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Involvement of N-type Ca^{2+} channel in microglial activation and its implications to aging-induced exaggerated cytokine response

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ABSTRACT

Voltage-dependent calcium channel (VDCC) is generally believed to be active only in excitable cells. However, we have reported recently that N-type VDCC (Cav2.2) could become functional in non-excitable cells under pathological conditions. In the present study, we show that Cav2.2 channels are also functional in physiological microglial activation process. By using a mouse microglial cell line (MG6), we examined the effects of a Cav2.2 blocker on the activation of MG6 cells, when treated with lipopolysaccharide (LPS) / interferon γ (IFNγ) or with interleukin-4 (IL-4). As a result, blocking the activation of Cav2.2 enhanced so-called alternative activation process of microglia (transition to *neuroprotective* M2 microglia) without changing the efficacy of the transition to *neuroinflammatory* M1 microglia. This enhanced M2 transition involved the activation of a transcription factor hypoxia inducible factor 2 (HIF-2), since a specific blocker of HIF-2 completely abolished this enhancement. We then examined whether Cav2.2 activation was involved in aging-related neuroinflammation. Using primary culture of microglia, we found that the efficacy of microglial M1 transition was enhanced but that M2 transition was reduced by aging, in agreement with a general notion that aging induces enhanced neuroinflammation. Finally, we show here that the moderate blockade of Cav2.2 expression in microglia restores this age-dependent reduction of microglial M2 transition and reduces the aging-induced exaggerated cytokine response, as revealed by a fast recovery from depressive-like behaviors in microglia-specific Cav2.2 deficient mice. These results suggest a critical role for microglial Cav2.2 channel in the aging-related neuroinflammation.

1. Introduction

Multiple types of VDCCs are known to be present in many excitable cells [1]. The VDCCs are composed of several subunits including α_1, α_2/δ and β subunits [2,3]. Of these subunits, α_1 is the most critical component, since it is the pore-forming subunit, harbors the voltage sensor regions, and determines the basic characteristics of each VDCC subtype [4–6]. In the mammalian genome, there are 10 genes encoding an α_1 subunit of the VDCC and they are classified into three families (Cav1, Cav2, and Cav3) according to their sequence similarities [2]. All the members of the Cav2 family (Cav2.1, Cav2.2, and Cav2.3) are expressed in the central nervous system (CNS) and play important roles in maintaining normal functions of the CNS [1,7]. We previously created a mutant mouse line carrying a knockout allele for the α_{1B} gene (*Cacna1b*) encoding α_1 subunit of Cav2.2 (N-type) channel. The Cav2.2 knockout mouse has shown several neurological abnormalities including pain sensitivities [8]. In the studies aimed at identifying the downstream factors responsible for the reduced pain symptoms in the Cav2.2 knockout mice [9–14], we have obtained evidence that Cav2.2 channels are expressed and functional in non-excitable microglial cells and that microglial Cav2.2 channels play a role in inducing allodynia accompanying neuropathic pain [15]. However, the exact role for Cav2.2 channels in microglial functions remains to be clarified.

Macrophages are the essential player of the cell defense system (natural immunity), and can change their characteristics dramatically depending on the surrounding microenvironment. To describe these multiple phenotypes of macrophages, the classification of pro-inflammatory M1 type and anti-inflammatory M2 type has originally been introduced [16–18]. Although substantial amounts of following reports have indicated the presence of other subtype states in macrophages [19], the M1/M2 classification of macrophages is still often used for convenience.

Microglia are often considered as the resident macrophages of the brain, since microglia and macrophages share many common features with respect to their functions. Therefore, the classification of *neuroinflammatory* M1 microglia and *neuroprotective* M2 microglia has

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emerged by analogy with the M1/M2 classification of macrophages [20], though their origins are completely different. Brain injury or brain infection initially induces the pro-inflammatory M1 type activation of microglia. The M1 microglia release several inflammatory cytokines including IL-1β and tumor necrosis factor α (TNF-α), activate inducible nitric oxide synthase (iNOS) to release nitric oxide for killing the pathogens such as bacteria and viruses, and engulf the debris for clearance. In resolving inflammation, microglia change their characteristics to adopt an alternatively activated M2 phenotype. The M2 microglia are known to release cytokines important for repairing and protecting damaged neurons [20]. Accumulated evidence indicates that failure of this conversion of microglial activation states (firstly M1 activation followed by M2 activation) occurs in the aging brain and thereby tends to prolong the microglial M1 states, leading to neuronal damages [21]. However, the mechanisms underlying the aging-related abnormality in microglial activation are not yet fully understood.

In the present study, we investigated the effects of blocking microglial Cav2.2 channels on microglial activation profiles and found that the microglial Cav2.2 is a negative regulator of M2 activation in microglia. We also investigated the effects of knockdown of Cav2.2 on the aging-related alterations in microglial activation patterns. We report here that Cav2.2 channels in microglia are critically involved in the aging-related changes in microglial properties.

