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ORIGINAL PAPER

Interleukin-6 inhibits L-type calcium channel activity of cultured cerebellar granule neurons

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Abstract Our previous work has shown that interleukin-6 (IL-6) implements its neuroprotective effect by inhibiting the intracellular Ca^{2+} overload in neurons. Here, we examined whether regulation of L-type calcium channels (LCCs) activities is involved in the neuroprotective action of IL-6. In cultured cerebellar granule neurons (CGNs), patch-clamp recording showed that the whole-cell Ca^{2+} current and LCC current were significantly reduced by IL-6 pretreatment (120 ng/ml, for 24 h). Calcium imaging data indicated that IL-6 significantly suppressed high K^+-induced intracellular Ca^{2+} overload and LCC Ca^{2+} influx. Moreover, expression of the LCC subunit, $Ca_v1.2$, was remarkably downregulated by IL-6 in cultured CGNs. These findings suggest that IL-6 exerts a neurotrophic effect by preventing Ca^{2+} overload, at least partly through inhibition of LCC activity in cultured CGNs.

Keywords Interleukin-6 · L-type calcium channels · Whole-cell recording · Calcium imaging · Cerebellar granule neurons

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Introduction

Interleukin-6 (IL-6), a member of pleiotropic cytokine family, has complex effects on the central nervous system (CNS) [1]. Under normal physiological conditions, the IL-6 level in the CNS is low. In neural functional disorders, such as brain diseases and injuries, IL-6 expression increases greatly [2–7]. The increased IL-6 may reflect a harmful process as an injurious mediator. For example, IL-6 is a detrimental player in the CNS, contributing to pathogenesis of neurodegenerative diseases, e.g., Alzheimer's and Parkinson's disease [8, 9]. However, the IL-6 increase may also represent a compensative mechanism for neural repair. For instance, IL-6 regulates neuronal function and development in the innate response of the CNS to injury and diseases [10, 11], and exerts neurotrophic and neuroprotective effects on glutamate- and *N*-methyl-D-aspartic acid (NMDA)-induced neuronal damage [12–15]. Hereby, further exploration is needed to understand the roles of IL-6 in brain physiology and pathology.

It is well known that Ca^{2+} is not only an important signaling molecule in neurons, but also a mediator leading to neuronal injury and death when it accumulates in the cytosol of cells, termed Ca^{2+} overload. Neuronal Ca^{2+} overload mainly involves three mechanisms: Ca^{2+} influx through ligand-gated channels, Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) activated by membrane depolarization, and Ca^{2+} release from intracellular store induced by an increase in cytosolic Ca^{2+} [16]. By means of confocal laser scanning microscope (CLSM), we previously found that IL-6 suppressed neuronal intracellular Ca^{2+} overload induced by glutamate or NMDA, and exerted a neuroprotective effect [13, 15]. However, the mechanism underlying the IL-6 suppression of intracellular Ca^{2+} overload is not clear. We hypothesized that IL-6 exerts its neuroprotective

function by suppressing the expression of VGCCs in cerebellar granule neurons (CGNs).

VGCCs are expressed in neurons and have multiple types, such as L-, N-, P/Q-, R-, and T-type Ca^{2+} channels [17–21]. Among these various types of VGCCs, L-type calcium channels (LCCs) are widely distributed on the cell body of neurons in mammalian CNS, including CGNs [22–24]. Calcium influx through LCCs in response to membrane depolarization serves essential functions in the regulation of intracellular Ca^{2+} homeostasis and neuronal excitability [25, 26]. Excessive Ca^{2+} influx through LCCs results in intracellular Ca^{2+} overload, which has been implicated in the pathogenesis of neurodegenerative disorders resulting from brain ischemia [16, 27, 28]. Therefore, in the present study, we firstly focused on LCCs to clarify the mechanism of the neuroprotective effect of IL-6 on LCCs by means of whole-cell patch clamp methods and calcium imaging.

