflammation cytokine (*TNF*α) and immediate early genes *ARC* were consistently changed with NR2B (E and F; *t*-test, * $p < 0.05$, mean ± SD, $n = 6$).

III-2. Pretreatment of VPA prevents the CREB phosphorylation in salicylate-induced excitotoxicity cells.

Activation of NR2B by salicylate increases intracellular calcium influx (Iacobucci and Popescu 2019). Increased Ca^{2+} induce excitotoxicity in nerve cells and it can cause about cell damages. The expression of CREB is phosphorylated in response to these cellular stresses. The phosphorylated levels of CREB was examined by immunocytochemical staining and immunoblot analysis with antibodies CREB and phospho-CREB (p-CREB) in SH-SY5Y cells. The phosphorylation levels of CREB were increased in salicylate-treated cells and significantly decreased in VPA treated cells (Fig. 3A and 3B). The experiments were repeated and identified significant differences between salicylate alone and VPA pretreatment with salicylate treatment group (Fig. 3C). These results indicate that VPA pretreatment reduces intracellular stress generation by protecting cells from salicylate-induced excitotoxicity.





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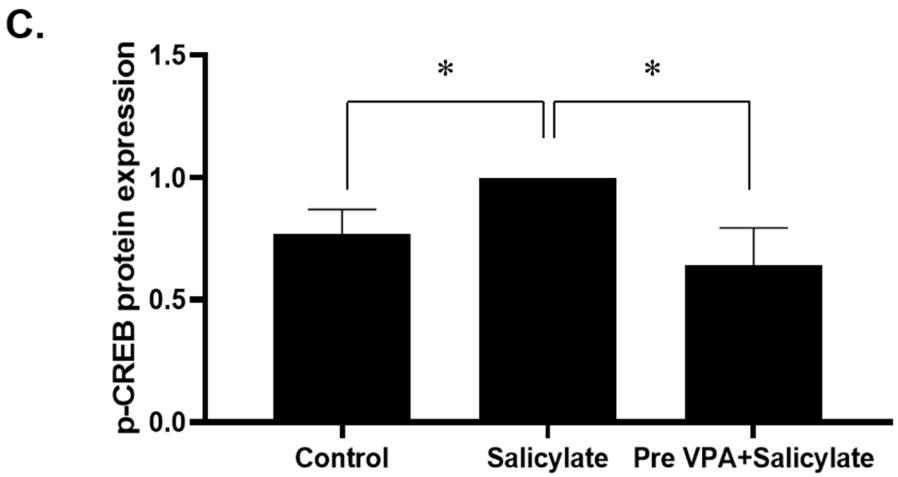
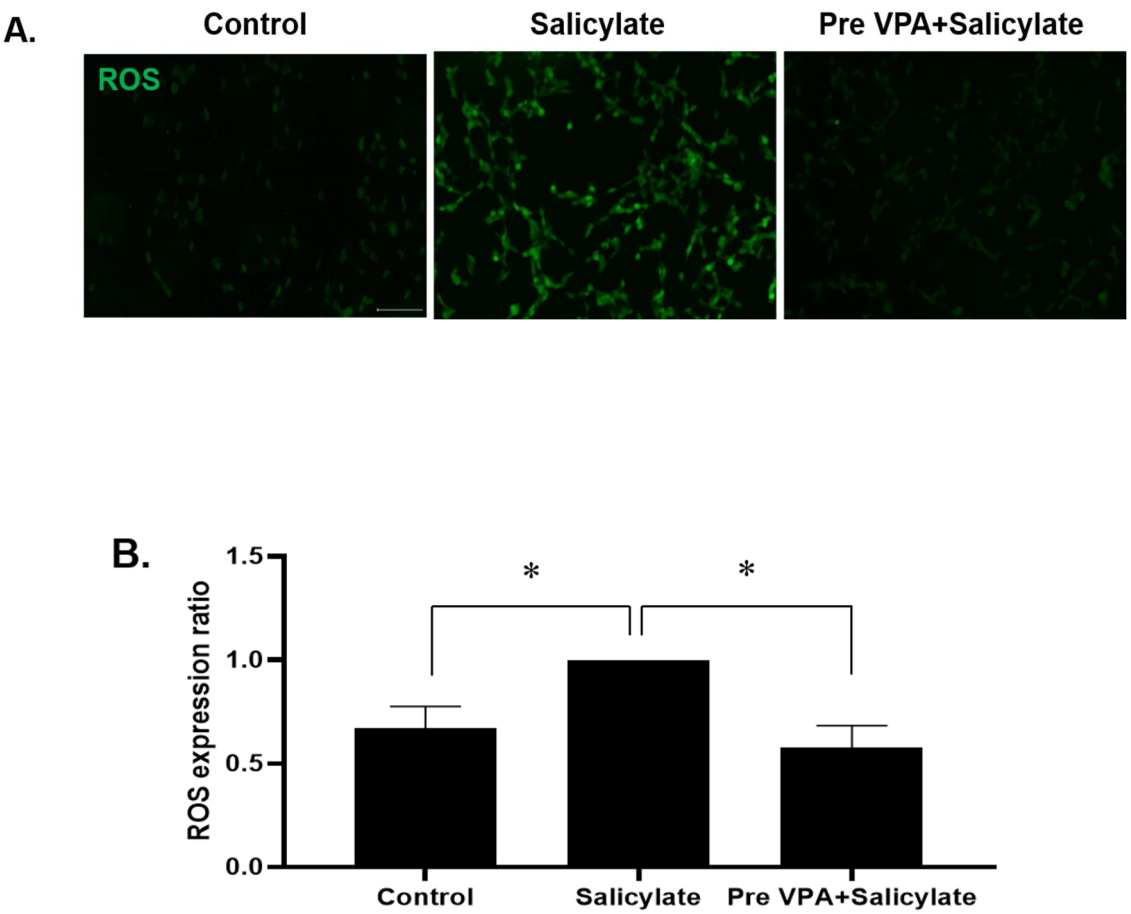


Fig. 3. VPA pretreatment can reduce CREB phosphorylation in salicylate-induced excitotoxicity SH-SY5Y.

Phospho- CREB (p-CREB) were labeled with green fluorescent and the nucleus were labeled with blue fluorescent by immunocytochemical staining (A). Consistent results were obtained from immunoblot analysis. The proteins were targeted with related antibodies specific for p-CREB, CREB. GAPDH was used as loading control for total fraction. The p-CREB immunoblot analysis bands were quantified using the Image J software and normalized by CREB (C; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$).