2. Materials and methods

2.1. Reagents

LPS, HIF-C2, and tamoxifen were purchased from Sigma-Aldrich (Cat. No. L4516, SML0883, and T5648, respectively). Murine IFNγ and murine IL-4 were purchased from PeproTech (Cat. No. 351-05 and 214-14, respectively). ω−conotoxin GVIA (ωCgTXGVIA) was purchased from Alomone Lab (Cat. No. C-300).

2.2. Antibodies

Rabbit anti-iNOS antibody was obtained from BD Biosciences (Cat. No. 610332), goat anti-arginase 1 (Arg1) antibody was obtained from Santa Cruz Biotechnology (Cat. No. SC-18354), Alexa Fluor 488 labeled anti-rabbit IgG antibody was obtained from Thermo Fisher Scientific (Cat. No. A-21206), Cy3-labeled anti-rabbit IgG antibody was obtained from Jackson ImmunoResearch (Cat. No. 111-165-144), and Alexa Fluor 555-labeled anti-goat IgG antibody was obtained from Thermo Fisher Scientific (Cat. No. A-21432).

2.3. Mice

Animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permission No. A2018-054A and A2018-093A). Transgenic mice where microglial Cav2.2 expression can be suppressed by tamoxifen treatment (Tg(Itgam-cre/ERT2,U6-RNAi:Cacna1b)17Ttan) were described previously [15]. In the present study, the transgenic mice with a C57Bl/6 background were used. In normal condition, ~70% of the microglia express Cav2.2 and after tamoxifen treatment, ~40% of the microglia still express Cav2.2. Thus, the knockdown efficiency was calculated to be ~43% [15]. On the other hand, in the pathological condition, knockdown efficiency was reduced to be ~24 [15].

2.4. Cell culture

MG6 cells were a generous gift from Dr. Kitani and were obtained from RIKEN BRC cell bank. Method of culturing MG6 cells was described previously [22]. Briefly, cells were cultured on petri dishes (Falcon 1007, BD) in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (BioWest), 10 μg/ml insulin, 1 mM L-glutamine and 100 μM 2-mercaptoethanol and incubated at 37 °C under 5% CO_2 in humidified air. For immunocytochemistry experiments, MG6 cells cultured on petri dish for 3 days, were reseeded onto poly-L-lysine coated 8-well glass slide (Lab-Tek II chamber slide system, Nunc) at the densities of 5×10^4 and 1.25×10^4 /well for LPS/IFNγ experiments and IL-4 experiments, respectively, and were cultured for one day. Cells were then pre-treated with 200 nM of ωCgTXGVlA and/or 10 μM of HIF-C2. After 1 h, LPS (25 ng/ml) + IFNγ (5 ng/ml) or IL-4 (50 ng/ml) were further added to stimulate the cells for 24 h. At the end of the incubation period, the culture medium was recovered for ELISA experiment and stored frozen at −80 °C until use and the cells were subjected to immunocytochemistry. For RNA experiments, cells were cultured in essentially the same way and at the end of the culture period they were removed from the slides by suspending them in HEPES-buffered saline containing 0.1 mM EDTA. Cells were collected by brief centrifugation, frozen in liquid nitrogen, and stored at –80 °C until use.

Primary microglial culture from adult mice was performed in essentially the same way as described previously [23]. In brief, mouse brain was excised after cardiac perfusion with ice-cold phosphate buffered saline (PBS) and minced by chopping it with a razor blade. Then cell suspension was obtained after papain treatment followed by trituration. The cell suspension was then subjected to Percoll density gradient centrifugation. Cell pellet containing microglia was rinsed with red blood cell lysis buffer and finally filtered through 10 μm nylon mesh (pluriStrainer, pluriSelect Life Science). Microglial cells were plated onto poly-L-lysine coated 8-well glass slide and cultured for 3 days. Treatment of microglial cells with LPS/IFNγ or IL-4 and subsequent immunocytochemical and RNA analyses were essentially the same as in the case of MG6 experiments described above. For unknown reasons, the viability of microglial cells from old mice (both WT and Cav2.2-MGKD) was 3–4 times higher compared to that from young mice, as assessed by the density of viable cells at the end of the culture period.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS, and then subjected to immunocytochemistry. Primary antibodies used were a rabbit anti-iNOS antibody (1:500 dilution) and a goat anti-Arg1 antibody (1:200 dilution). Primary antibodies were incubated overnight at 4 °C, were washed with PBS containing 0.1% Triton X-100, and were then detected with appropriate fluorescent secondary antibodies. Hoechst33258 was used for nuclear staining. Photographs were taken by BZ-9000 Fluorescence Microscope (Keyence Corp.) equipped with a 20X objective lens. Image analysis was performed with Hybrid Cell Count software (Keyence Corp). To obtain one quantification datum point, images of MG6 cells from randomly selected two areas in one culture well of a chamber slide were analyzed. In the case of microglial cells of primary cultures, images from randomly selected three areas were analyzed in the same way.

2.6. Measurement of IL-10

The amount of IL-10 in culture medium (100 μl) was measured by an ELISA kit (R&D) according to the manufacturer's instructions.

