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THE CROSS-TALK BETWEEN NEURONS AND MICROGLIA

THROUGH INTERLEUKIN-4 AFTER ISCHEMIC INJURY

by

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THE CROSS-TALK BETWEEN NEURONS AND MICROGLIA

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A

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for the Degree of

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by

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Houston, Texas

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Abstract

THE CROSS-TALK BETWEEN NEURONS AND MICROGLIA

THROUGH INTERLEUKIN-4 AFTER ISCHEMIC INJURY

Shun-Ming Ting, M.S.

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After ischemic stroke, the loss of blood supply to the affected region of the brain leads to a series of pathological events known as ischemic cascades, that include excitotoxicity and microglia/macrophage over-activation resulting in damaging inflammatory responses. Studies from our research group suggest that the viable neurons in the ischemic penumbra (location receiving less profound damage, as compare to ischemic core) produce and release a potent anti-inflammatory cytokine, interleukin(IL) -4. We propose that this neuronal response to sublethal ischemia is designed to guide surrounding microglia/macrophages to a reparative and anti-inflammatory (M2) phenotype (1).

The research included in this study is designed to establish the mechanisms that underlie neuronal production and secretion of IL-4 under ischemic injury. To investigate this process we used primary rat cortical neurons in culture and a well-validated *in vitro* ischemic injury model that is based on transient oxygen- and glucose- deprivation, (OGD). In this model, only longer durations of OGD result in neuronal death. We discover that short-duration, sublethal OGD, as determined by LDH release assay, more LDH release correspond to greater damage) induces neuronal IL-4 production at the gene (RT-qPCR) and protein (ELISA) level. Furthermore, we show that mild excitotoxic stress produced by N-methyl-D-aspartate (NMDA) receptor (NMDAR)

activation (process that normally occurs in the cerebral ischemia) triggers IL-4 production and release by neurons in the primary neuron culture. We also implicated calcineurin and nuclear factor of activated T cell (NFAT) as potential players in the transcriptional regulation of the IL-4 synthesis in neurons. Finally, using neuron conditioned medium transfer to microglia, we find that neuronal IL-4 is capable of polarizing microglia toward a restorative, anti-inflammatory, and phagocytic phenotype.

For the first time, this study demonstrates that the ischemia-evoked NMDAR activation, through calcineurin/NFAT pathway induces IL-4 production by neurons, and that this neuron-secreted IL-4 is capable of regulating microglia phenotype change. This cross-talk between neurons and microglia could represent a therapeutic target for cerebral ischemia.

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Chapter 1 Introduction

Ischemic stroke

Ischemic stroke is a devastating disease that occurs when brain tissue cannot receive oxygen or nutrients from the blood due to a sudden blockade of a blood supplying vessel. Stroke is the fifth leading cause of death in the United States, and the leading cause of long-term disability (2). Right now recombinant tissue plasminogen activator (rt-PA)-mediated reperfusion (thrombolysis) and endovascular thrombectomy, mechanical blood clot retrieval, are the only FDA-approved therapies to treat ischemic stroke. The major limitations of these approaches are the risk of bleeding and short therapeutic time window, which for rt-PA is 4.5 hours and for thrombectomy is 16 to 24 hours after stroke onset (3, 4). Also, there is no neuroprotective treatment that allows neurons to survive longer so the reperfusion could occur at a later time. Moreover, there is no effective therapy for long-term post-stroke recovery.

At the onset of ischemic stroke, the lack of blood supply leads to energy failure, which makes ATP-dependent ion pumps stop working, leading to anoxic depolarization. Depolarization of damaged neurons, and consequent increase in concentration of intra-neuronal calcium triggers the synaptic release of excitatory neurotransmitters, especially glutamate and aspartate, leading to over-excitation of glutamate receptors with further increase in intracellular calcium, that initiates pathological process referred to as excitotoxicity. Primarily through NMDAR, the excitotoxicity increases calcium influx and initiates the activation of calcium-dependent intracellular enzymes, production of reactive oxygen and nitrogen species (ROS/RNS). These free radicals can directly damage proteins, lipids, nucleic acids, and cell membranes. Damage to mitochondria also contribute to apoptotic or necrotic cell death (5). With severe injury, cells die through necrosis, a cell death that involves loss of cell integrity, release of toxic

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intracellular content, and damage-associated molecular patterns (DAMPs) that initiate inflammation that can further exacerbate the local injury. On the other hand, cells with less severe injury may likely die through apoptosis, cell death that permits for more homeostatic, phagocytosis-mediated, elimination of dead cells by microglia or blood-derived macrophages, without causing a surge of potentially harmful pro-inflammatory responses. In the ischemic core, area of the brain with dramatic loss of blood supply, cells are irreversibly damaged and die through necrosis within minutes. In the ischemic penumbra, location with more modest reduction of blood supply, the affected cells are initially well but then progressing over time into irreversible damage due to sustain ischemia and the harmful environment caused by the necrosis-associated cell lysis in the ischemic core. Penumbral neurons are the cells that can eventually be rescued by reperfusion therapy.

Microglia responses after brain injury

Microglia are the tissue-resident macrophages of the brain and the first responders to injuries in the central nervous system. Similar to all the macrophages, microglia are professional phagocytes capable of engulfment and eliminating sources of inflammation including toxic cellular debris and damaged or displaced cells from the site of injury. However, when activated in a fashion resembling responses to microbial attack, they produce pro-inflammatory cytokines, reactive nitrogen and oxygen species, and damaging proteases that could further aggravate the injury; although, microglia can be activated toward different phenotypes. In response to pro-inflammatory cytokines (interferon-γ or tumor necrosis factor-α), microglia are driven towards the classical pathway to an M1 phenotype (6). In this phenotype, they secrete pro-inflammatory

cytokines like tumor necrosis factor-α, and interleukin (IL)-1β and proteases such as matrix metalloproteinase-9 (MMP-9), which will enhance inflammation and disrupt the blood brain barrier (7, 8). On the other hand, in response to IL-4 and IL-13, microglia are activated through the alternative pathway toward an M2 "healing" phenotype (9, 10). In this phenotype, microglia preferentially secrete anti-inflammatory cytokines like transforming growth factor-β and IL-10 and growth factors, such as vascular endothelial growth factor and brain-derived neurotropic factor. Also, scavenger receptors (cluster of differentiation(CD) 36, CD204, and CD163 are upregulated, so that their phagocytic abilities are enhanced. Therefore, microglia in an M2 phenotype effectively eliminate the source of inflammation and enhance tissue recovery.

Interleukin-4

IL-4 is a pleiotropic cytokine that is classically considered anti-inflammatory because of its ability to suppress type I (classical) inflammation (11). In the brain, IL-4 also plays important roles in macrophage/microglia activation (9, 10) and modulation of learning and memory (12). IL-4 signals through type I and type II IL-4 receptors that are expressed on microglia and macrophages. Type I IL-4 receptors consist of one alpha chain (IL-4Rα) and one common gamma chain (γC) while type II receptors consist of one IL-4Rα and one IL-13 receptor α1 chain. IL-4 binding to IL-4Rs activates the transcription factor signal transducer and activator of transcription 6 (STAT6), which will then go through homo-dimerization, nuclear translocation, and initiation of transcription of downstream genes (13). In microglia/macrophages, IL-4-dependent STAT6 signaling pathway results in upregulation of peroxisome proliferator-activated receptor-γ (PPARγ) a key transcription factor in regulating macrophage function (14). PPARγ, as a nuclear

receptor, has to dimerize with another nuclear receptor retinoid-x-receptor (RXR) to form a dimer capable of promoting the transcription of genes involved in the M2 phenotype. The IL-4-STAT6-PPARγ signaling axis is crucial to macrophage/microglia alternative activation (15).

Glutamate receptor-mediated excitotoxicity

Glutamate is an amino acid and a major excitatory neurotransmitter in the central nervous system. Excitatory neurons use glutamate as a neurotransmitter that is released from synaptic vesicles to stimulate postsynaptic neurons for synaptic plasticity. In response to ischemia, glutamate is released in high concentrations by neurons and astroglia. Glutamate receptors are diverse and consist of ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). The mGluRs couple with G-protein and modulate intracellular biochemical cascades, while the iGluRs are ligand-gated ion channels mediating fast synaptic transmission. The iGluRs can be further subdivided into three main classes: N-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors.