Materials and methods

Isolation and culture of rat CGNs

Primary cultures of CGNs were obtained from neonatal Sprague-Dawley rats (The Center of Experimental Animals, Nantong University, China) at 8 days of age using previously described procedures [29]. Briefly, the cerebellum was removed from rats and minced with sterile surgical blades. The minced cerebellum was chemically dissociated in the presence of trypsin (Amresco, USA) and DNase I (Worthington, USA), and resuspended in the following culture medium: basal Eagle's medium (Sigma, USA), 10 % fetal bovine serum (Amresco, USA), 25 mM KCl, 0.1 g/l gentamicin, and 2.2 g/l $NaHCO_3$, 2.385 g/l HEPES. The samples were plated onto poly-L-lysine-coated glass coverslips (0.32×10^6 cells/ml) for electrophysiological recording, or seeded at a density of 0.8×10^6 cells/ml in 96 wells for calcium imaging or at 2.0×10^6 cells/ml in 6 wells for Western blot, respectively. The cells were incubated at 37 °C with a humidified 5 % CO_2/95 % air atmosphere in an incubator (ESPEC BNA-311, Japan). To inhibit glial proliferation, cytosine arabinoside (Sigma, USA, 10 μM) was added to the cultures 18–24 h after the cells were plated. Rat recombinant IL-6 (R&D Systems, USA) at a concentration of 120 ng/ml was added to the cultures of CGNs for at least 24 h incubation. All experiments described below were performed using the CGNs cultured for 8 days.

Electrophysiological recording

Current through the Ca channel was isolated by blocking the Na channel with TTX and recorded using an Axopatch 200B patch-clamp amplifier (Axon, USA) at room temperature (20–22 °C). The bath solution was composed of TEA-Cl 144, $BaCl_2$ 10, $MgCl_2$ 2, CsCl 3, HEPES 10, glucose 10, 4-aminopyridine 2, and TTX 0.001 (all in mM), and adjusted to pH 7.4 with TEA-OH. Patch pipettes were pulled on a micropipette puller (pp830, Narishige, Japan) to a tip resistance of 3–5 MΩ when filled with internal solution. The pipette solution contained CsCl 140, HEPES 10, EGTA 10, TEA-Cl 5, and Na_2-ATP 2 (all in mM), and was adjusted to pH 7.2 with CsOH. Current responses were low-pass filtered at 1 kHz and analyzed with pClamp10.2 (Axon, USA). Linear components of capacitive and leak currents were subtracted using the P/4 protocol. I_{Ca}, carried by Ba^{2+}, was elicited by a series of command potentials from −60 to +40 mV for 250 ms in 10-mV steps from a holding potential of −80 mV. The whole-cell current densities were defined as peak current amplitude divided by cell capacitance. Nifedipine (Sigma), a blocker for LCCs, was used to determine the proportion of LCC current in the whole-cell current. It was added to 2 ml of bath solution with a final concentration of 10 μM, and 2-min later, the non-L-type channel current was recorded [30]. To determine the voltage-dependent activation property of LCCs, values of currents obtained were normalized to conductance with the form $g = I/(V_m - V_{rev})$, and fitted to a single Boltzmann function of the form $g/g_{max} = 1 - \{1 + \exp[(V_m - V_{1/2})/K]\}^{-1}$, where g is conductance, I is the amplitude of whole-cell LCC current, V_m is the membrane voltage, V_{rev} is the reversal potential, k is the slope factor, and g_{max} is the maximal conductance.

Measurement of intracellular Ca^{2+} fluorescence intensity

Intracellular Ca^{2+} level was quantified by single cell fluo-3 fluorescence intensity as described previously [29] with a small modification. Briefly, cultured CGNs were rinsed twice with balanced salt solution (BSS), then incubated at 37 °C for 45 min in the presence of 5 μM fluo-3/acetoxymethyl ester (Fluo-3/AM, Calbiochem), washed twice again with BSS, and incubated for an additional 20 min prior to imaging. The BSS was composed of (in mM): 145 NaCl, 5.6 KCl, 5 HEPES, 3.6 $NaHCO_3$, 5.6 glucose, and 2.3 $CaCl_2$. Calcium imaging was recorded by CLSM (Leica TCS SPE, Germany). Successive images were collected at 5-s intervals. Fluo-3 fluorescence was excited at 488 nm, and emitted light was measured at 530 nm. Quantification of the fluorescence intensity was performed using TCS-SPE software from Leica. To depolarize neurons and activate VGCCs, neurons were stimulated with high K^+-solution (150 mM KCl), whose composition was the same as that of BSS, but Na^+ was replaced by K^+. When