III-3. Protective effects of VPA in salicylate-excitotoxicity cells.

Glutamate excitotoxicity induced by excessive activation of NMDA receptor causes various damage to cells, which leads to cell death. In previous studies, increased ROS levels were detected in the spiral ganglion neuron of salicylate-treated mice model (Deng, et al. 2013). I tested whether VPA pretreatment can control the ROS accumulation induced by salicylate. As shown in Figure 1, ROS levels were increased in salicylate-treated cell. However, the VPA pretreatment group were showed similar levels to the control group. (Fig. 4A and 4B). To examine the cell protection effects by VPA, immunoblot analysis were performed with antibodies p53 and cleaved caspase-3, and confirmed to observe apoptosis by pretreatment of VPA (Fig. 4C). These results indicate that VPA pretreatment has the effect of protecting nerve cells from oxidative stress and apoptosis pathways induced by salicylate.



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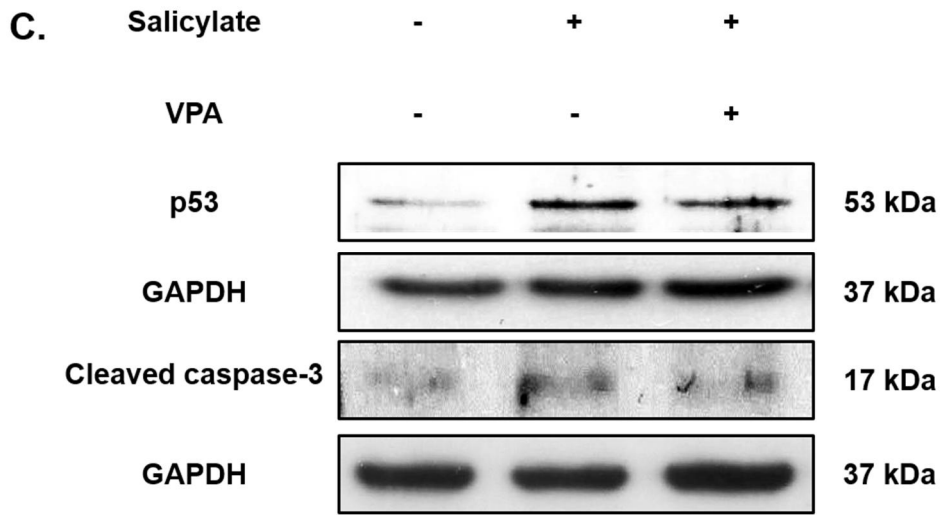
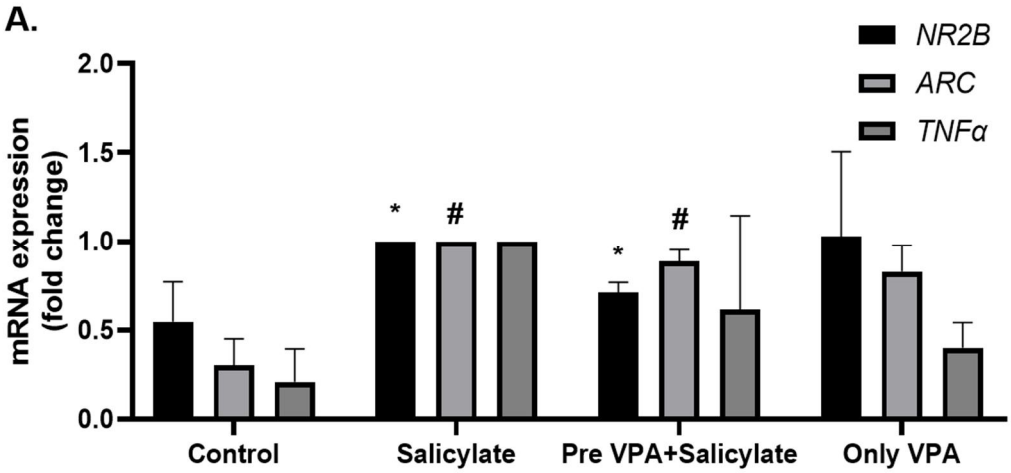


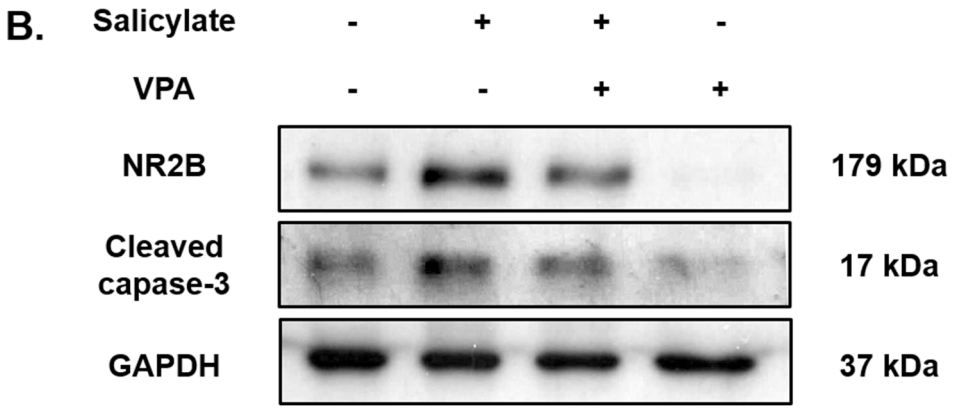
Fig. 4. VPA prevents oxidative damage and cell death of salicylate-induced excitotoxicity SH-SY5Y.

Intracellular ROS were stained by DCFH-DA and were labeled green fluorescent. The stained cells were observed under fluorescent microscopy. The production of ROS was increased in salicylate-treated SH-SY5Y cells, and significantly decreased in VPA pretreatment cells (A; scale bar=100 μm). The ROS levels were quantified using the Image J software and present in the graph. The graph was normalized by Hoechst 33342 staining (B; *t*-test, * $p < 0.05$, mean ± SD, $n = 4$). Immunoblot analysis were performed with antibodies for p53, cleaved caspase-3. The protein band of VPA pretreatment group was higher than that of control group. The tested proteins were decreased by VPA treatment. GAPDH was used as internal standard (C).

III-4. Pretreatment of VPA prevent salicylate-induced excitotoxicity in rat cortical neuron.