NMDA receptors have long been proposed as mediators of glutamate-induced excitotoxicity, due to their high permeability to calcium ions (16). NMDA receptors are hetero-oligomers composed of the constitutive NR1 subunit and one or more NR2 subunits (NR2A-D). The receptor is activated when there is binding at both the NR2 subunit by glutamate and the NR1 subunit by either glycine or D-serine co-activators. At resting membrane potential, the channel pore of NMDA receptor is blocked by magnesium. When the magnesium is removed by depolarization, the activated NMDA receptor allows the influx of calcium, leading to excitatory postsynaptic current.

Intracellular calcium is tightly regulated by neurons with the typical difference in cytosolic calcium being four orders of magnitude lower than outside of the cell. The activated postsynaptic iGluRs, especially NMDA receptors, open ion channels, allowing the overflow of extracellular calcium into the cell. Furthermore, the influx of calcium depolarizes the cell membrane and activates voltage-gated calcium channels, further enhancing intracellular calcium overload.

Activity-dependent signaling pathways

In the brain, the homeostatic gene transcription in neurons is often regulated by the integration of synaptic activities through a myriad of signaling pathways. These lead to transcriptional expression of a set of genes that are of key importance for proper functioning, survival, differentiation, and long-lasting plasticity (17-19). Nuclear factor of activated T cells (NFAT) family members are calcium-dependent transcription factors. While NFAT family members are recognized for their essential role in regulating T cell development and activation, they are also present in neurons (20). In response to synaptic activity, calcium influx mediated by NMDA receptors activates the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. Activated calcineurin dephosphorylates NFAT, an essential step in transcriptional activation of NFAT. The target genes of NFAT in neurons remain to be characterized.

Hypothesis:

In response to sublethal ischemia, neurons produce IL-4 that acts as a signal to local microglia to prevent their over-activation, and, through alternative activation pathway, enhance their healing phenotype.

Chapter 2 Materials and Methods

Primary cortical neuron cultures

The cortices of E18 fetal Sprague Dawley rat embryos were dissected and dissociated by trituration. The dissociated cells were plated on poly-L-lysine-coated 6-well plates in Neurobasal medium with B27 at a density of 5 x10^5 cells/mL. The cells were maintained in a CO_2 incubator (5% CO_2, 21% O_2) at 37.0±0.5°C. Half of the culture medium was changed every 3 days. Neurons were ready for experiments after 11 days in culture, as determined by extensive axonal and dendritic networks. Neurons constitute more than 90% of living cell population, as confirmed by both microtubule associated protein 2 (MAP2) immunofluorescence staining and RT-PCR (1).

Primary brain glial culture and microglia isolation

The cortices of E18 fetal Sprague Dawley rat embryos were dissected and dissociated by trituration. The dissociated cells were plated in 75 cm^2 tissue culture flasks in DMEM with 10% fetal bovine serum, and maintained in a CO_2 incubator (5% CO_2, 21% O_2) at 37.0±0.5 ºC. Half of the culture medium was changed every 3 days. After a total of 12-15 days in culture, the astrocytes form a confluent layer signifying that the culture was ready for microglia isolation. The loosely adherent microglia were harvested by shaking at 220 rpm for 30 minutes. The detached microglia were collected and re-plated onto poly-L-Lysine coated 6-well plates with or without German-glass inserts at a density of 1-4 x10^5 cells/mL (1).

Oxygen-glucose deprivation injury model

Eleven-day-old primary cortical neurons in culture were subjected to oxygen-glucose deprivation (OGD) injury. The culture media were replaced with Neurobasal medium without glucose. The cultures were placed in a gas-tight humidified chamber and flushed with 5% CO_2/95%N_2 for 10 minutes. After flushing, the cultures were kept in OGD status for the designated time in the incubator. At the end of OGD, glucose at 4.5mg/mL was added into the cultures, and the cells were then returned to their original culture condition and incubated for 6 hours for reperfusion (1).
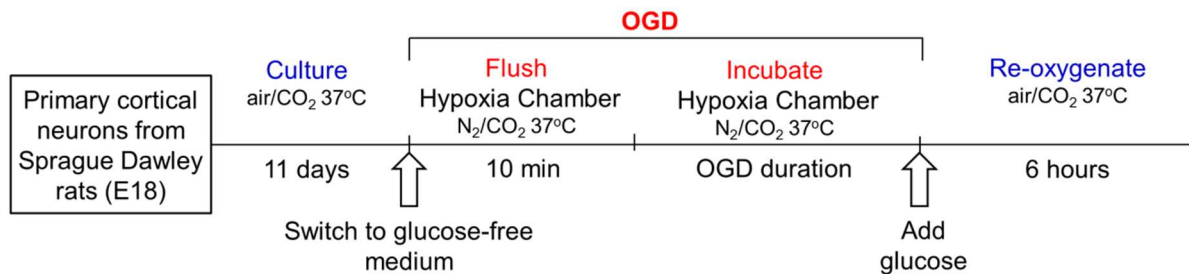
Figure 1. A schematic timeline of the induction of OGD injury in primary neuron cultures.

Immunofluorescence of MAP2

MAP2 immunofluorescence was used to label neurons. The primary neuron cultures grown on German glass were fixed with 95% methanol containing 5% acetic acid for 10 minutes at room temperature, and incubated in mouse anti-MAP2 (M4403; Sigma-Aldrich) overnight at 4°C. Rabbit anti-mouse IgG–Alexa Fluor 488 (Invitrogen) was used to visualize MAP2-labeled neurons. The images of MAP2-labeled neurons were captured using Zeiss Axioskop 2 fluorescence microscope (1).

Cytotoxicity assay

The degree of cell injury was assessed by determining the amount of lactate dehydrogenase (LDH) released into the culture medium using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). The assays were performed following the manufacturer's manual. Briefly, 50μl of medium from cultured cells was collected and mixed with 50μl of CytoTox 96 reagent in a 96-well flat clear bottom plate. The plate was incubated for 30 minutes at room temperature and then recorded the absorbance at 490 nm. The result of each sample was measured by averaging the results from triplicate wells. The cell injury index was determined by comparing the LDH release of each condition to the vehicle cells control.

RNA isolation and reverse transcription-quantitative polymerase chain reaction

Total RNA from harvested cells were isolated using Trizol reagents. Complimentary DNA (cDNA) was synthesized from 2μg of RNA using amfiRivert Platinum One (GenDEPOT) following manufacturer's protocol. IL-4 mRNA level analysis was performed on a Mastercycler ep realplex (Eppendorf) with Taqman probe-based real-time PCR system (Thermo Fisher Scientific). For the gene-expression profile of microglia, SYBR Green-based real-time PCR system was used. Each cycle consisted 5 seconds of denaturation at 95℃, 15 seconds of annealing at 60℃, and 10 seconds of extension at 72℃ (40 cycles). Glyceraldehyde-3-dehydrogenase (GAPDH) was used as an internal control. The expression level was calculated using the delta-delta Ct method.

Enzyme-linked immunosorbent assay (ELISA)

The amount of IL-4 in the culture medium was measured using Rat IL-4 Platinum ELISA kit (eBioscience) following the manufacturer's manual. Briefly, 50μl of medium from cultured cells was collected and incubated with Biotin-Conjugate in the supplied 96-well plate for overnight at 4ºC. The wells were then washed 3 times and Streptavidin-HRP added, incubating for 1 hour at room temperature. After removing Streptavidin-HRP, TMB substrate solution was added to the wells, incubating 10 minutes for color development. After adding stop solution, the absorbance of each well was read on a spectraphotometer at 450nm. The standard curve was established with the concentration of 0-100pg/mL. The result of each sample was measured by averaging the results from duplicate wells.

Electrophoretic mobility assay (EMSA) for NFAT

The activity of transcription factor NFAT was assessed by using NFAT EMSA kit (Signosis) following the manufacturer's protocol. Briefly, the nuclear extract from the cells was acquired using Nuclear Extraction kit (Signosis) and was incubated with NFAT probe. The protein/DNA complexes were then separated by a non-denaturing polyacrylamide gel. After transferring the gel to a nylon membrane, the probe was detected using Streptavidin-HRP and chemiluminescent substrate. Optical density was determined using Kodak Analysis (EDAS) 290 system.