2.7. Quantitative RT-PCR (qRT-PCR)

RNA was prepared from MG6 cells by using RNeasy® Mini Kit (Qiagen) and from primary microglial cultures by RNeasy micro kit (Qiagen) according to the manufacturer's instructions. First strand cDNA synthesis was performed using the Superscript VILO kit (Thermo Fisher Scientific). qRT-PCR was performed using TaqMan® Gene expression assays (Mm01333678_m1 for *Cacna1b* and Mm99999915_g1

for *GAPDH*; Thermo Fisher Scientific) on a 7500 real time PCR system (Applied Biosystems). Absolute copy number of each cDNA was measured using plasmids containing a cDNA of respective genes as a standard sample. Then the ratio of *Cacna1b/GAPDH* was calculated for normalization.

2.8. Behavioral experiments

Mice (> 2-year-old, female) were pre-treated with tamoxifen (40 mg/kg, ip) for 5 days, and then they received LPS injection (Day 0, 800 μg/kg, ip) several days after the last tamoxifen injection. Body weight and food consumption were measured daily from Day –1 for 7 days. Behavioral tests assessing social interaction were performed once before the LPS injection and 3 times after the LPS injection (6, 24 and 72 h post-LPS) [24,25]. Social interaction test was performed under 650 lx room lighting in a sound-proof room after acclimatization for at least 1 h. Mouse behaviors were recorded with a video camera set above the home cage. After recording spontaneous locomotor activity of the host mouse for 5 min, a female mouse with a smaller size (intruder) was put into the home cage of the host mouse and their behaviors were recorded for 10 min. The spontaneous locomotor activity was analyzed by counting the number of line crossings made by the mouse. Upon analyses of the videos, mouse cage was divided into 2 equal compartments and the number of translocations (from one compartment to the other) made by each mouse was counted as line-crossings. The social interaction behavior was evaluated by measuring the total duration when the host mouse followed or sniffed at the intruder.

2.9. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). For multiple comparisons, Tukey-Kramer test was performed to evaluate the statistical significance. Differences between two groups were analyzed by Student's *t*-test or Welch's *t*-test. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Blockade of microglial Cav2.2 enhances M2 activation

Treatment of the microglial cells with LPS and IFNγ induces M1 activation and expression of M1-specific genes like iNOS and TNF-α. On the other hand, treatment of the cells with IL-4 induces M2 activation and expression of genes like Arg1 and IL-10. We first tested whether the N-type VDCC is involved in the microglial activation process, by applying an N-type channel blocker, ωCgTXGVIA, to a mouse microglial cell culture. When the MG6 cells [22] were treated with LPS/IFNγ for 24 h, cell density was decreased compared to the control possibly because of the cell death induced by the released inflammatory cytokines but almost all the cells that survived expressed iNOS (Fig. 1A-C). Pretreatment of the same cells with ωCgTXGVIA had no effects on this process (Fig. 1A-C). When the MG6 cells were treated with IL-4 for 24 h, Arg1 expression was induced in some of the cells (~21%), with the cell density almost the same as control (Fig. 1D-F). However, pretreatment with ωCgTXGVIA greatly enhanced the Arg1 expression (~70% of the total cells), with the cell density unaffected (Fig. 1D-F). Concentration of IL-10, another M2 marker, in the conditioned medium was also significantly higher in the ωCgTXGVIA-treated samples (Fig. 1G). Thus, the activation of Cav2.2 may control the microglial activation by blocking the microglial cell transition to M2 type. Interestingly, IL-4 induced significantly higher expression of *Cacna1b* encoding Cav2.2 in MG6 cells, as revealed by qRT-PCR experiments, though LPS/IFNγ and ωCgTXGVIA had no effects on the expression of *Cacna1b* (Fig. 1H). The increase in the Cav2.2 expression induced by IL-4 may represent a kind of negative feedback mechanism controlling the M2 type activation, though the physiological implications of this phenomenon await further studies.

3.2. HIF-2 is responsible for the enhanced M2 transition by Cav2.2 blockade

Hypoxia inducible factors (HIFs) are transcription factors, which have been known to be involved in controlling the expression of inflammation-related genes [26]. Since HIF-1 is known to induce expression of M1 markers such as iNOS in macrophages and since HIF-1 and HIF-2 often have opposite functions despite their sequence similarities [27], we examined the effect of HIF-C2, a HIF-2 inhibitor [28], on the enhanced M2 transition induced by ωCgTXGVIA. When the MG6 cells were treated with ωCgTXGVIA, they showed a stronger expression of Arg1 after stimulation with IL-4 compared to the control (Fig. 2A, B). However, pretreatment with HIF-C2 prevented this ωCgTXGVIA-induced enhancement of Arg1 expression almost completely, though HIF-C2 by itself exhibited virtually no effects on the basal response (Fig. 2A, B). Furthermore, ωCgTXGVIA–induced increase in the concentration of IL-10 in the culture medium was also reversed by a pretreatment with HIF-C2 (Fig. 2C). These results suggest that in the presence of IL-4, which induces M2 activation state, Cav2.2 may control the function of HIF-2 by blocking the HIF-2 transcriptional activity. However, further rigorous studies would be necessary to understand the details.