the high-K^+ solution was applied to stimulate neurons, 100 µl of solution containing 150 mM KCl was added to 100 µl of BSS, and therefore the high K^+ concentration was about 75 mM. Because the concentration of other constituents than K^+ in the high-K^+ solution was the same as that in BSS, the addition of the high-K^+ solution to BSS did not alter the concentration of other constituents such as HEPES, $NaHCO_3$, glucose, and $CaCl_2$. Nifedipine (10 µM) was applied to neurons 25 min before high K^+ stimulation. In one-scanned visual field, 30 neurons were randomly selected to obtain their dynamic intracellular Ca^{2+} levels. Neuronal basal Ca^{2+} fluorescence intensity before high K^+ stimulation was firstly recorded for about 90 s, and then these neurons were stimulated by high K^+ and scanned for 6 min. Neuronal maximal fluo-3 fluorescence intensity after high K^+ stimulation was statistically analyzed. The same experiment was repeated four times.

Western blot assay

For measurement of expression of the LCC subunit, pore-forming α_{1c} (also known as $Ca_v1.2$), the cultured CGNS were lysed by boiling sample buffer (125 mM Tris-HCl, pH 6.8, containing 4 % SDS, 12 % β-mercaptoethanol, and 20 % glycerol). The cell extracts were boiled for 5 min and loaded onto gels in each electrophoresis. After SDS-PAGE, the separated proteins in the gel were electrotransferred onto a PVDF membrane (Millipore) in tris-glycine-methanol buffer. The membrane was blocked in blocking solution (5 % non-fat dry milk in TBS), and then incubated with primary antibody in blocking solution (rabbit anti-α_{1c}, 1:200; Alomone) overnight at 4 °C. After washing with TBS/Tween-20, the membrane was incubated in secondary antibody (1:5,000 dilution) coupled to HRP, washed as above, and visualized by chemiluminescence using the ECL system.

Statistical analysis

Data were analyzed using pClamp 10.2 (Axon Instruments). One-way analysis or Student's t test was used for comparisons, with $p < 0.05$ indicating statistical difference. All data were presented as mean ± standard deviation ($M \pm SD$).

Results

Influence of IL-6 on whole-cell LCC current

Under the condition of Ba^{2+} instead of Ca^{2+} in the bath solution, which reduced the influence of Ca^{2+} current rundown [31], the whole-cell current through the Ca channel, evoked by depolarization from −60 to +40 mV at a holding potential of −80 mV, in neurons pretreated with IL-6 (120 ng/ml) was smaller than that in control neurons (Fig. 1a, b). Statistical analysis of current density displayed that the effect of IL-6 diminishing Ca-channel current was significant between −20 and +10 mV of depolarization (Fig. 1c).

The effect of IL-6 on LCC current was examined using the selective LCC antagonist, nifedipine. In control neurons, depolarization from a holding potential of −80 mV to a test potential of −10 mV evoked an inward Ca-channel current, and perfusion with nifedipine (10 µM) diminished the Ca-channel current (Fig. 2a). This demonstrated that opening of LCCs contributed to the inward current through the Ca channel. In IL-6-pretreated neurons, the depolarization from −80 to −10 mV also evoked an inward Ca-channel current, but the current was smaller than that in control neurons (Fig. 2a), demonstrating an inhibitory effect of IL-6 on Ca-channel current. The nifedipine perfusion also decreased the current through Ca-channel in IL-6-pretreated neurons (Fig. 2a). However, between IL-6-treated and control neurons, the nifedipine-insensitive