Conditioned medium transfer

The medium from the cultured neurons exposed to different experimental conditions was harvested and centrifuged at 400g for 5 minutes. 500μl of the

supernatant (conditioned medium combined with 500μl of fresh DMEM (total 1mL) was transferred to the culture wells containing microglia and incubated for 24 hours before harvesting microglia for gene expression analysis. For the naïve and positive controls, 500μl of fresh neurobasal medium with or without rat recombinant IL-4, respectively, combined with 500μl of fresh DMEM (total 1mL) was added to the wells containing microglia (1).
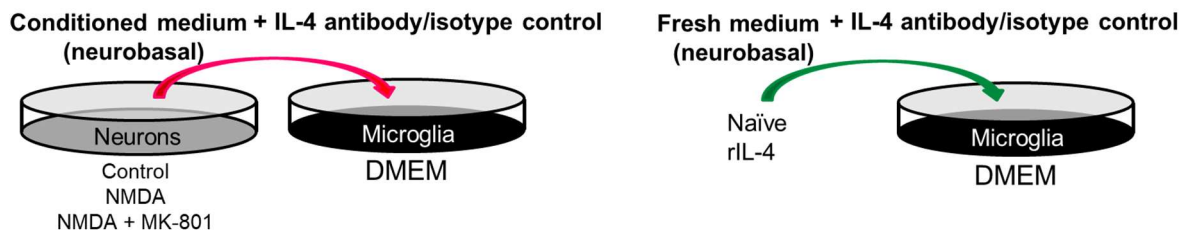
Figure 2. A diagram of conditioned medium transfer experiment. Conditioned medium transferred from the neuron conditioned cultures to microglia cultures (21). Fresh neurobasal medium was used for naïve and positive control groups, with positive control containing IL-4 (right).

Statistical analysis

All statistical analyses, including correlation analysis between IL-4 mRNA expression and NFAT activity, were performed using the GraphPad Prism 7 and InStat programs. Two-way analysis of variance (ANOVA) was used to assess data with two grouping variables. Remaining data were analyzed using one-way ANOVA. The Tukey test was used for pairwise comparisons. Non-paired t-test was used when two groups were compared.

Chapter 3 Results and Analysis

A well validated model of primary cortical neuron-enriched culture

To study the interaction between neurons and microglia through neuron-generated IL-4 in response to ischemia, we employed rat primary cortical neuron cultures from E18 embryos and subjected them to OGD. We utilized 11-day-old cultures to allow neurons to reach maturity and form extensive synaptic networks including the expression of NMDA receptors that is necessary to study excitotoxic processes (Figure 3A). The purity of our neuronal culture was validated using RT-PCR (Figure 3B). Using RNA extracted from our cultured neurons and using agarose gel electrophoresis, the PCR products (40 cycles) showed very strong expression of neurofilament long (NFL, marker for neurons) with little presence of myelin basic protein (MBP, marker for oligodendrocytes), glial fibrillary acidic protein (GFAP, marker for astrocytes), and CD68 (marker for microglia), indicating high purity of our neuron-enriched culture (Figure 3B).

A

B

Neuron cultures
1   2   3

NFL
MBP
GFAP
CD68
GAPDH
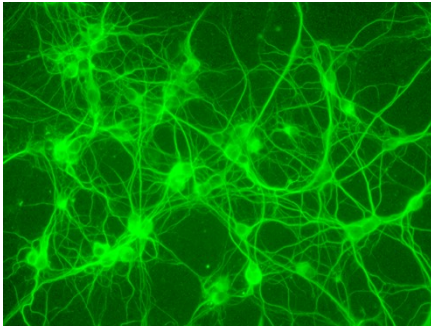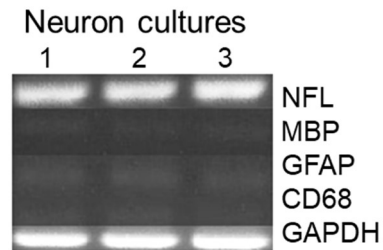
Figure 3. Immunohistochemistry and RT-PCR to validate primary neuron cultures.

(A) A representative image of MAP2 (M4403; Sigma-Aldrich) staining of neurons at 11 days in culture, showing extensive dendritic networks. (B) Representative RT-PCR (40 cycles) results analyzing three different samples of primary cortical neurons in culture to establish the purity of neurons in our cultures. Each sample was pooled from two wells from 6-well plates with total of 2 x10^6 cells. Expression of NFL, MBP, GFAP, CD68, and GAPDH were used as markers for neurons, oligodendrocytes, astrocytes, microglia and internal control, respectively. NFL: neurofilament long, MBP: myelin basic protein, GFAP: glial fibrillary acidic protein, CD68: macrosialin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Sublethal oxygen-glucose deprivation triggers neuronal IL-4 production and release

To study the mechanisms underlying the production and release of IL-4 by neurons in response to ischemia, we utilized rat primary cortical neuron cultures from E18 embryos. OGD was used to simulate ischemic stroke-induced insult. We tested 5 and 20 minute durations of exposure to OGD to achieve mild and more pronounced level of injury, respectively. Six hours after OGD, culture medium was collected for measurement of LDH to gauge the level of injury, and for IL-4 released to the surrounding media (ELISA). Cells were harvested and processed for gene expression analysis with TaqMan real-time PCR. The results of the LDH assay revealed two levels of injury caused by the 5 and 20 minutes of OGD (Figure 4A). The results showed that both the gene (Figure 4B) and protein (Figure 4C) levels of IL-4 significantly increased under exposure to the sublethal duration of 5 minutes of OGD, the insult that induced only modest LDH release. Comparing to 5 minutes of OGD, 20 minutes of OGD (lethal insult) had significantly lower expression in both protein and gene levels. These results support the hypothesis that sublethal injury to neurons induces neuronal IL-4 translation, production, and release.
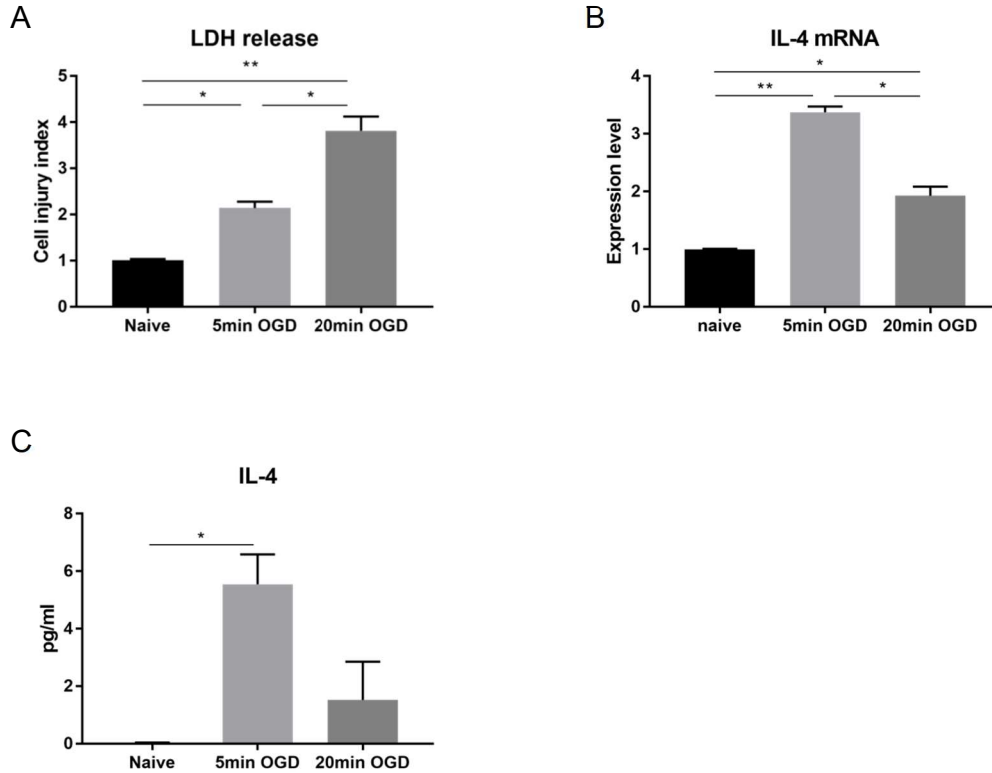
Figure 4. Sublethal ischemic injury (OGD model) triggers neuronal IL-4 production and release. Eleven-day-old primary neuron cultures were subjected to two different durations of OGD injury, 5 and 20 minutes. Six hours after reperfusion, culture media were collected for LDH release via CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) (A) and IL-4 protein analysis via ELISA (eBioscience) (C). The cell injury index was determined by comparing the LDH release of each condition to the naïve control. The cells were collected for IL-4 mRNA analysis using Taqman probe-based real-time PCR system (B). The data are mean±SEM (n=3). *P<0.05. **P<0.01. One-way ANOVA followed by Tukey test.