3.3. Knockdown of microglial Cav2.2 restores the attenuated M2 transition in aged microglia

Microglia play a major role in the innate immune system and reduce brain damage caused by various infections or diseases [21]. However, it is also known that aging induces malfunction of microglia and that these abnormal microglia exacerbate the brain damage [29]. We found that the efficacy of the microglial M1 transition was enhanced but the M2 transition was reduced by aging, using primary cultures of microglia (Fig. 3). When the primary cultures of microglia prepared from wild-type (WT) mice of young (2~3 months) and old ages (> 2 years) were stimulated with LPS/IFNγ, the expression of iNOS was induced in ~13% and ~28% of total cells, respectively, with almost no iNOS expression in unstimulated controls (Fig. 3A-D). The degree of iNOS induction was higher in the aged WT mice compared to the young WT, suggesting that microglia from aged mice tend to adopt an M1 activation phenotype. On the other hand, IL-4-induced Arg1 expression in the aged mouse was attenuated greatly, compared to the robust induction in microglia from the young mouse (Fig. 3E-H). To test whether this reduced M2 transition found in aged mice can be restored by modulating Cav2.2 expression, we utilized the conditional transgenic mice where the Cav2.2 channel expressed in microglia can be moderately (about 20~40) reduced without showing any effects on neuronal Cav2.2 (Cav2.2-MGKD mice, where MGKD stands for microglia-specific knockdown) [15]. To avoid possible developmental compensation, we started to reduce the expression of microglial Cav2.2 a couple of weeks before conducting the experiments. As in the WT microglia, primary cultures were treated with IL-4 and Arg1 expression was assessed. We found that the reduced M2 transition, which was induced by IL-4, in aged mice was restored by blocking the expression of microglial Cav2.2, however, the expression level of Cav2.2 in microglial cells tended to be decreased during aging (Fig. 1S). Thus, age-dependent malfunction of microglia may be induced by the activation of Cav2.2 rather than the alteration of its expression level in aged mice.

3.4. Knockdown of microglial Cav2.2 facilitates the recovery from depressive-like behaviors after peripheral LPS challenge

It is well known that the symptoms of illness caused by infections and brain damages become severer by aging and that the recovery also become slower [30]. These age-dependent changes have been speculated to be caused by the exaggerated cytokine response [31], where