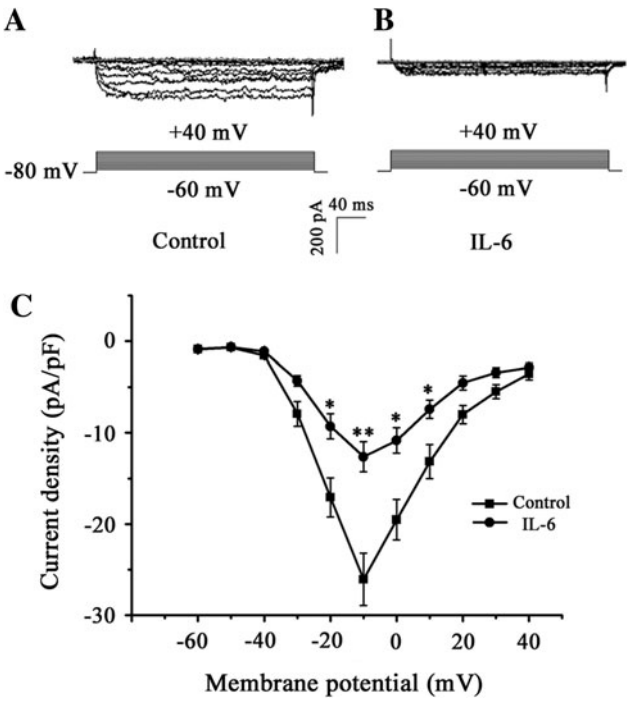

Fig. 1 Effect of IL-6 on whole-cell Ca-channel current in cultured CGNs. The whole-cell inward currents through the Ca channel were evoked by depolarization from −60 to +40 mV at a holding potential of −80 mV. A typical whole-cell inward Ca-channel current in control neuron (**a**) and in IL-6-pretreated neuron (**b**) was exhibited. Statistical analysis of current density displayed that the effect of IL-6 diminishing the Ca-channel current was significant between −20 and +10 mV of depolarization (**c**). $*p < 0.05$, $**p < 0.01$, compared with relative membrane potential of control ($n = 10$)