Neuronal IL-4 production is triggered by a sublethal excitotoxic insult

Cerebral ischemia-induced injury is a composite of various biochemical events that among others include oxidative stress, apoptosis, or excitotoxicity (22, 23). OGD-induced injury shares with the *in vivo* situation many of the *in vivo* features, including oxidative stress, apoptosis, or excitotoxicity (24, 25). Thus, to dissect what event of ischemic injury is involved in IL-4 gene induction in neurons, we utilized primary neuron cultures and exposed them to hydrogen peroxide to mediate oxidative stress, staurosporine to mediate pro-apoptotic stimulation, and glutamate to mediate excitotoxicity. We established dosages that produce incremental damage to neurons, including a minimal injury insult, based on LDH release (Figure 5A). To establish the impact of these agents on IL-4 induction, we added the agent into the cultured neurons and, at 6 hours of incubation, collected the neurons for RNA analysis by RT-PCR (Figure 5B) and culture medium for IL-4 protein analysis by ELISA (Figure 5C). Out of these three approaches, we found that only glutamate-mediated excitotoxicity promoted neuronal IL-4 gene induction and protein level increase in the culture medium. In agreement with our hypothesis, only a sublethal concentration of 50μM glutamate induced neuronal IL-4 production and release. These results suggest that IL-4 production is not a universal response of neurons to harmful stimuli and that glutamate-mediated responses, likely mediated through activation of glutamate receptors, underlies the induction of IL-4 in neurons.

A

LDH release

B

IL-4 mRNA

C

IL-4
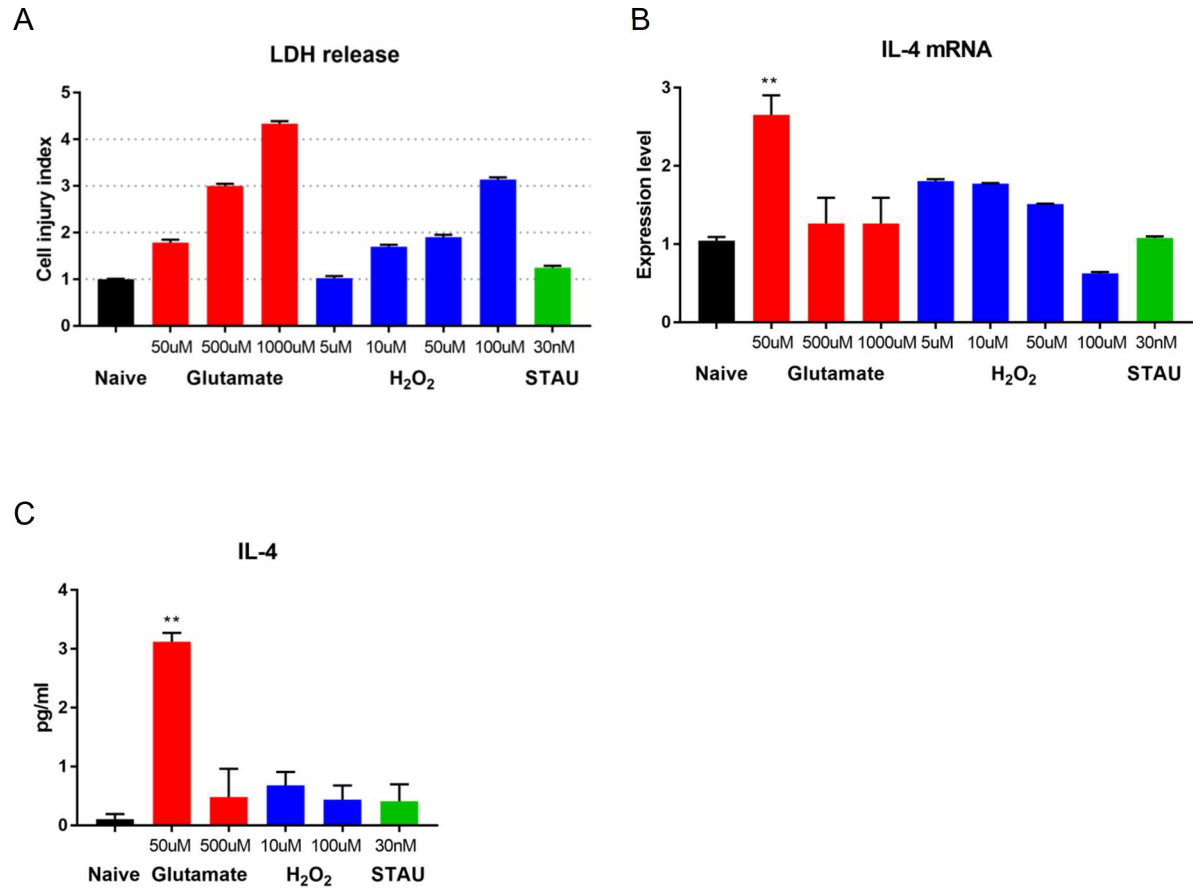
Figure 5. Mild excitotoxicity triggers neuronal IL-4 production. Eleven-day-old primary neuron cultures were incubated with glutamate, hydrogen peroxide (H_2O_2), or staurosporine (STAU) at the indicated concentrations for 6 hours. Culture medium was collected for LDH release via CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) (A) and IL-4 protein analysis via ELISA (eBioscience) (C). The cell injury index was determined by comparing the LDH release of each condition to the naïve control. The cells were collected for IL-4 mRNA analysis using Taqman probe-based real-time PCR system (B). The data are mean±SEM (n=3 independent experiments). **$P<0.01$, compared with the naïve group. Two-way ANOVA followed by Tukey test.

NMDA receptor mediates neuronal IL-4 production

Glutamate receptors include the ionotropic receptors, such as NMDA, AMPA, and kainate receptors, and metabotropic glutamate receptors. Among these receptors NMDAR is recognized as an early and central component of glutamate-mediated ischemic injury (26). Thus, to further investigate the glutamate-mediated neuronal IL-4 production, we investigated two ionotropic glutamate receptors – the NMDA and kainate (KA) receptors. The KA receptor does not appear to play a role in directly promoting ischemic injury (27). Again, we utilized primary neuron cultures and exposed them to varying doses of NMDA and KA. We established the range of concentrations of NMDA and KA that induces lethal or sublethal injury (Figure 6A). In subsequent experiments, we exposed neurons to these predetermined concentrations of NMDA and KA. Six hours later, we harvested the neurons and culture medium for IL-4 gene expression and protein analysis. We found that the sublethal concentration of $50\mu M$ NMDA significantly increased neuronal IL-4 gene expression (Figure 6B) and protein release (Figure 6C) in the culture medium. However, both the lethal concentration of NMDA, and sublethal ($50\mu M$) or lethal ($500\mu M$) doses of KA did not induce neuronal IL-4 production, despite the fact that the sublethal concentration of KA produced similar injury level as the sublethal concentration of NMDA.
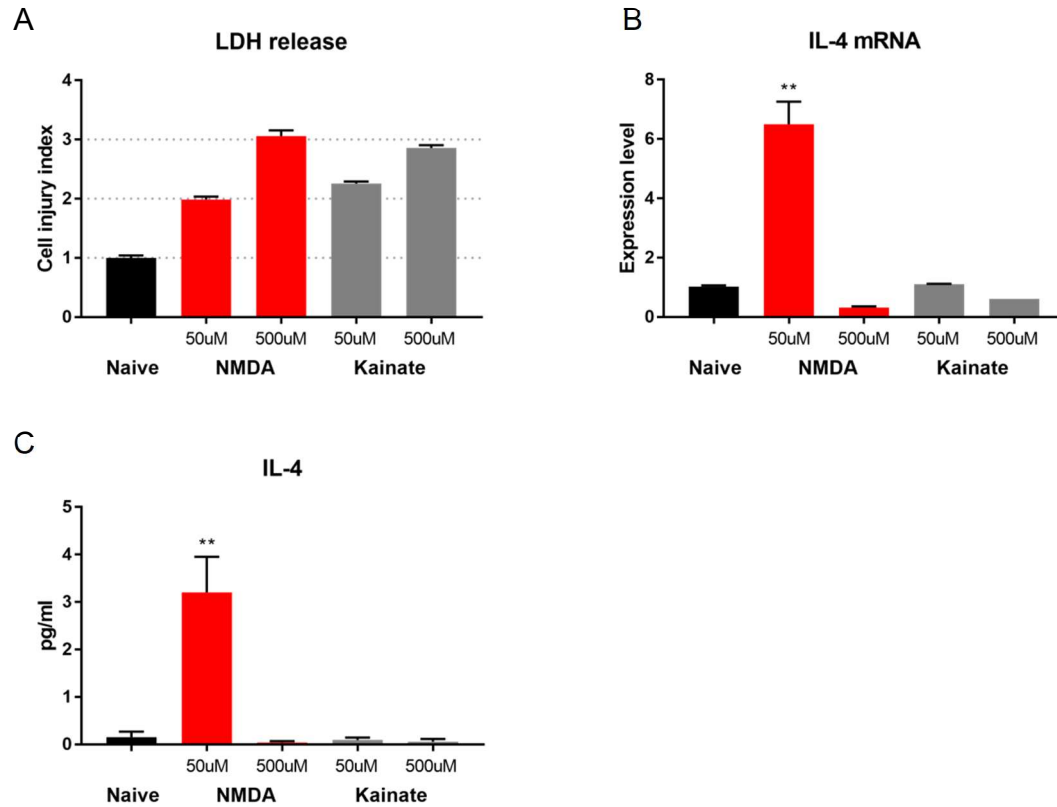
Figure 6. NMDA but not kainate receptor mediates neuronal IL-4 production.
Eleven-day-old primary neuron cultures were incubated with NMDA or kainate at indicated concentrations for 6 hours. At that time, neurons and culture medium were collected for LDH release via CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) (A) and IL-4 protein analysis via ELISA (eBioscience) (C). The cell injury index was determined by comparing the LDH release of each condition to the naïve control. The cells were collected for IL-4 mRNA analysis using Taqman probe-based real-time PCR system (B). The data are mean±SEM (n=3). **$P<0.01$, compare with the naïve group. Two-way ANOVA followed by Tukey test.