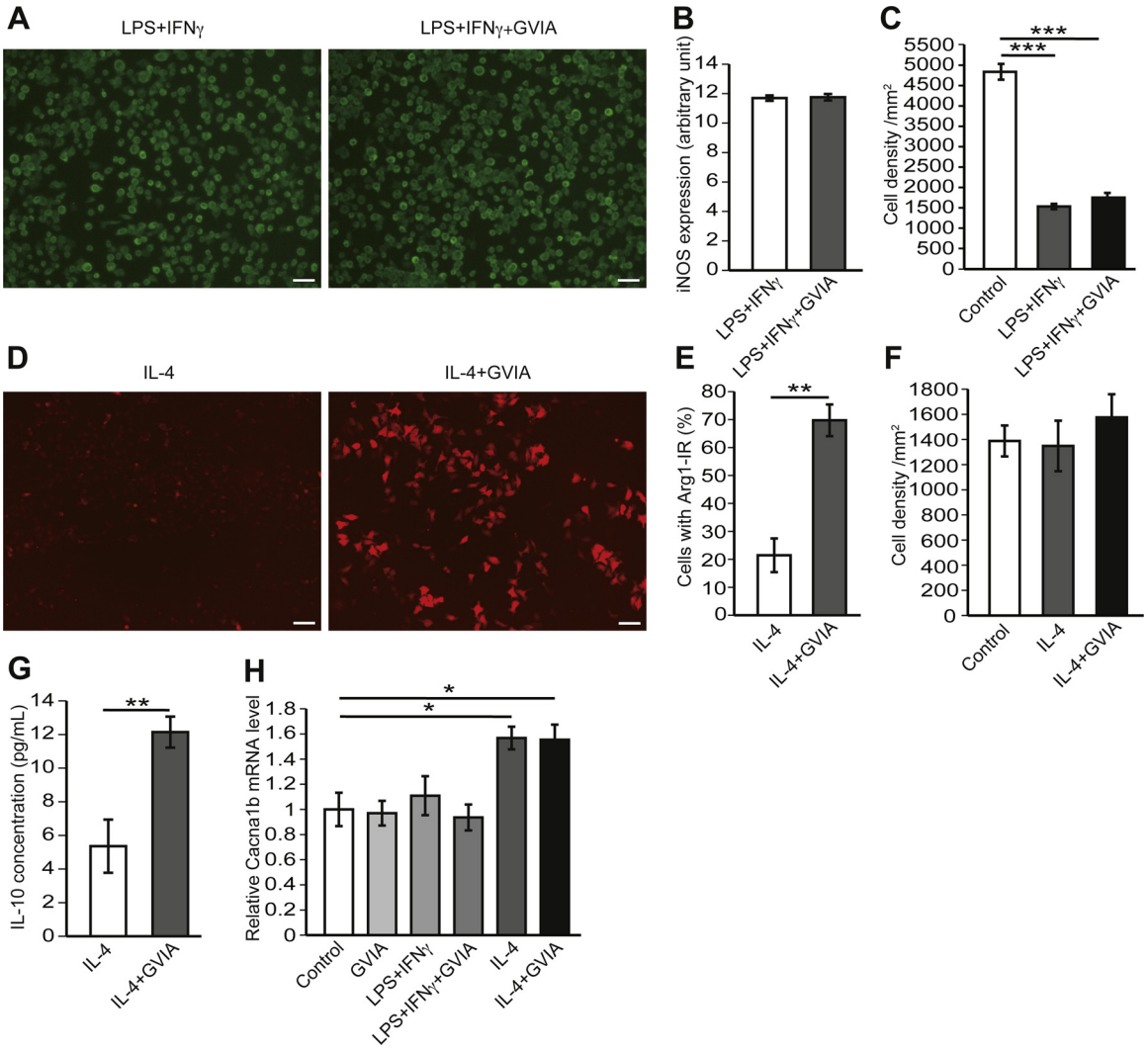

Fig. 1. Blockade of N-type VDCC in microglial cells enhances M2 type transition. **(A)** MG6 cells activated with LPS + IFNγ in the absence or presence of ωCgTXGVIA (GVIA) were immunostained with an iNOS antibody. **(B)** Quantification of signal intensity of iNOS immunoreactivity (IR) in **(A)**. **(C)** Density of cells that survived after 24 h of treatment with LPS + IFNγ. **(D)** MG6 cells were activated with IL-4 in the absence or presence of GVIA and assessed for Arg1 expression by immunocytochemistry. **(E)** Quantification of the results in **(D)**. Percentage of cells showing Arg1 IR is shown. **(F)** Cell density after 24 h of IL-4 treatment. **(G)** IL-10 concentration in the conditioned medium determined by ELISA. **(H)** *Cacna1b* mRNA expression level normalized with *GAPDH* as determined by qRT-PCR. Results are expressed as relative value, with the control set to one. Scale bars, 50 μm. Data are presented as mean ± SEM. (n = 6 from 3 independent cultures in **B**, **C**, and **H**. n = 4 from 4 independent cultures in **E**, **F**, and **G**). *p < 0.05, **p < 0.01, ***p < 0.001 with Student's *t*-test (**B**, **E**, **G**) and Tukey-Kramer test (**C**, **F**, **H**).

age-dependent changes of microglia may play a major role. It has been shown previously that low level of LPS (low enough to avoid sepsis induction) induces inflammation, which in turn causes behavioral deficits including lethargy, anorexia, and decreased social interaction. These deficits are known as depressive-like behaviors, which are thus thought to be causally related to the cytokine production in the brain [32,33]. It is also known that aging exacerbates the depressive-like behaviors [21], indicative of the exaggerated cytokine response. We therefore tested whether aged Cav2.2-MGKD mice show decreased depressive-like behaviors, because the M2 transition was recovered in the microglia from these mice, as shown in Fig.3.

Mice were injected with LPS (800 μg/kg) and their depressive-like behaviors were assessed by the changes in body weight, food consumption, spontaneous locomotor activity, and social interaction with an unfamiliar mouse brought into their home cages, 6, 24, and 72 h after the LPS injection. As shown in Fig. 4, aged Cav2.2-MGKD mice showed less severe symptoms in all the assessment methods to evaluate the depressive-like behaviors. Especially, the spontaneous locomotor activity, as judged by the number of line-crossings, was significantly higher compared to the WT at 24 h after LPS injection. Thus, the aged Cav2.2-MGKD mice recovered from LPS-induced illness more quickly than the control old mice (Fig. 4).

4. Discussion

We previously reported that the microglial N-type VDCC was functional under some pathological conditions, as revealed by reduced neuropathic pain symptoms in the Cav2.2-MGKD mice [15]. In the present study, we have clarified a physiological function of the microglial N-type VDCC in the process of M2 activation. When microglia were stimulated with IL-4 in the presence of ωCgTXGVIA to block the N-type VDCC, a marked increase in M2 marker expression was observed. Therefore, the N-type VDCC in microglia is suggested to be a negative regulator of M2 transition under a normal condition. Moreover, we have demonstrated that the ωCgTXGVIA-induced enhancement of M2 transition involves the HIF-2 transcriptional pathway. To date, several transcription factors are known to be involved in the control of microglial polarization toward M1 and M2 types. For example, signal transducer and activator of transcription 6 (STAT6) is known to be activated downstream of the IL-4 signaling and to augment the