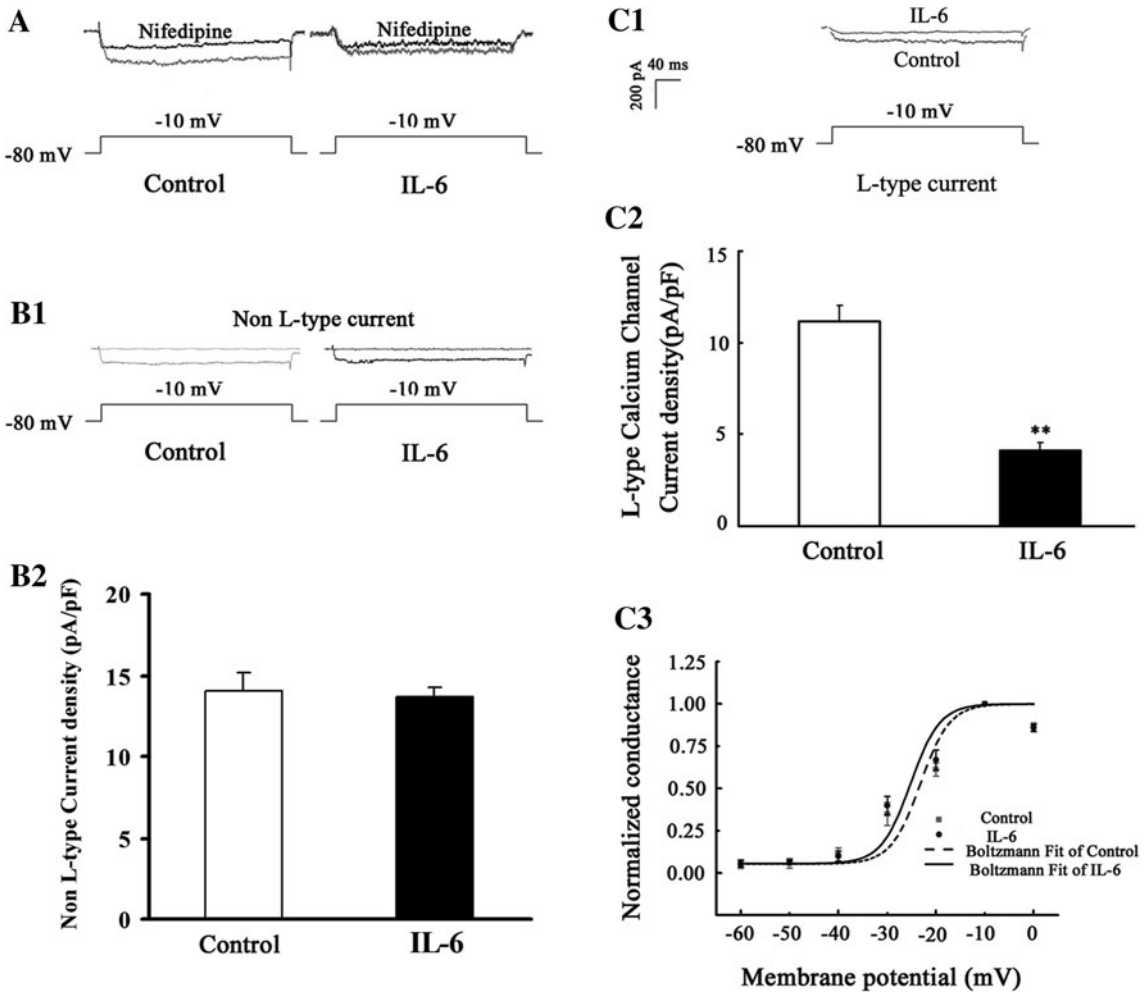

Fig. 2 Influence of IL-6 on whole-cell LCC current in cultured CGNs. Depolarization voltage was set to −10 mV from a holding potential of −80 mV, and whole-cell inward current through the Ca channel was recorded in control and IL-6-exposed neurons. Perfusion of control or IL-6-exposed neurons with 10 μM of nifedipine, a blocker for LCCs, reduced the inward current through the Ca channel (**a**). The inward current after nifedipine action was non-L-type Ca-channel current (**b1**), and it was not significantly different between IL-6-pretreated and control neurons (**b2**). The inward current blocked by nifedipine was the LCC current (**c1**). The LCC current density was evidently lower in IL-6-exposed neurons than in controls (**c2**). $^{**}p < 0.01$, compared with controls ($n = 8$). Voltage-dependent activation curves were obtained by the Boltzmann equation, $g/g_{max} = 1 - \{1 + \exp[(V_m - V_{1/2})/K]\}^{-1}$. The fitted values of $V_{1/2}$ were −25.05 ± 1.93 and −26.84 ± 1.64 mV, and the k (slope factor) was −5.84 ± 1.81 and −4.75 ± 1.30 in control and IL-6-treated neurons, respectively. No significant differences in the data were found between IL-6-treated and control neurons (**c3**, $n = 6$)

Ca-channel current was not significantly different (Fig. 2b), indicating that IL-6 did not alter the non-L-type Ca-channel current. On the other hand, the nifedipine-sensitive Ca-channel current was remarkably suppressed by IL-6 exposure (Fig. 2c). This revealed that the suppressive effect of IL-6 on the Ca-channel current was a result of its inhibition of LCCs. Moreover, to examine whether the voltage-dependent activation property of I_{LCC} was modified by IL-6 exposure, we calculated normalized conductance of LCCs using Boltzmann's equation. The value of the reversal potential was close to 60 mV. The fitted values of $V_{1/2}$ were −25.05 ± 1.93 and −26.84 ± 1.64 mV, and the slope factors were −5.84 ± 1.81 and −4.75 ± 1.30 in control and IL-6-treated neurons, respectively. These data showed that neuronal voltage dependence on activation was not changed following incubation of the neurons with IL-6 (Fig. 2c3).

Effect of IL-6 on high K^+-evoked $[Ca^{2+}]_i$ increase

To further demonstrate the effect of IL-6 on LCCs, we measured dynamic changes of intracellular Ca^{2+} fluorescence intensity in cultured CGNs by CLSM. In control neurons, depolarization stimulation by high K^+ evoked an acute elevation of intracellular Ca^{2+} level (Fig. 3). In IL-6-pretreated neurons, high K^+ stimulation evoked significantly less elevation of the intracellular Ca^{2+} level than in control neurons (Fig. 3), indicating that IL-6 suppressed