I have shown that salicylate stimulated NR2B expression and increased intracellular ROS level (Fig. 2 and Fig. 4). VPA pretreatment prevented those harmful effects in SH-SY5Y cells. Neuronal damage caused by salicylate had been shown in various area of tinnitus model's brain. To confirm these results in cortex area neuron cells, rat cortical neurons were used. The expression levels of NR2B were increased in salicylate-treated cortical neurons and decreased in VPA treatment, measured by qPCR and immunoblot analysis (Fig. 5A to C). Consisted results were obtained in qPCR of *ARC* and *TNFα* (Fig. 5A). The expression of cleaved caspase-3 also decreased by VPA pretreatment in salicylate-induced cortical neurons. VPA only group without salicylate treatment had a reduced level of NR2B protein compared to the control group. It was confirmed that VPA affects cortex neurons even when there isn't any excitotoxic effects (Fig. 5B and 5D). These results showed that VPA pretreatment blocked the NMDA receptor in neuronal cells as well as in the neurologically differentiated SH-SY5Y. VPA also have protective effects about salicylate-induced excitotoxicity and apoptosis signaling.





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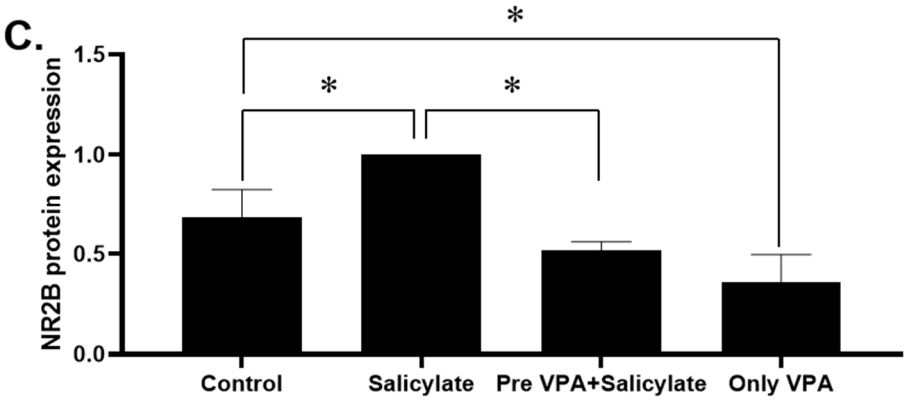


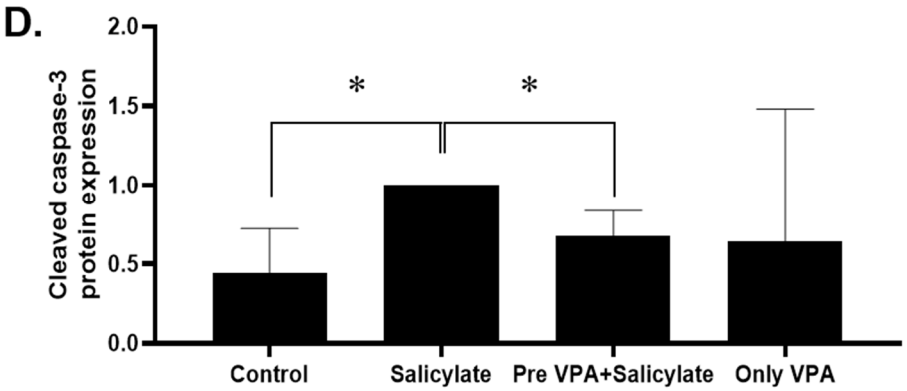


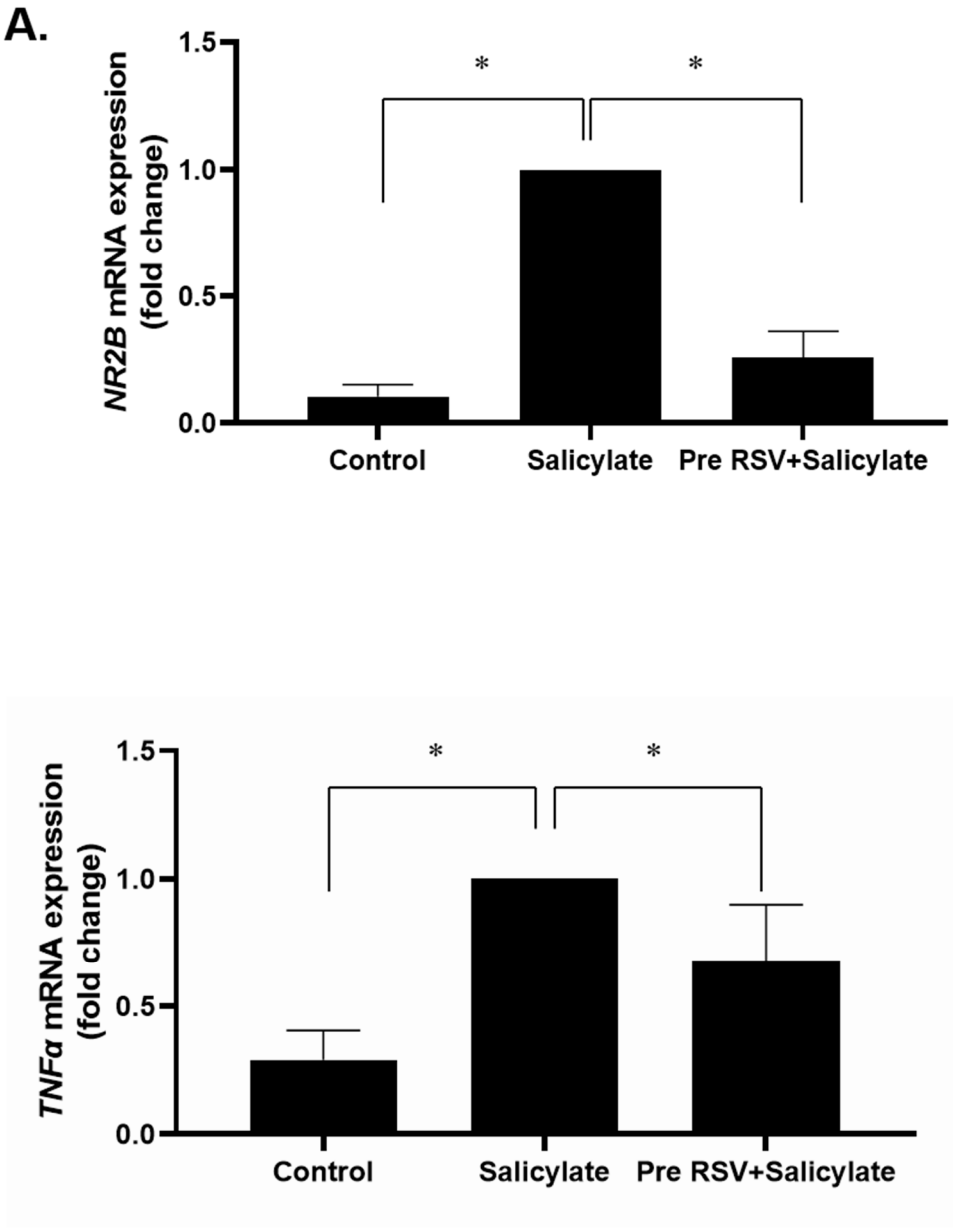
Fig. 5. The expression of NMDA receptor and apoptosis marker VPA in rat cortical neurons