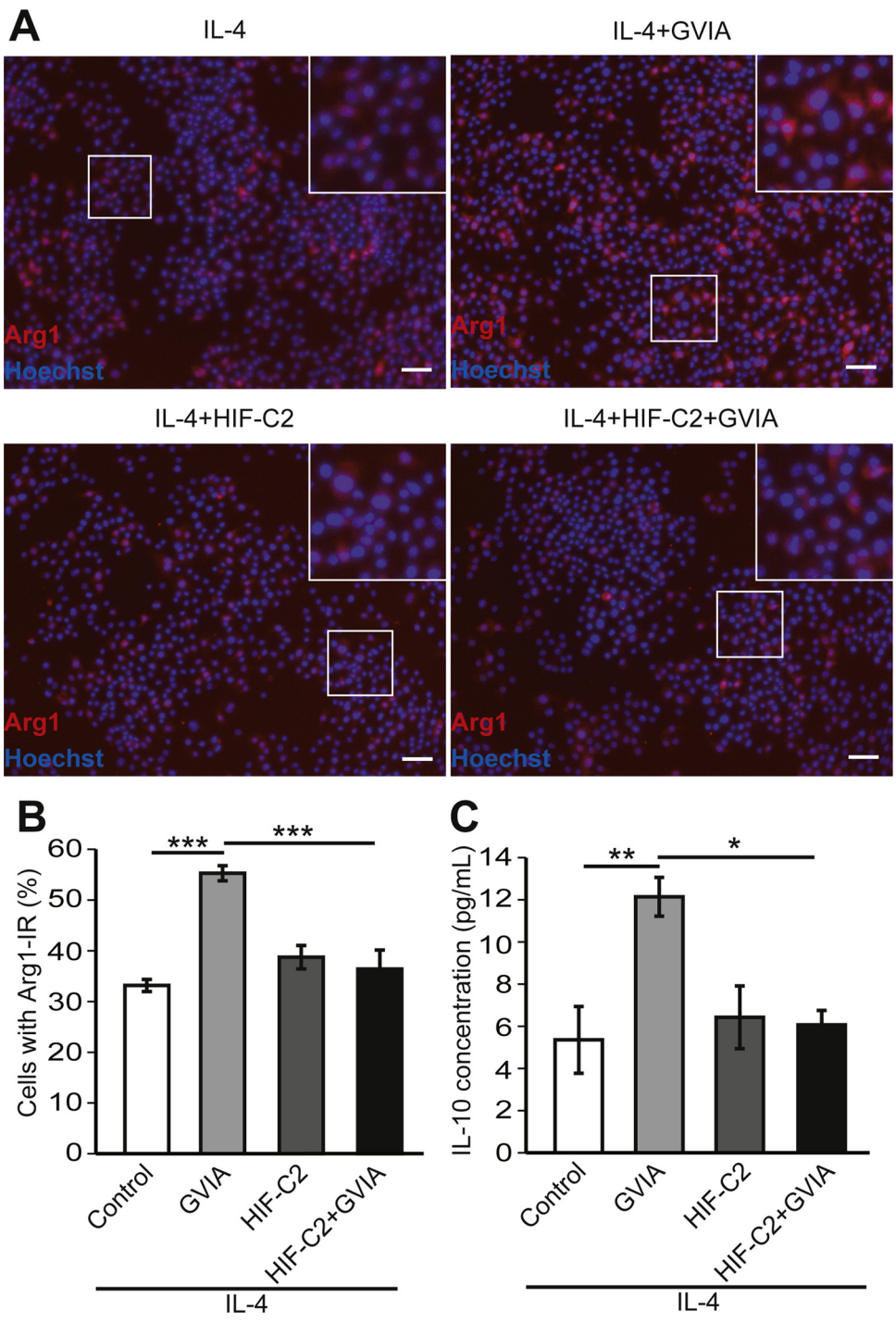

Fig. 2. Enhanced M2 transition of microglial cells by N-type channel block was attenuated by treatment with HIF-C2. **(A)** MG6 cells pretreated with GVIA and/or HIF-C2 were treated with IL-4 for 24 h in the presence of these drugs and assessed for Arg1 expression by immunocytochemistry. Boxed areas are shown as enlargements in the insets. **(B)** Quantification of results in **(A)**. Percentage of cells showing Arg1 expression is shown. **(C)** ELISA measurement of IL-10 in the conditioned medium from each sample. Scale bars, 50 μm. Data are presented as mean ± SEM. (n = 6 from 3 independent cultures in **B** and **C**). *p < 0.05, **p < 0.01, ***p < 0.001 by Tukey-Kramer test.

expression of M2-related genes [34]. The Arg1 promoter contains several STAT6-binding sites and actually its expression is controlled by STAT6 [35]. To our knowledge, however, the cross-talk between HIF-2 and IL-4/STAT6 pathways has not been known, nor involvement of calcium in the control of the IL-4/STAT6 pathway has been known. Thus, HIF-2 seems to be a novel player in the control of the expression of M2-related genes in microglia, though the detailed mechanism underlying the activation of HIF-2 upon blockade of the microglial N-type VDCC deserves further studies.