high K^+-induced intracellular Ca^{2+} overload. After exposure to nifedipine (10 μM), an LCC antagonist, for 25 min, high K^+ stimulation resulted in a reduction of intracellular Ca^{2+} overload compared with control neurons lacking nifedipine exposure (Fig. 3). This suggests that the reduction of intracellular Ca^{2+} was attributable to a reduction of Ca^{2+} influx through LCCs. However, the inhibitory effect of nifedipine on high K^+-induced intracellular Ca^{2+} overload did not have a notable difference in the presence and the absence of IL-6 (Fig. 3). This indicated that IL-6 did not significantly alter nifedipine-resistant Ca^{2+} overload components and therefore suggested that IL-6 exerted its suppressive effect on high K^+-evoked intracellular Ca^{2+} overload by attenuating nifedipine-dependent LCC Ca^{2+} influx.

IL-6 downregulates protein expression of LCC subunit

Expression of the LCC subunit, pore-forming α_{1c} (also known as $Ca_v1.2$), in cultured CGNs was measured in order to reveal the mechanism underlying IL-6 suppression of the LCC current and LCC Ca^{2+} influx. The LCC subunit protein expression was remarkably downregulated by IL-6 pretreatment (Fig. 4). This showed that via the downregulation, IL-6 carried out its inhibitory effect on LCC function.

Discussion

In this study, IL-6 pretreatment of cultured CGNs significantly reduced the inward current through the Ca channel evoked by depolarization from −20 to +10 mV at a holding potential of −80 mV, suggesting that IL-6 inhibits VGCC opening. To examine the contribution of LCCs, a type of VGCCs, to the inward Ca-channel current, we used nifedipine to block LCCs and found that the inward Ca-channel current was diminished. This suggests that depolarizing stimulation causes opening of LCCs and consequent influx of Ca^{2+} current in cultured CGNs. The report that extracellular Ca^{2+} influx occurs not only directly through the glutamate-activated membrane channel, but also indirectly through activated VGCCs by membrane depolarization [32] supports our present results. Importantly, after neurons were pretreated with IL-6, the effect of the nifedipine-sensitive inward Ca-channel current was significantly suppressed. The result suggests that IL-6 inhibits LCC activity. Some other cytokines, such as interleukin-1β, tumor necrosis factor α, and ciliary neurotrophic factor, have been reported to modulate various types of VGCC currents in neurons [30, 33, 34]. Thus, our present data provide more evidence for IL-6 regulating the LCC current in cultured CGNs.

To further demonstrate the modulation of LCC activity by IL-6, we observed the influence of IL-6 on intracellular Ca^{2+} overload evoked by high K^+-depolarization stimulation in cultured CGNs. The IL-6 pretreatment significantly reduced the high K^+-evoked intracellular Ca^{2+} overload. The result is consistent with the data obtained from the patch-clamp experiments and demonstrates that IL-6 inhibits VGCC activity. In our previous work, we indicated that IL-6 suppresses glutamate- or NMDA-induced intracellular Ca^{2+} overload and neuronal apoptosis in cultured CGNs, and therefore suggest that IL-6 has a neuroprotective effect [13, 15, 29]. Here we add evidence for the IL-6 neuroprotection at the profile of its suppression of VGCCs. Further, we hypothesized that the inhibitory effect of IL-6 on VGCC-dependent Ca^{2+} influx is mediated by LCC-activity suppression. We observed that nifedipine attenuated intracellular Ca^{2+} overload triggered by high K^+-depolarization stimulation, demonstrating that LCC opening is involved in the high K^+-induced intracellular Ca^{2+} overload. The inhibitory effect of nifedipine on intracellular Ca^{2+} overload occurred similarly in IL-6-exposed and control neurons. It indicates that IL-6 does not significantly alter the nifedipine-insensitive Ca^{2+}-influx component. Therefore, the suppression of intracellular Ca^{2+} overload by IL-6 is attributed to its suppression of the nifedipine-sensitive Ca^{2+}-influx component. These findings are consistent with the conclusion from the whole-cell recording that IL-6 suppresses LCC activity. Thus, we suggest that IL-6 neuroprotection through suppression of intracellular Ca^{2+} overload is implemented, at least partly, by the inhibition of the LCC current.