The expressions of *NR*2B, *TNF*α and *ARC* genes were measured by qPCR (A; *t*-test, * $p < 0.05$, # $p < 0.05$, mean ± SD, $n = 3$). Protein expressions of NR2B and cleaved caspase-3 were measured by immunoblot analysis (B). The expressions of NR2B (C; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$) and cleaved caspase-3 (D; *t*-test, * $p < 0.05$, mean ± SD, $n = 4$) were quantified using the Image J software. The graphs were normalized by GAPDH.

Part II. Protective effect of resveratrol on excitotoxicity caused by salicylate.

III-5. RSV blocks the increased the NR2B by salicylate-treated in neuronal cell line.

RSV activates several neuroprotection factors and has a neuronal cell protective effect. In addition, RSV has a neuroprotective effect on glutamate-induced excitotoxicity by activating SIRT1. Therefore, I confirmed whether RSV can protect neuronal cells from excitotoxicity caused by salicylate. First, I checked that pretreatment of RSV prevents excessive NR2B activity induced by salicylate. SH-SY5Y cells were differentiated into neuronal cells and then treated with RSV or salicylate according to described in materials and methods section. The expression mRNA levels of *NR2B*, inflammatory cytokine *TNFα* and immediate early gene *ARC* were increased their expression in salicylate treated group, and were blocked in RSV pretreated cells (Fig. 6A). Expression of NR2B protein were confirmed and consistent results were obtained by immunocytochemical staining and immunoblot analysis (Fig. 6B to D). RSV pretreatment prevents the increase of NR2B induced by salicylate at both RNA level and protein level. The role of RSV was to prevent immoderate activation of NMDA receptor.



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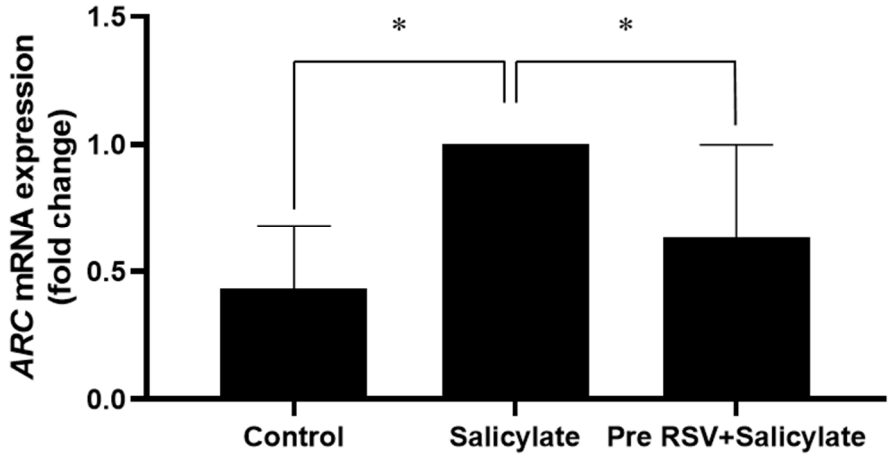

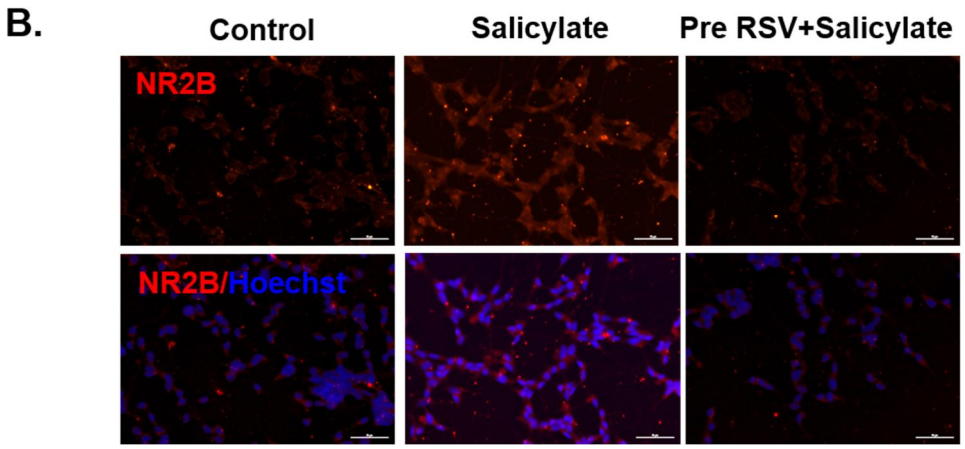

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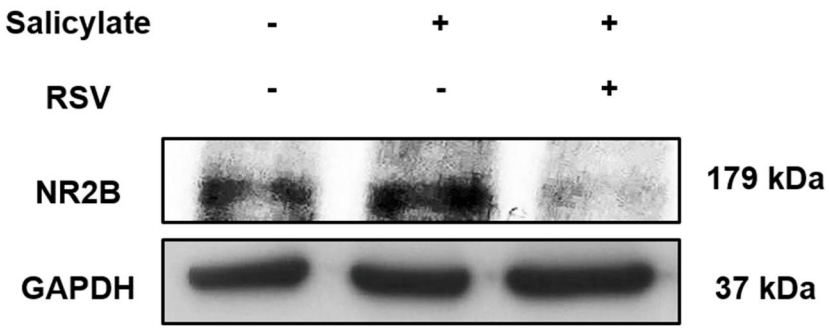