It is generally believed that microglia from aged mice tend to adopt an inflammatory phenotype [36], though a study on microglial gene expression profile suggests an M2-type predominance in the aged murine microglia [37]. Our results of primary culture of microglia were in favor of the former notion. Microglia from aged WT mice tended to adopt M1 phenotype compared to those from young WT mice, as indicated by a higher percentage of iNOS-expressing cells after treatment with LPS/IFNγ. In addition, M2 activation was attenuated in the microglia from aged WT mice, since induction of Arg1 expression was not very much conspicuous compared to the microglia from young WT mice. Consistent with the data on MG6 cell activation pattern in the presence of ωCgTXGVIA, primary microglial culture from the Cav2.2-MGKD mice showed almost the same iNOS expression pattern compared to the WT control, whereas induction of Arg1 expression was robustly increased compared to the WT control. Therefore, microglial N-type VDCC may be activated to suppress M2 activation during the course of normal aging. Overall, pharmacological blockade of

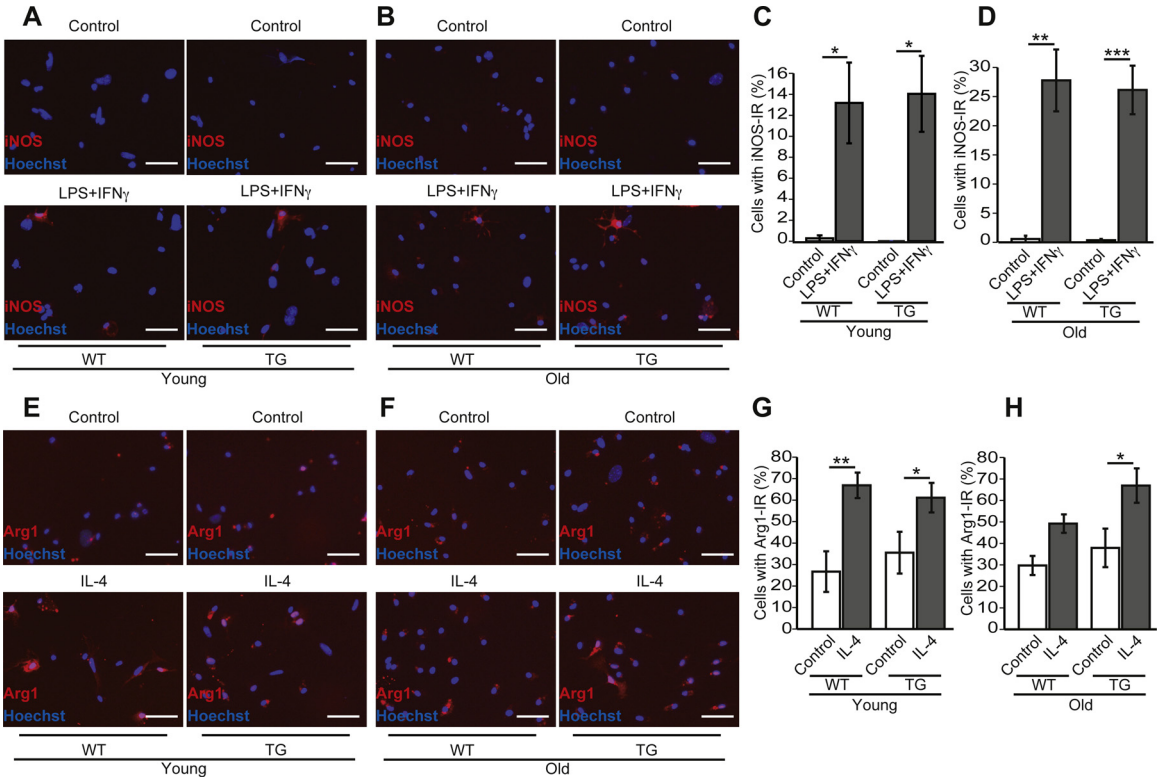

Fig. 3. Attenuated M2 transition in microglia from aged WT mice is reversed by decreasing expression of microglial Cav2.2 channel. **(A)**, **(B)** Primary culture of microglia from young (**A**) and aged (**B**) mice of both genotypes (WT and TG) was treated with LPS + IFNγ and immunocytochemistry was performed using an anti-iNOS antibody. **(C)**, **(D)** The results of analysis of images in **(A)** and **(B)**, respectively. **(E)**, **(F)** Primary culture of microglia from young (**E**) and aged (**F**) mice was treated with IL-4 and stained with an antibody against Arg1 to evaluate the M2 activation of the cells. **(G)**, **(H)** The results of analysis of images in **(E)** and **(F)**, respectively. Scale bars, 50 μm. TG, Cav2.2-MGKD mice. Data are mean ± SEM. (n = 3–8 from 3 or 4 independent cultures in **C**, **D**, **G**, and **H**). *p < 0.05, **p < 0.01, ***p < 0.001 by Tukey-Kramer test.