Next, to further establish our findings about the essential role of NMDARs in IL-4 induction by neurons, we tested if an NMDA competitive inhibitor, MK-801, can block the effects of NMDA on neurons. We also sought to see if the co-activators of NMDAR, D-serine or glycine, are required. The neuronal cells in culture were pre-incubated with MK-801, D-serine, or glycine for five minutes prior to adding the sublethal concentration of $50\mu M$ NMDA to the cells. The neurons and culture medium were harvested for gene, LDH, and protein analysis at 6 hours after treatment with NMDA. The cytotoxicity index (Figure 7A) showed that 10nM MK-801 was effective in blocking the detrimental effect of NMDA. On the other hand, D-serine or glycine, alone did not cause toxicity to the neurons. However, when combined with $50\mu M$ NMDA, D-serine further exacerbated the excitotoxic insult, while glycine did not. This may be due to the higher affinity of D-serine to the glycine binding site than glycine (28). The results of RT-qPCR (Figure 7B) and ELISA (Figure 7C) showed that MK-801 prevented the neuronal induction of both IL-4 mRNA and protein. D-serine showed no effect on mRNA expression, but it enhanced the presence of IL-4 protein in the medium, which suggests enhanced release. Although not significant, combining NMDA and D-serine further increased IL-4 secretion without elevating mRNA levels. However, glycine did not present similar effects. These results suggest the role of NMDARs in mediating neuronal IL-4 production.

A LDH release

B IL-4 mRNA

C IL-4
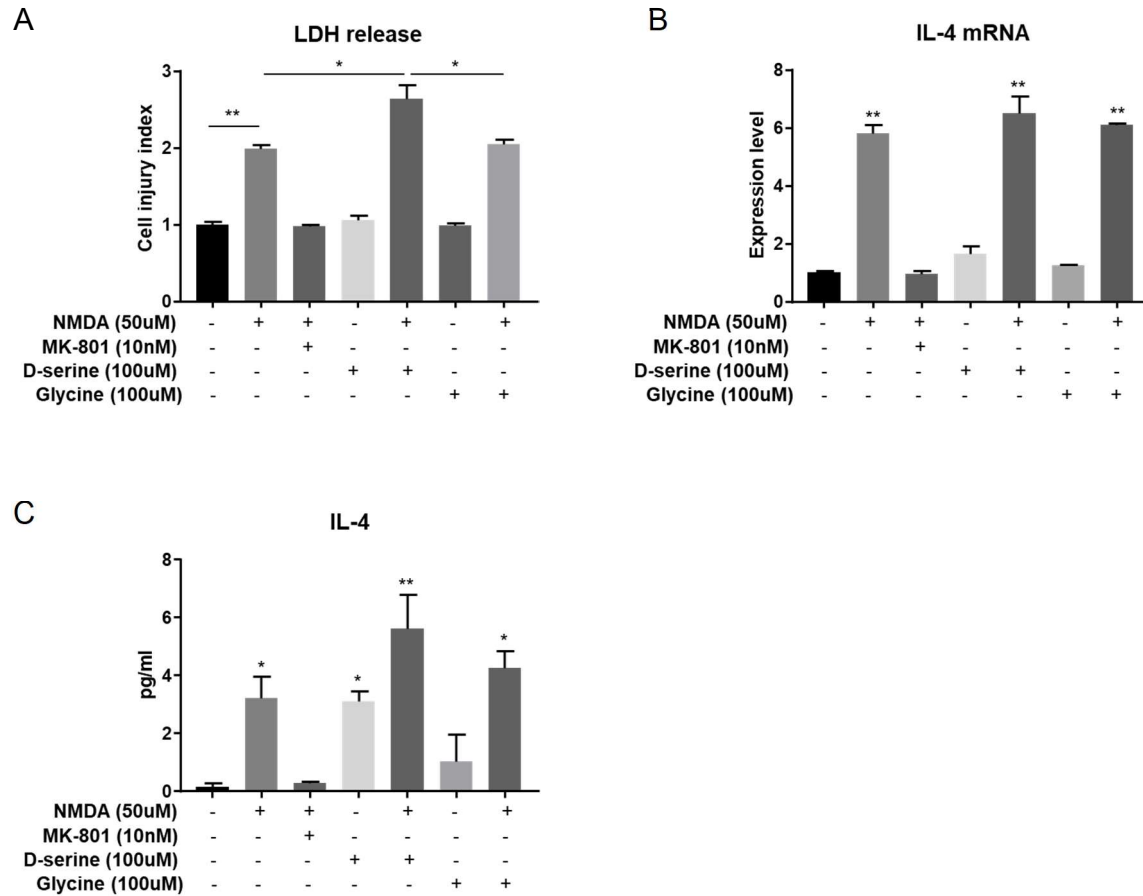
Figure 7. NMDA receptor antagonist, MK-801, prevents the effects of NMDA on neuronal IL-4 induction. Eleven-day-old primary neuron cultures were pretreated with MK-801, D-serine, or glycine and then incubated with 50μM NMDA for 6 hours before harvesting. Culture medium was collected for LDH release via CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) (A) and IL-4 protein analysis via ELISA (eBioscience) (C). The cell injury index was determined by comparing the LDH release of each condition to the naïve control. The cells were collected for IL-4 mRNA analysis using Taqman probe-based real-time PCR system (B). The data are mean±SEM (n=3). *P<0.05, **P<0.01, compare with the naïve group unless indicated in the figure. One-way ANOVA followed by Tukey test.

Nuclear factor of activated T cells (NFAT) regulates neuronal IL-4 production

After establishing the causal role for NMDARs in neuronal IL-4 production, we next sought to explore the mechanisms downstream of the NMDAR that could regulate this process. One potential pathway leading to IL-4 transcription, as shown for some lymphocytes, is through the transcription factor nuclear factor of activated T cells (NFAT) (29). Interestingly, neurons express NFAT family members as part of activity-dependent gene transcription (30) and the activation of NFAT is modulated through dephosphorylation by the calcium-dependent phosphatase calcineurin, an enzyme that, in neurons, could be activated in response to NMDA (31). NFAT activation is also known to be inhibited by calcineurin inhibitors (32).