Since the voltage-dependent property of I_{LCC} was not modified by IL-6 pretreatment in the current study, the mechanism underlying the IL-6 inhibition of LCC activity needs to be explained. We found that expression of the LCC pore-forming subunit $Ca_v1.2$ was significantly downregulated by IL-6 exposure in cultured CGNs. The downregulation reached 60 %, and it was quite consistent with the reduction in I_{LCC} peak current density in IL-6-treated neurons. On the basis of these findings, we suggest that the suppression of LCC function by IL-6 is related to a decrease in LCC protein expression.

As we previously reported [13, 15, 29], the present study represents a neuroprotective role of IL-6. However, since IL-6 is a pleiotropic cytokine, it exerts neurotrophic and neuroprotective effects, and yet can also function as a mediator of inflammation, demyelination, and astrogliosis, depending on the cellular context [35]. Therefore, the dosage of IL-6, degree of neuronal damage, type and environment of neurons, and existence of soluble IL-6 receptors can influence IL-6 effects [36, 37]. For example, Nelson et al. [38] showed that a lower dose of IL-6 (5 ng/ml) exposure enhances the mean amplitude of the Ca^{2+} signal

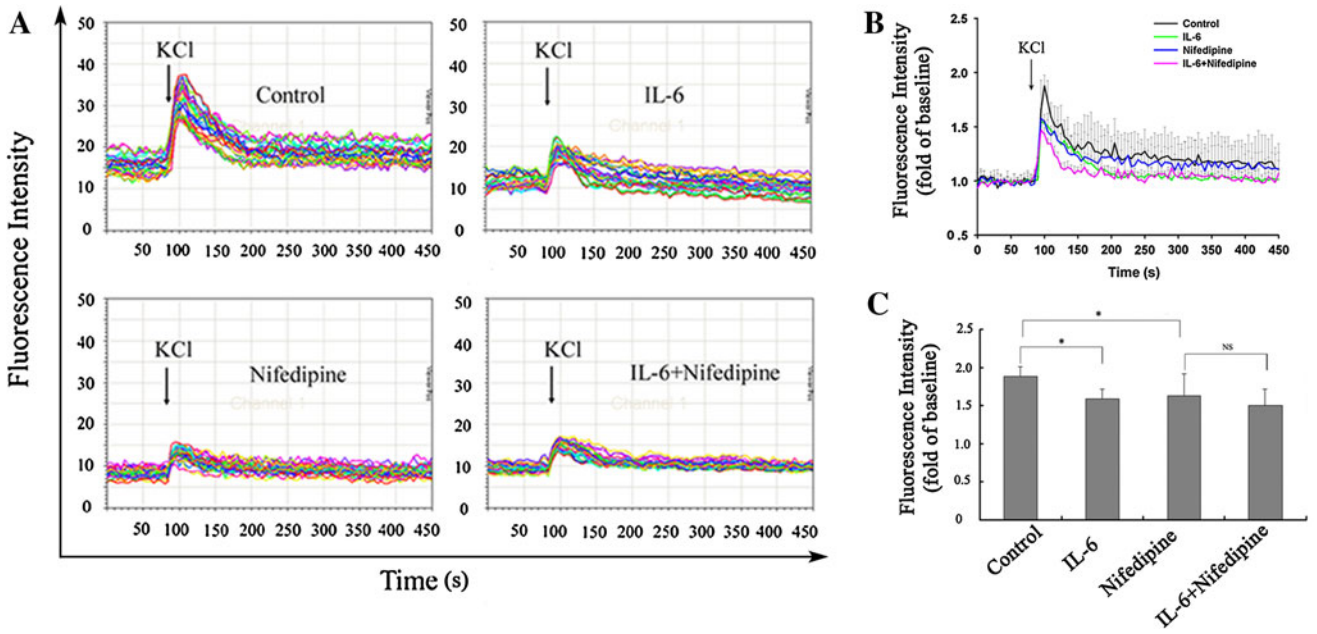

Fig. 3 Role of LCCs in IL-6 suppressing high K^+-trigged intracellular Ca^{2+} overload. LCC blocker nifedipine (10 μM) treated neurons for 25 min before high K^+-stimulation. The neurons were incubated at 37 °C for 45 min in the presence of 5 μM of Fluo-3/AM, and then dynamic changes in intracellular Ca^{2+} levels were tested by CLSM during the whole 6-min high-K^+ stimulation. In each treatment, 30 neurons were randomly selected to analyze dynamic intracellular Ca^{2+} levels (**a**). The compilation of data for the mean and SD of four separate experiments as in **a** is presented in **b**. The peak intracellular Ca^{2+} levels following high K^+ stimulation were compared for statistical significance of the differences between the various treatments (**c**). The *arrows* denote the beginning time when KCl was applied. $*p < 0.05$ and *NS* means no significant difference

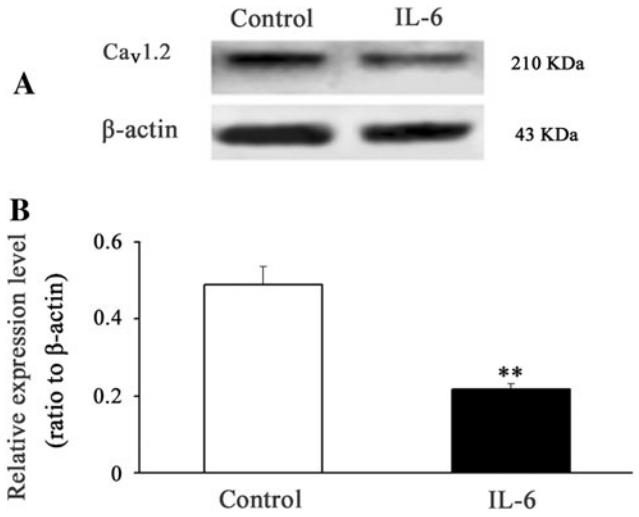