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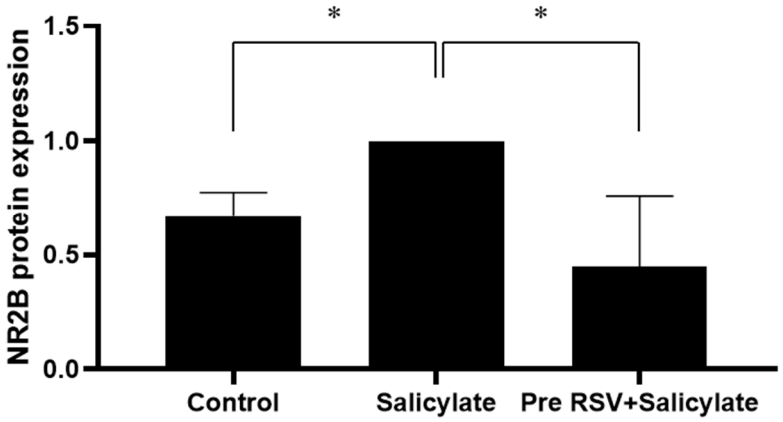
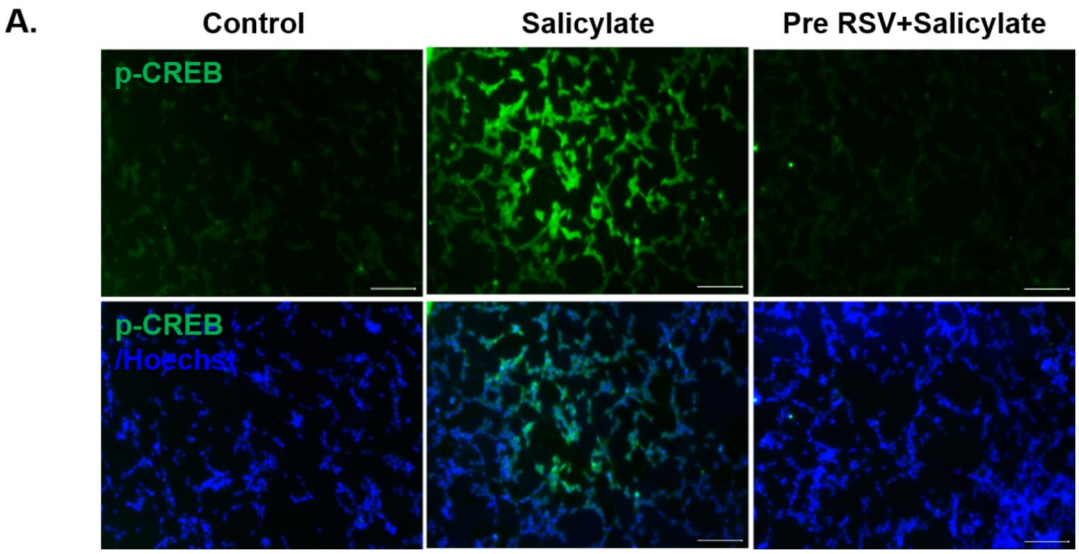


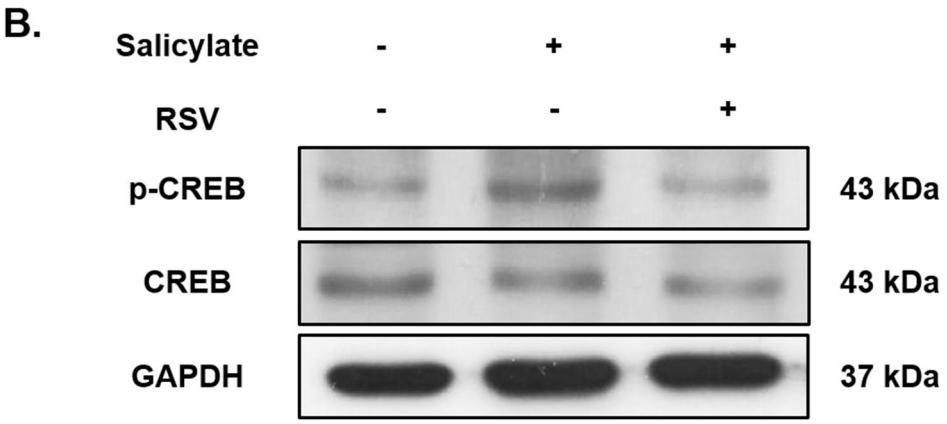
Fig. 6. Expression of NR2B, inflammation cytokine and immediate early gene

Salicylate significantly increased the expression of *NR2B* and inflammation cytokine (*TNFα*) and *ARC* as immediate early genes (IEGs) mRNA expression differentiated SH-SY5Y cells (A; *t*-test, * $p < 0.05$, mean ± SD, $n = 6$). The expression was significantly decreased by treatment with RSV. Expression NR2B at protein levels (B, C, D; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$). Salicylate increased the expression of NR2B protein and the expression were blocked by the treatment of RSV.

III-6. RSV pretreatment prevents stress in salicylate-treated neuronal cells.

Previous studies have shown that activated CREB were increased in salicylate-induced tinnitus mice model (Zhao, et al. 2018). Due to the over-activated NMDA receiver, a large amount of Ca^{2+} influx causes intracellular stress. Increasing of p-CREB by glutamate excitotoxicity is due to stress response via CAMKII/CREB signaling. I confirmed that RSV pretreatment shuts off excessive activation of the NMDA receptor (Fig. 2). In the salicylate-treated cell model the phospho-CREB (p-CREB) was increased in salicylate-treated cell and obstructed in RSV pretreated group, which were tested by immunocytochemical staining and immunoblotting (Fig. 7A and 7B). Immunoblot were repeated three more times and quantified (Fig. 7C). RSV pretreatment inhibits the inflow of Ca^{2+} by blocking the NMDA receptors. These results showed that RSV pretreatment can prevent excessive activation of the NMDA receptor and lowers the resulting cell stress.