Thus, in the first experiment we probed a potential role of the NFAT-dependent pathway in synthesis of IL-4 by neurons. We treated cortical neurons in culture with a calcineurin inhibitor, FK-506 (with anticipation to block the activity of NFAT), prior to exposing them to the sublethal concentration or 50μM NMDA. At 6 hours, we collected neurons and culture medium for IL-4 transcription determination and viability assay. The cell viability assay (Figure 8A) revealed that FK-506 alone is not toxic to neurons, even under high concentrations of 100nM. Moreover, in a dose-dependent manner, FK-506 partially blocked excitotoxic stress produced by NMDA. The RT-qPCR analysis (Figure 8B) demonstrated that FK-506 in a dose-dependent manner significantly reduced IL-4 mRNA expression induced by NMDA. IL-4 ELISA (Figure 8C) also showed that FK-506 abolished the presence of IL-4 in the media of cells treated with NMDA.
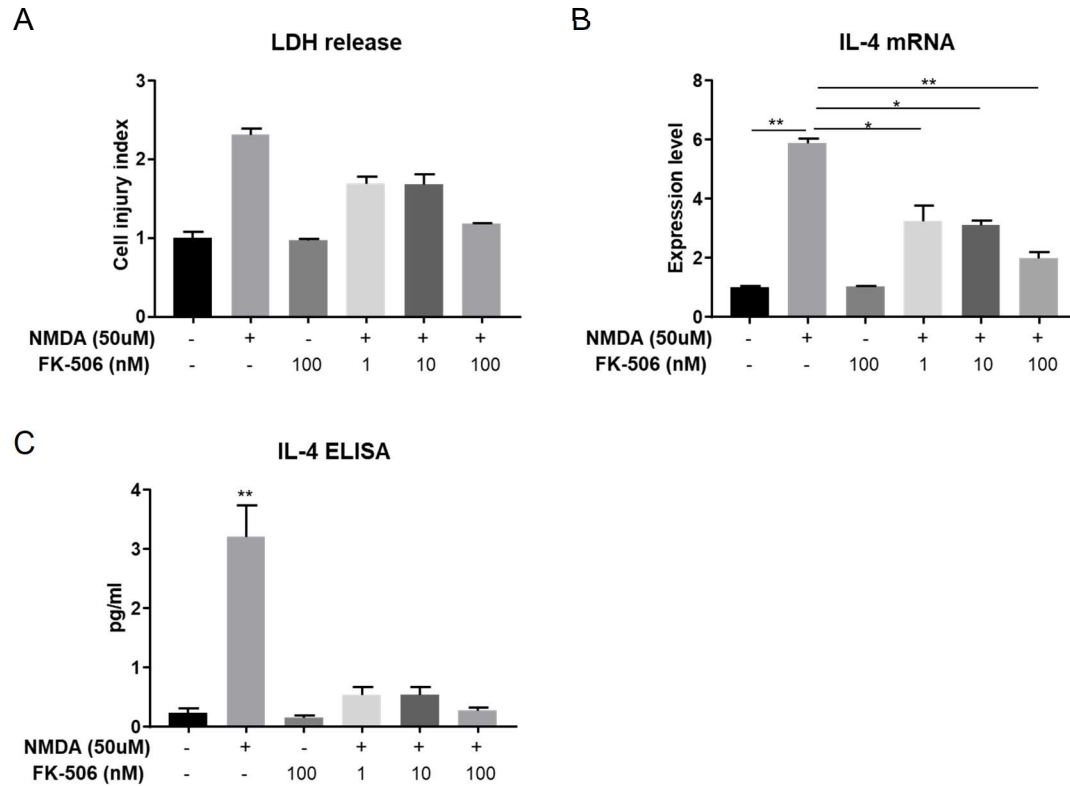
Figure 8. Calcineurin inhibitor, FK-506, prevents IL-4 expression induced by NMDA. Eleven-day-old primary neuron cultures were pretreated with FK-506 and then incubated with 50μM NMDA for 6 hours before harvesting. Culture medium was collected for LDH release via CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) (A) and IL-4 protein analysis via ELISA (eBioscience) (C). The cell injury index was determined by comparing the LDH release of each condition to the naïve control. The cells were collected for IL-4 mRNA analysis using Taqman probe-based real-time PCR system (B). The data are mean±SEM (n=3). *P<0.05, **P<0.01, compared with the naïve group. One-way ANOVA followed by Tukey test.

Our next step was to directly measure the activity of NFAT in neurons to establish whether the calcineurin-NFAT pathway indeed induces neuronal IL-4 gene expression. To achieve this task, we pre-treated neurons in cultures with MK-801, D-serine, or FK-506 before exposing them to NMDA or to sublethal OGD to trigger IL-4 production. The neurons were harvested 6 hours after the treatment with NMDA or OGD. Neuronal nuclei were extracted for electrophoresis mobility shift assay (EMSA) to measure the activity of NFAT which also is the indicative of calcineurin-mediated dephosphorylation of NFAT (prerequisite for activation). This experiment showed that NMDA alone, NMDA with D-serine, or OGD significantly enhanced the activity of NFAT in neurons (Figure 9A). Both MK-801 and FK-506 effectively abolished the NFAT activation caused by NMDA. Also, we found that the activity of NFAT in neurons correlated with IL-4 mRNA expression (R^2=0.926), strongly suggesting that the calcineurin-NFAT pathway acts as a potential regulator to IL-4 gene transcription in neurons (Figure 9B and 9C).
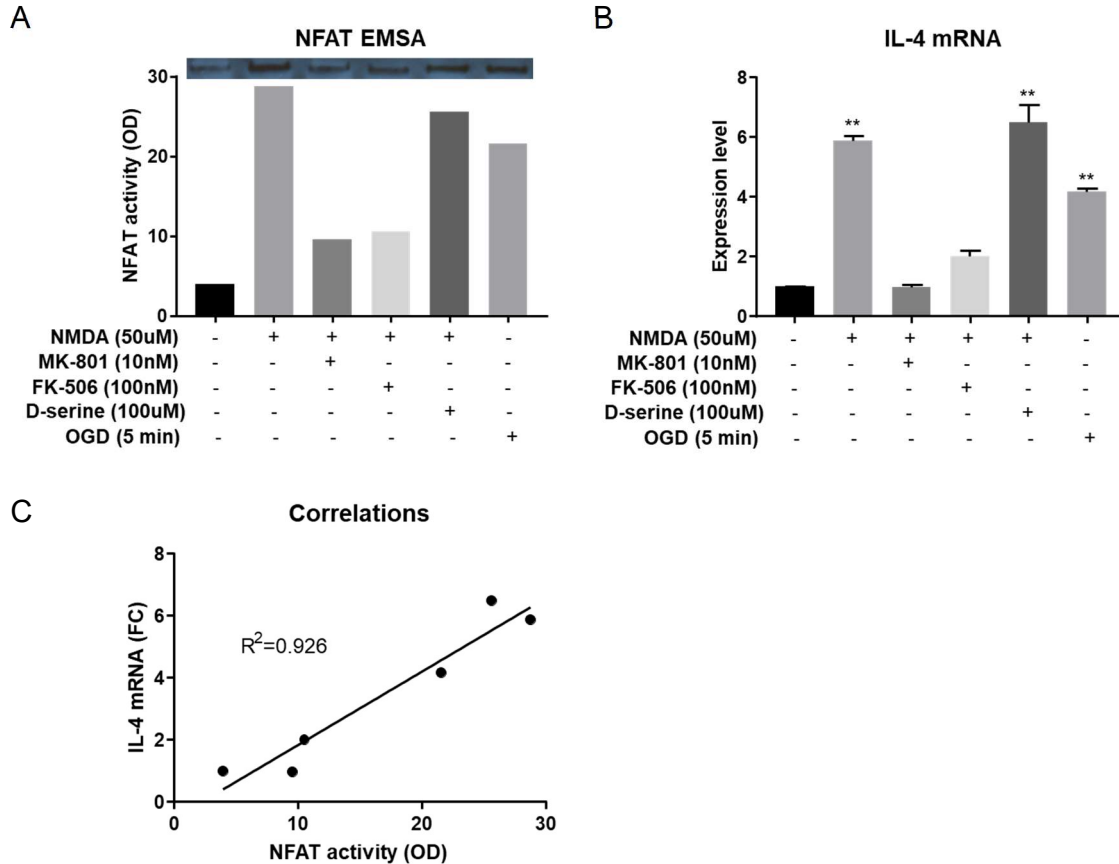
Figure 9. NFAT as a positive modulator of neuronal IL-4 production. (A) The activity of NFAT under various conditions was measured by NFAT EMSA kit (Signosis). (B) IL-4 mRNA expression in neurons using Taqman probe-based real-time PCR system. (C) The expression of IL-4 mRNA showed a strong correlation to the activity of NFAT. The data of IL-4 mRNA expression are mean±SEM (n=3). *P<0.05, **P<0.01, compare with the naïve group. One-way ANOVA followed by Tukey test. Correlations between NFAT activity and IL-4 mRNA, R^2=0.926, p<0.01.