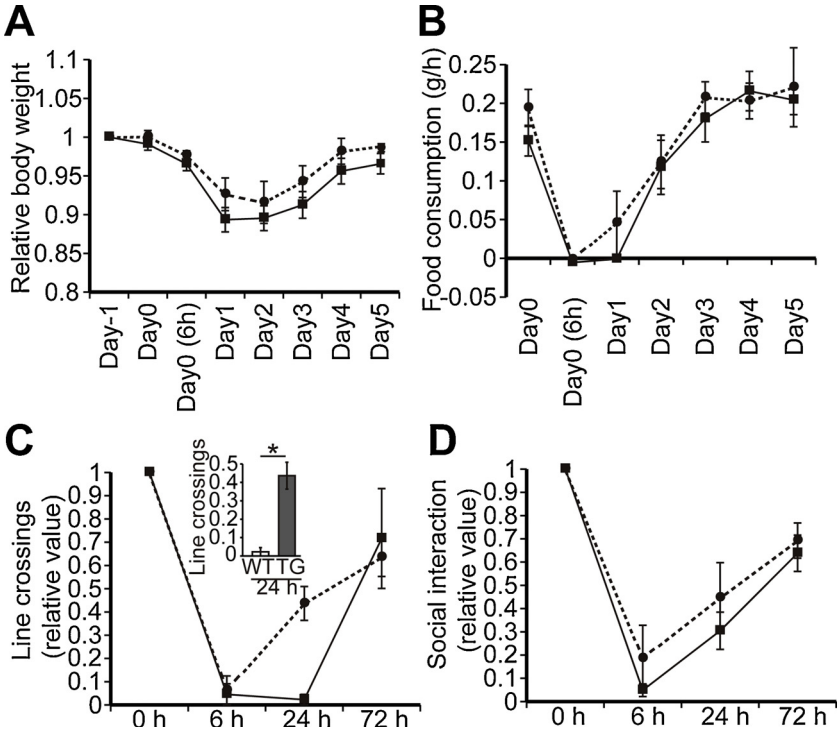

Fig. 4. Depressive-like behaviors in aged Cav2.2-MGKD mice are less severe. **(A)**, **(B)**, **(C)** and **(D)** Depressive-like behaviors were assessed by changes in body weight (**A**), food consumption (**B**), line crossings as a sign of spontaneous locomotor activity (**C**), and time spent in social interactions (**D**). LPS injection was performed on day 0. Body weight was normalized by that measured one day before the injection in (**A**). Line crossings and time spent in social interactions were normalized by the day 0 data obtained just before the LPS injection. The inset in (**C**) shows the line crossing results at 24 h after LPS injection, which indicates a significantly higher locomotor activity in Cav2.2-MGKD mice. Solid lines, WT (n = 4); dotted lines, Cav2.2-MGKD mice (n = 4). Data are presented as mean ± SEM. *p < 0.05 by Welch's *t*-test.

microglial Cav2.2 N-type channel and genetic knockdown of microglial Cav2.2 expression resulted in enhanced M2 activation. The underlying mechanism may involve both decreased level of Cav2.2 channel activity and deficits in the possible channel-independent functions of Cav2.2. Recently we have reported that blockade of microglial Cav1.2 L-type channel enhances M1 but decreases M2 activation in microglia, effects opposite to that of Cav2.2 blockade [38]. Therefore, it seems plausible that modulation of the Ca^{2+} influx, rather than the channel-independent functions of VDCCs in microglia may greatly affect the activation profiles of microglia, though rigorous studies will be required to conclude this hypothesis.

We finally demonstrated the behavioral consequence of the above-mentioned aging-dependent suppression of M2 activation. By a low dose of LPS challenge, the Cav2.2-MGKD mice displayed diminished symptoms of depressive-like behaviors. The results of LPS challenge experiments are well explained, if we assume that the recovery phase after the LPS injection is mainly dependent on the M2 microglial functions, because in the Cav2.2-MGKD mice, microglia resumed the M2 transition efficacy. Therefore, Cav2.2 may be the core factor controlling the aging-induced exaggerated cytokine response. The underlying mechanism is suggested to be as follows: aging-induced activation of Cav2.2 reduces the transition of M2 microglia, skews the M1/M2 balance toward M1, and possibly (in a relative way) enhances the population of M1 microglia and the production of cytokines specific to M1 microglia, inducing exaggerated cytokine response.

Compromised M2 activation in aged WT mice may also lead to a situation termed “non-resolving inflammation” [39], which is known to cause many inflammatory diseases including neurodegenerative diseases. Since IL-10 is one of the cytokines, which are critically involved in the mechanism to resolve inflammation, blockade of Cav2.2 in microglia may be beneficial in improving the non-resolving inflammation situation. This is because the blockade of Cav2.2 in microglia enhanced IL-4-induced IL-10 production with no effects on the initial M1 activation. Thus, Cav2.2 blockers, if delivered properly, would be a plausible drug for preemptive protection of the aged brain.

5. Conclusions

In the present study, we have demonstrated that blocking the microglial Cav2.2 channel enhances the M2 activation induced by IL-4, and this enhanced M2 activation is dependent on the HIF-2 function. The microglial Cav2.2 channel is critically involved in aging-related changes in microglial activation profiles, since microglia from aged mice show an attenuated M2 activation, which is restored by knockdown of the microglial Cav2.2.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ceca.2019.102059.

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