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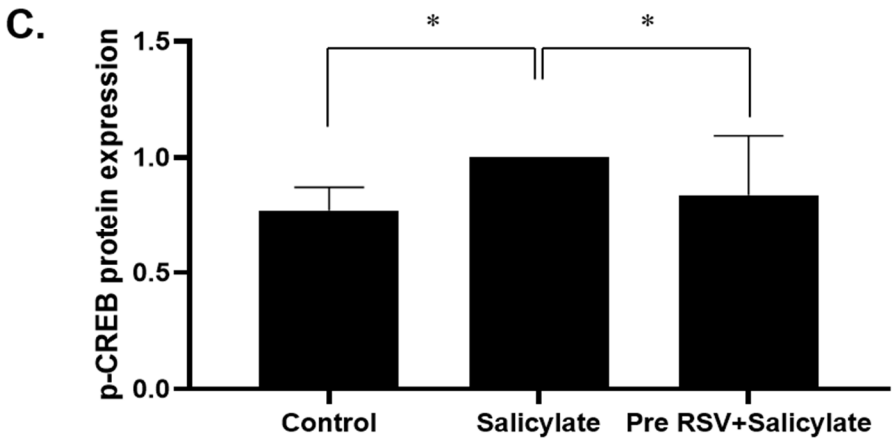
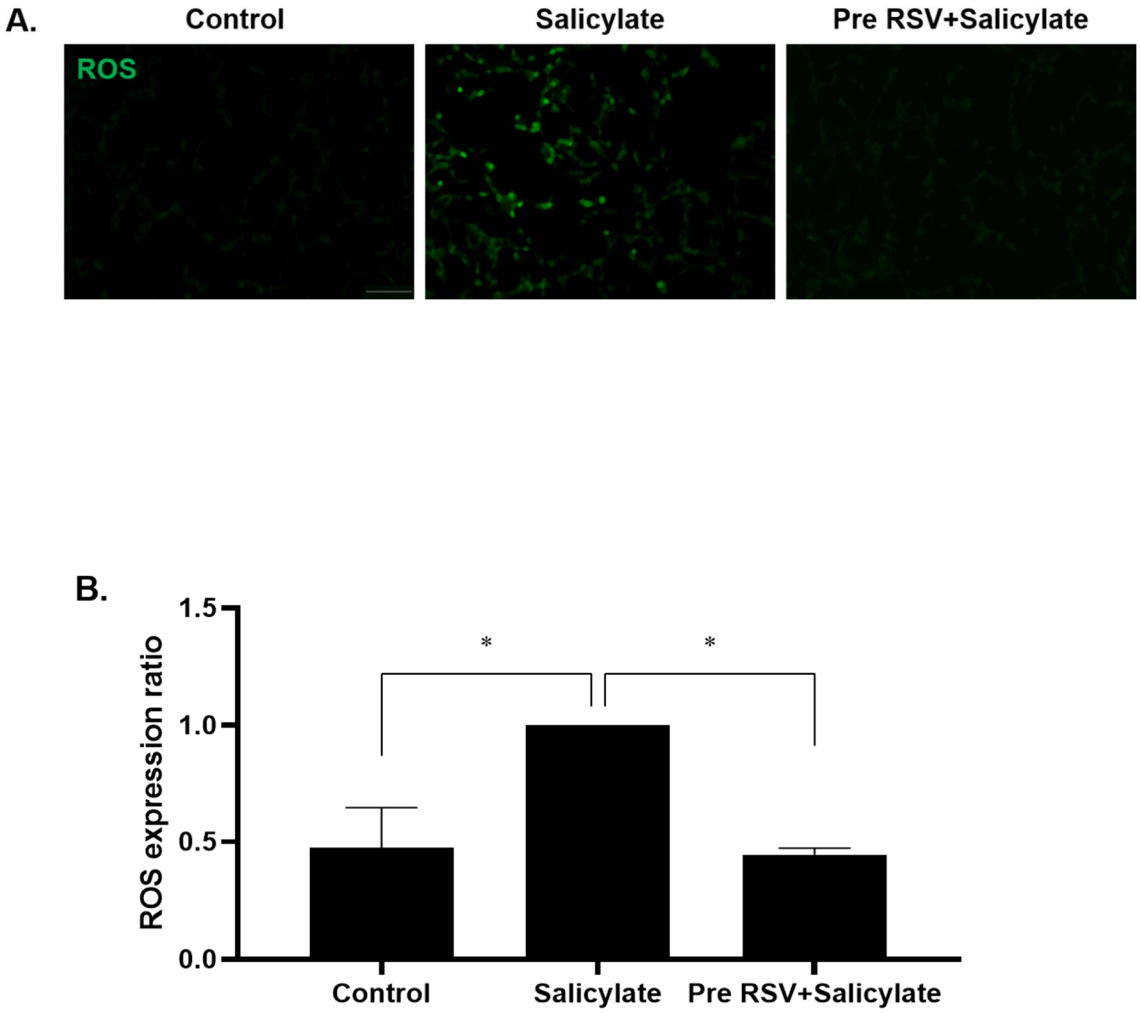


Fig. 7. RSV pretreatment declines p-CREB protein expression levels in neuronal cell line.

Salicylate increased the expression of p-CREB, tinnitus marker, in the SH-SY5Y cells and the expression were reduced by the treatment of RSV which was checked by immunocytochemistry staining (A; scale bar=100 μm) and immunoblot analysis (B). The protein levels were quantified using the Image J software (C; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$).

III-7. Protective role of RSV in Salicylate-treated neuronal cells.

Immoderate activation of NMDA receptor by high concentration of salicylate can induce mitochondria dysfunction with large quantity Ca^{2+} influx. Abnormal mitochondria are closely related to ROS production (Fig. 1). Intracellular ROS levels were highly increased in salicylate-treated cells, but RSV pretreatment was similarly with control group (Fig. 8A and 8B). In addition, I confirmed the apoptosis signaling by glutamate excitotoxicity. The apoptosis markers, p53 and cleaved caspase-3 were investigated through immunoblot analysis. The expression of apoptosis markers was significantly reversed through RSV pretreatment (Fig. 8C) These results indicate that RSV pretreatment protects neurons from salicylate induced oxidative damage and apoptosis signaling.