Neuronal IL-4 drives microglia toward a healing type

After investigating the mechanisms underlying the induction of IL-4 production by neurons, we sought to elucidate the potential function of this neuron-secreted IL-4, especially regarding neuron-microglia crosstalk. It is well defined that IL-4 induces alternative activation of macrophages (33), suggesting that the neurons under NMDA stimulation can modify microglia to their alternative type via secretion of IL-4. To test this notion, we performed conditioned medium transfer experiments. The cultures were transiently exposed to NMDA for 30 minutes, followed by immediate wash to remove NMDA, and then the medium was conditioned by neurons for another 6 hours to allow for IL-4 secretion. The conditioned medium, or medium from untreated neurons, was transferred to a primary microglia culture. Fresh neurobasal medium with supplemented recombinant IL-4 (rIL-4) served as a positive control and medium alone was used as a naïve control. In subsequent experiments, to further validate the effect of neuronal-secreted IL-4 on microglia and not another factor, we added an IL-4 neutralizing antibody or IgG isotype control antibody to the conditioned medium. After 24 hours of incubation with conditioned media, the microglia were harvested for gene analysis of selected genes allowing to establish the *status quo* of microglia polarization. The results of relative gene expression are presented as $\log_2 FC$ compared to medium transferred from untreated neuron cultures. GAPDH (Figure 10A) was used as an internal control.

In Figure 10B, CD68 was used as a marker for microglia and remained unchanged throughout all conditions, suggesting that the microglia cell number remained constant. The expression of pro-inflammatory genes, including IL-1β (a pro-inflammatory cytokine, Figure 10C), MMP-9 (a pro-inflammatory matrix protease, Figure 10D), and c-rel (a subunit of NF-κB, Figure 10E), were unchanged in fresh neurobasal

medium and rIL-4-supplemented medium with or without IL-4 antibody, suggesting that IL-4 did not affect pro-inflammatory machinery in microglia. However, all conditioned medium groups had a similar level of expression in these pro-inflammatory genes. IL-1β and MMP-9 expression was higher in the conditioned medium groups than in fresh medium groups. This could be attributed to other substances in the neuron culture medium that activated microglia.

As expected, the expression of PPARγ (Figure 10F), a downstream gene regulated by STAT6 through the IL-4 receptor, was enhanced in the positive control, and the enhancement was abolished by an IL-4 neutralizing antibody. NMDA conditioned medium also enhanced the expression of PPARγ in microglia, which was abolished by an IL-4 neutralizing antibody, suggesting that it was the IL-4 in the NMDA conditioned medium that enhanced the expression of PPARγ in microglia. As we demonstrated previously, pre-treatment of MK-801 suppressed the production of neuronal IL-4 induced by NMDA; therefore, MK-801 pre-treated NMDA conditioned medium failed to induce the expression of PPARγ in microglia. CD36 (Figure 10G), CD204 (Figure 10H), and CD163 (Figure 10I) are scavenger receptors of microglia facilitating phagocytosis, but they are also important markers for the M2 healing phenotype of microglia. The results of gene analysis demonstrated that the expression of these genes followed the same pattern as PPARγ. NMDA conditioned medium enhanced the expression of these scavenger receptors, and IL-4 neutralizing antibody abolished this enhancement. Conditioned medium with the pre-treatment of MK-801 before NMDA also failed to induce CD36, CD204, and CD163 gene expression. Our data demonstrated the function of neuron-derived IL-4 in driving microglia toward a healing type.

A   GAPDH

B   CD68

C   IL-1b

D   MMP9

E   c-rel

F   PPARg
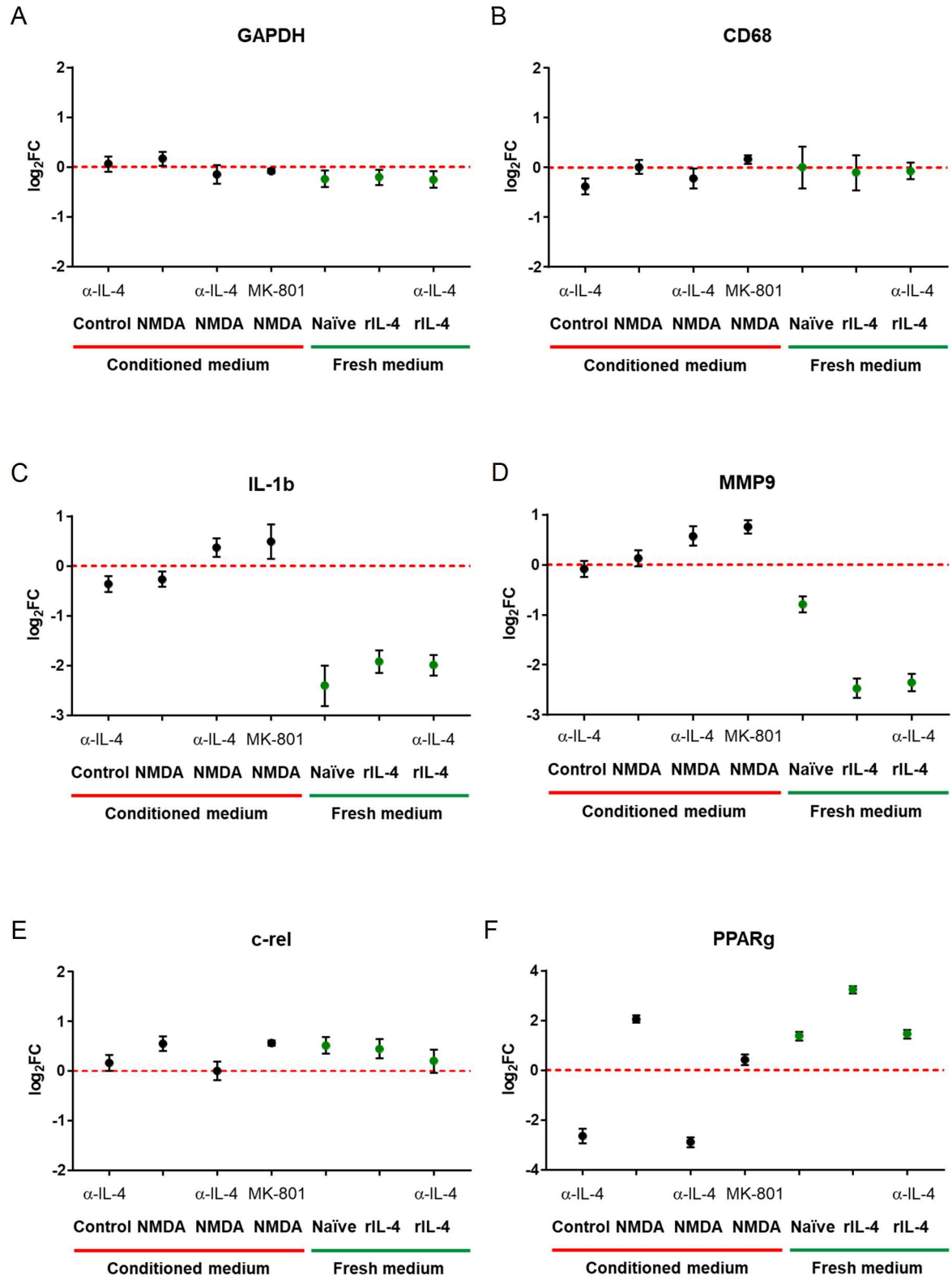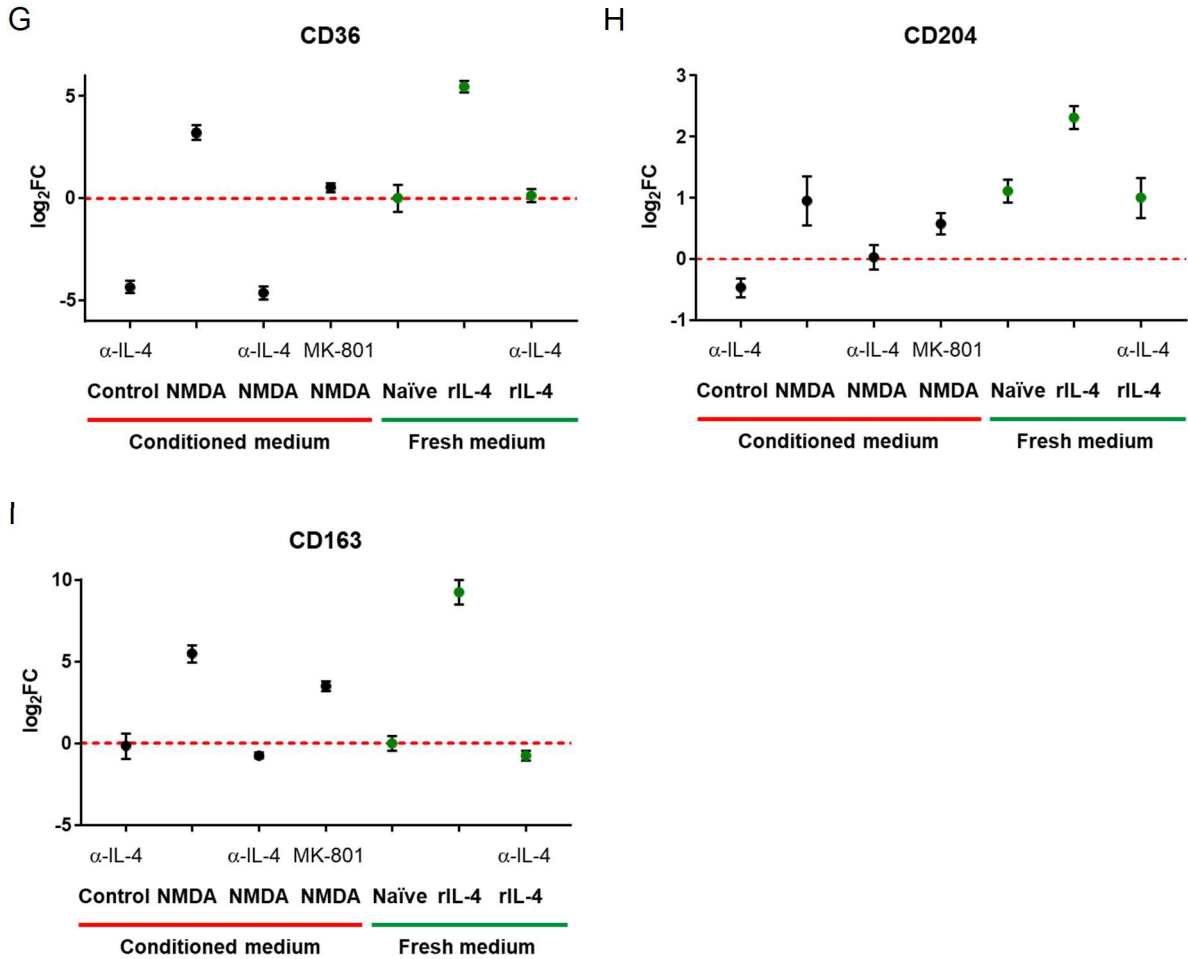
Figure 10. Gene expression profiles of microglia induced by conditioned medium from neuron cultures that were exposed to 50μM NMDA with or without MK-801 pretreatment. IL-4 neutralizing antibody (α-IL-4) or an IgG isotype control was added to the conditioned medium to validate the function of IL-4. Primary microglia cultures were incubated with conditioned medium from neuron cultures (conditioned medium groups) or fresh medium (naïve and positive controls) for 24 hours before being harvested for RT-qPCR. The data are presented as log_2FC (n=3) compared to medium transferred from untreated neuron cultures. GAPDH was used as internal control.