Fig. 4 IL-6 downregulates LCC subunit expression in cultured CGNs. The CGNs from 8-day-old rats were incubated for 7 days and then exposed to IL-6 (120 ng/ml) for 24 h. The protein expression of the LCC subunit, pore-forming α_{1c} (also known as $Ca_V1.2$), was significantly downregulated by IL-6 pretreatment (**a**). The data are from three separate experiments (**b**). $**p < 0.01$, compared with control

in response to glutamate receptor agonists in cultured cerebellar Purkinje neurons, whereas a higher concentration of IL-6 (10 ng/ml) has no effect on the Ca^{2+} signal in response to the same agonists. On the other hand, Vereyken et al. [39] report that transient high-K^+ stimulation (0.5 s) enhances the Ca^{2+} signal, but longer high-K^+ stimulation (>1 s) attenuates the Ca^{2+} signal in IL-6-treated neurons. In addition, NMDA infusion into rat striatum results in a decrease in striatal cholinergic and GABAergic neurons, and co-infusion of IL-6 and NMDA reduces the loss of cholinergic neurons, but fails to prevent the loss of GABAergic neurons [37]. These differences of response to IL-6 among different IL-6 dosages, neuron-damaged degrees, and neuronal types explain the distinct and complex effects of IL-6, neuroprotective, neuroinjured, or non-effective. Further exploration is needed to clarify the mechanisms underlying the different effects of IL-6.

In general, in the presence of IL-6 receptor, IL-6 acts on target cells and promotes dimerization of gp130, a signal-transducing subunit coupled with IL-6 receptor. CGNs have been reported to express IL-6 receptor and gp130 signal protein [40, 41]. In our previous work, anti-gp130 antibody blocked the inhibitory effect of IL-6 on glutamate-induced intracellular Ca^{2+} overload, indicating that the IL-6 receptor is involved in the neuroprotective effect of IL-6 [29]. On the basis of these findings, we suggest that the suppressed LCC activity caused by IL-6 is mediated by the IL-6 receptor.

In conclusion, we revealed that IL-6 inhibits the activity of LCCs in cultured CGNs and this inhibition is associated with downregulation of LCC protein expression. These results imply that a neuroprotective role of IL-6 in the CNS is implemented, at least partially, by suppression of the neuronal LCC current and therefore a reduction in intracellular Ca^{2+} overload.

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