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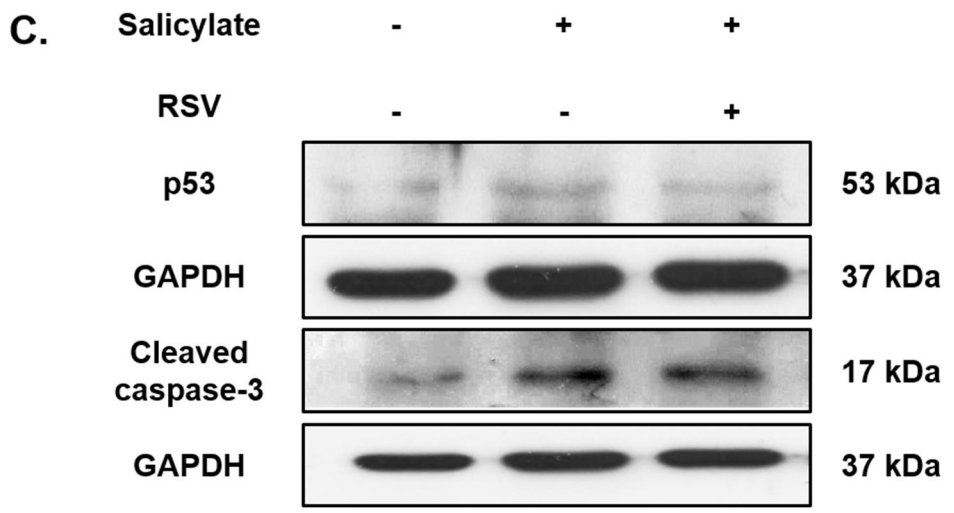
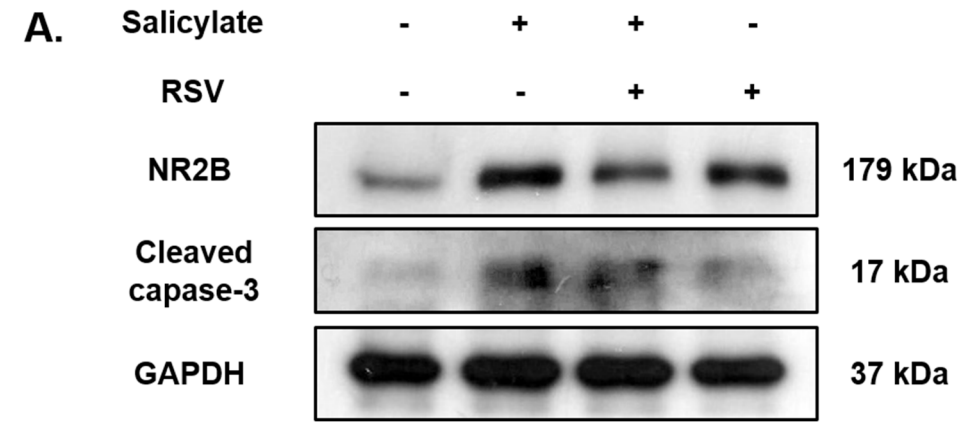


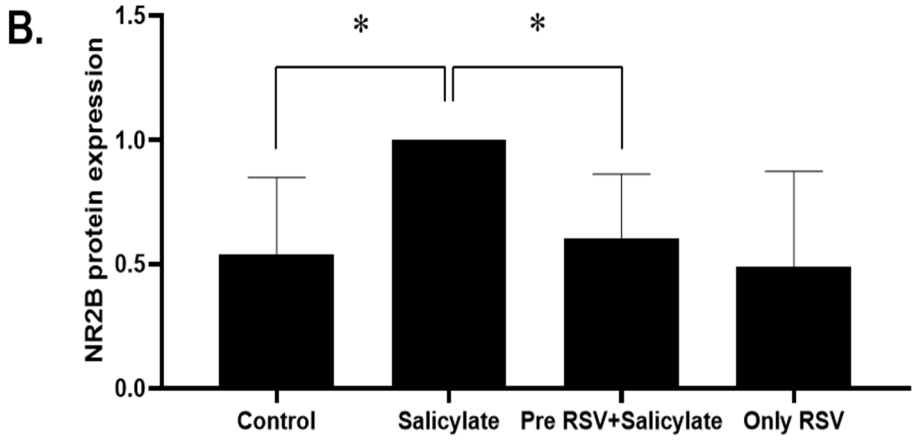
Fig. 8. RSV pretreatment reverses excitotoxicity ROS and apoptosis levels neuronal cell line.

To examine the intracellular ROS levels in treated cells, ROS detectable fluorescent DCFH-DA was used. RSV treated SH-SY5Y cells were treated with salicylate and their ROS levels were observed by fluorescence microscopy (A; scale bar=100μm). The ROS levels were quantified using the Image J software (B; *t*-test, * $p < 0.05$, mean ± SD, $n = 3$). Salicylate increased the expression of cleaved caspase-3 and p53 in the SH-SY5Y cells and the expression were decreased by the treatment of RSV which was confirmed by immunoblot analysis(C).

III-8. The effects of RSV in rat cortical neurons.

Though all the RSV effects were examined in neuronal cell line, I confirmed those effects in the primary culture cortical neurons. Rat cortical neurons were isolated and cultured in conditioned media and subjected with immunoblot analysis with antibodies NR2B and cleaved caspase-3. The NR2B and cleaved caspase-3 protein levels were increased in salicylate-treated cell (Fig. 8A). RSV pretreatment Apoptotic protein cleaved caspase-3 was significantly reduced by RSV treatment. In the only RSV group without salicylate, NMDA receptor activation and apoptosis markers were no different compared to the control group (Fig. 8B and C).





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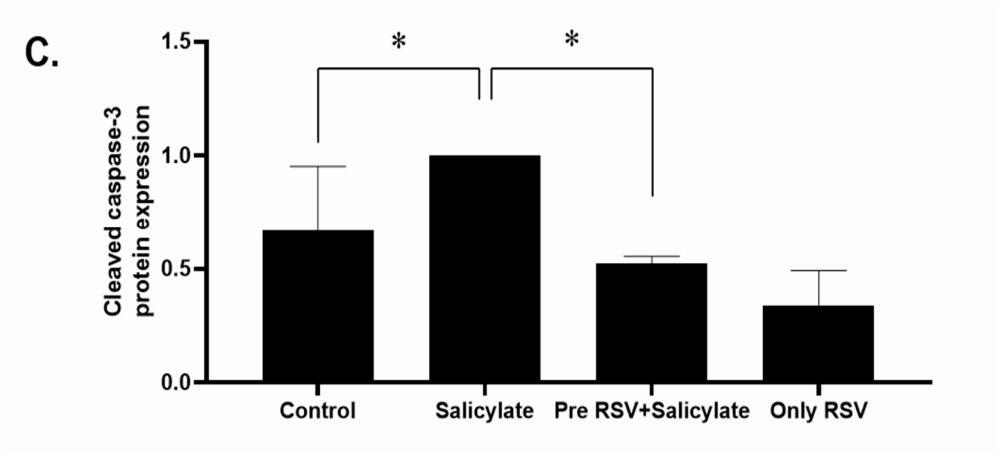

Fig. 9. RSV pretreatment protects excitotoxicity in primary rat cortical neuron.