Chapter 4 Conclusions and Discussion

In this study, we propose a new model of interactions between stressed neurons and microglia. We demonstrated that in response to sublethal ischemia, neurons secrete IL-4 as a signal to communicate with microglia to modulate their type. By exploring ischemic injury in neurons (utilizing an in vitro model of ischemia, oxygen glucose deprivation), we found that the excitatory responses mediated by NMDA receptors induces IL-4 transcription through the calcineurin-NFAT pathway. By using neuron-conditioned medium transfer to cultured microglia in presence or absence of IL-4 neutralizing antibody, we showed that neurons indeed produce sufficient amounts of IL-4 to promote microglia polarization. Based on gene-expression profiles, it appears that neuronal IL-4 contributes to the induction of microglial M2 healing phenotype. This type in microglia have been implicated in enhanced phagocytic and tissue healing properties.

It has been reported that neurons subjected to excitotoxic insult (induced by higher concentrations of NMDA) secrete pro-inflammatory cytokines including tumor necrosis factor-α, transforming growth factor-β and IL-6 to communicate with glial cells (34). This study also showed that neurons also secrete IL-4 under sublethal levels of OGD or excitotoxicity. Calcium overload via NMDA receptor is a key contributor to excitotoxic neuronal death (35) and blockade of this receptor during OGD protects neurons from the injury. Normally, the NMDA receptor is activated when both glutamate and the co-activator binding sites are occupied. Thus, prolonged exposure to D-serine in the presence of glutamate could potentially enhance glutamate-induced toxicity to neurons (36).

NFAT is a phosphoprotein and a transcription factor that is activated upon dephosphorylation by Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin. NFAT, through cooperation with many other transcription factors, promotes expression of many target genes (37). The calcineurin-NFAT pathways has also been shown to play an important role in neuronal plasticity and can be inhibited by FK-506 (38, 39). Although the target genes of NFAT in neurons are largely unknown, we showed a strong correlation between the activity of NFAT and IL-4 gene expression, suggesting that IL-4 is a product of NFAT transcriptional regulation in neurons. Specifically, we demonstrated that NMDAR activation with sublethal concentrations of NMDA leads to NFAT activation that also involves calcineurin, as calcineurin inhibition with FK-506 suppressed this process. Interestingly, the calcineurin-NFAT pathways also play an important role in neuronal plasticity and can be inhibited by FK-506 (38, 39).

IL-4 has been recently proposed to be involved in regulatory mechanisms in learning and memory, suggesting an important role of IL-4 in tuning some neuronal activities (40). IL-4 was also demonstrated to have a trophic effect on neurons and increase neuronal survival after *in vitro* axotomy (41). Furthermore, IL-4 could provide therapeutic effects in animal models of ischemic stroke (1, 42, 43). It is reported that brain-residing or infiltrating T cells could also represent a major source of IL-4 at the injury site in the central nervous system (44). Indeed, upon stimulation, T cells in culture produce IL-4 in a range of 500 to 2000 pg/mL (45). However, the recruitment of T cells into the ischemia-affected brain is delayed and occurs within 24 hours from the onset of injury in experimental animal models (46). This T-cell mediated response is significantly delayed as compared to the marked production of IL-4 in less than 6 hours by neurons in the present study and thus may play a more important role during early hours of stroke pathogenesis progression. Considering the complexity of the environment in the

brain, where cells are highly interconnected and the milieu of the extracellular space is rigorously regulated, production of IL-4 by neurons in pathological conditions in response to glutamate (under pathological conditions such as stroke or under conditions involving plasticity such as learning and memory) may be necessary to regulate microglia function regarding their "healing" role in stroke and trophic function in synaptic plasticity. The present study also demonstrates the effect of neuronal IL-4 on M2 differentiation in microglia by our media transfer experiment.

In the M2 "healing" phenotype, microglia functionally produce more trophic factors and possess enhanced phagocytic capacity (47, 48) that could be important to neuronal remodeling and repair. In the area of ischemic injury, the abundance of pro-inflammatory molecules and cytokines may activate microglia and infiltrating hematogenous macrophages toward the classical pathway leading to an M1 polarization (49). My present results show that neurons that suffer sublethal ischemic injury (e.g. simulating ischemic penumbra) secrete IL-4 as a "protect me" signal to potentially promote microglia polarization toward an M2 healing phenotype. This process could help neurons to escape being targeted and damaged by the M1 microglia. M2 microglia secrete trophic factors and clear out ischemic debris to decrease the source of toxicity and inflammation, thereby enhancing tissue healing. Therefore, this study provides a potential target for ischemic stroke treatment.
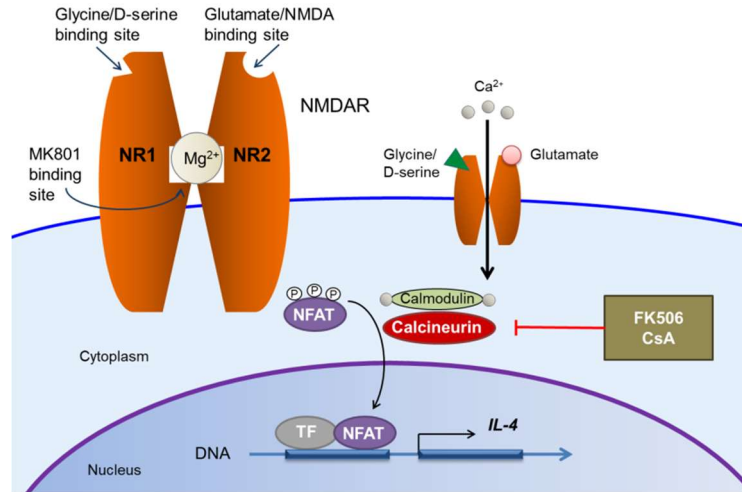
Figure 11. An illustration of proposed mechanisms of neuronal IL-4 production.

The IL-4 produced by neurons under sublethal ischemia is mediated by NMDA receptor

signaling and regulated through the Ca^{2+}/Calmodulin/calcineurin/NFAT pathway.

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