Protein expression of NR2B and apoptosis markers by western blotting (A). The level of protein was quantified using the Image J software. Salicylate increased the expression of NR2B and cleaved caspase-3 in the primary rat cortical neuron and the expression were decreased by the treatment of RSV which was confirmed by western blotting (B, C; *t*-test, * $p < 0.05$, mean ± SD, $n = 4$).

IV. DISCUSSION

Salicylate has been widely prescribed as an analgesic and anti-inflammatory drug (Jastreboff, et al. 1988). However, a long term or high doses usage may accompany by side effects such as tinnitus (Qin, et al. 2019). In animals, high dose of salicylate treatment can cause tinnitus and hearing loss by inducing the structural abnormalities in the central auditory system and frontal cortex (Niu, et al. 2018). The tinnitus condition also occurred through the abnormal activity of NMDA receptors in the synapse of inner hair cells (Cui, et al. 2019). Salicylate abuse induces the excessive activation of NMDA receptor and accelerates intracellular Ca^{2+} influx which causes excitotoxicity at a region of auditory nervous system and eventually leads to damage to the cells (Gupta, et al. 2013). In the present study, I examined the effect of salicylate in neuronal cells and observed that increased expressions of NMDA receptor subunit NR2B, and its related genes *TNFα* and *ARC* in neuronal cells (Fig. 2). High concentration of salicylate excessively activated NMDA receptor so a large amount of Ca^{2+} inflow. This change causes excitotoxicity in nerve cells. Neuronal hyperactivity by NMDA receptors could also stimulate stress response such as a CREB phosphorylation (Yi, et al. 2018; Amidfar, et al. 2020) (Fig. 3).

In the part I, I have determined the beneficial effect of VPA in the salicylate-induced tinnitus cell model. VPA was widely used as a drug for bipolar disorder, epilepsy and migraine headaches and was known as an excitatory inhibitor of prefrontal cortical neurons (Nanau and Neuman 2013). The expression level of NR2B and CREB phosphorylation, were reduced upon treatment with VPA but not in salicylate alone. salicylate was reduced (Fig. 2 and 3). Salicylate stimulated the NR2B activation and

increased CREB phosphorylation, which were associated with intracellular stress by glutamate excitotoxicity. VPA can prevent the unnecessary stimulation of NMDA receptor and protect the neurons from the salicylate induced excitotoxicity.

Chronic glutamate excitotoxicity induces stress to neurons, eventually causes cell death (Hernández, et al. 2018). In the previous study, salicylate treatment bring to increase the intracellular ROS level in the spiral ganglion neuron of tinnitus-model mice (Deng, et al. 2013), and auditory neuronal cells death through the intrinsic apoptosis pathway (Feng, et al. 2011). Similarly, in neuronal cells, intracellular ROS levels were increased in the salicylate group, while were contrary in VPA pretreated group (Fig. 4A and 4B). The apoptosis related protein cleaved caspase-3 was also reduced in pretreatment of VPA (Fig. 4C).

High concentrations of salicylate produce excess activity in auditory cortex and the plasticity of these cortical extrapolation neurons is involved in the production of salicylate-induced excitotoxicity(Wu, et al. 2018). First, I confirmed that VPA pretreatment in the neuronal cell line reduces the activity of NMDA receptor and also the apoptosis signaling increased by salicylate (Fig. 2 and 4). Similarly, results were obtained in the primary rat cortical neurons (Fig. 5). Also, VPA, which was treated alone without salicylate, had no significant effect on cells. In order words, the protective effect of VPA is induced by averting excitotoxicity and oxidative stress.

In the part II, I examined the protective effect of RSV on excitotoxicity caused by salicylate. A natural polyphenol compound RSV is well known to anti-inflammatory and anti-aging effects (Nunes, et al. 2018). It has been reported that RSV has neuroprotective efficacy from glutamate excitotoxicity. I expected that RSV may have an effect on neurotoxicity caused by salicylate treatment. Increased expression of NR2B and related

genes were blocked in treatment of RSV (Fig. 6).

CREB is a major molecule that control plasticity, neurogenesis and survival of neurons (Pardo, et al. 2017). Previous studies have shown that increasing of Ca^{2+}/CaMKII/CREB signaling in salicylate-induced tinnitus in rats.(Yi, et al. 2018; Zhao, et al. 2018). Similar to the previous report, I also observed an increase in the phosphorylation of CREB in salicylate-treated neuronal cells, and reversed back when RSV was pretreated (Fig. 7).

Excessive ROS production is one of the important causes of hair cell damage (Scasso, et al. 2017). Salicylate produces excessive ROS as a side effect in long-term users and induce ototoxicity of peripheral/central auditory neurons (Tabuchi, et al. 2011). The cumulative ROS by salicylate was confirmed in neuronal differentiated SH-SY5Y cells, and down-regulated upon the pretreatment of RSV (Fig. 8A and 8B).

Ca^{2+} influx caused by unnecessary stimulation of NMDA receptor and accumulated ROS contributes to the instability of SIRT1 mRNA (Abdelmohsen, et al. 2007). Destruction of SIRT1 mRNA promotes apoptosis of nerve cells (Yang, et al. 2020). Also, RSV attenuates neurodegenerative disorders through the activation of SIRT1 (Corpas, et al. 2018). Activation of SIRT1 by RSV treatment inhibits p53 acetylation in neurotoxicity induced by NMDA receptor, preventing apoptosis and exhibiting neuroprotective effect (Yang, et al. 2017). Based on this, I have observed that the group which displayed excitotoxicity upon salicylate increased the activation of p53 and those which are pretreated with RSV showed reduction of p53 (Fig. 8 C).

A high dose of salicylate leads to hyperactivity in hippocampus, cerebral cortex including auditory cortex, and the plasticity of these cortical excitatory neurons is involved in salicylate-induced tinnitus (Wu, et al. 2018). I observed that NR2B overexpression and excessive ROS production in salicylate-treated cells were maintained

like control during RSV treatment. These preventive effects of RSV upon salicylate treatment were confirmed with the primary cortical neurons, which also yielded similar results (Fig. 9).

In conclusion, I examined the increasing of tinnitus markers, ROS and apoptosis in vitro model of excitotoxicity induced by salicylate. This can cause nerve cell damage and cell apoptosis and can finally lead to tinnitus. Pre-treating the cells with VPA or RSV blocked damages induced excitotoxicity by salicylate. These results suggest that VPA and RSV have the ability to protect neurons from excitotoxicity by preventing the unnecessary activation of NMDA receptors. This study proposes that VPA and RSV as therapeutic agent for tinnitus induced by excitotoxicity.

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