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Neuron-Microglia Interaction Induced Bi-directional Cytotoxicity Associated with Calpain Activation

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Abstract

Activated microglia release pro-inflammatory factors and calpain into the extracellular milieu, damaging surrounding neurons. Mechanistic links to progressive neurodegeneration in diseases such as Parkinson's disease (PD) and multiple sclerosis (MS) remain however obscure. We hypothesize that persistent damaged/dying neurons may also release cytotoxic factors and calpain into the media which then activate microglia again. Thus, inflammation, neuronal damage, and microglia activation, i.e. bi-directional interaction between neurons and microglia, may be involved in the progressive neurodegeneration. We tested this hypothesis using two in vitro models: (1) the effects of soluble factors from damaged primary cortical neurons upon primary rat neuron-microglia and (2) soluble factors released from CD3/CD28 activated peripheral blood mononuclear cells (PBMCs) of MS patients on primary human neurons and microglia. The first model indicated that neurons injured with pro-inflammatory agents (IFN-γ) release soluble

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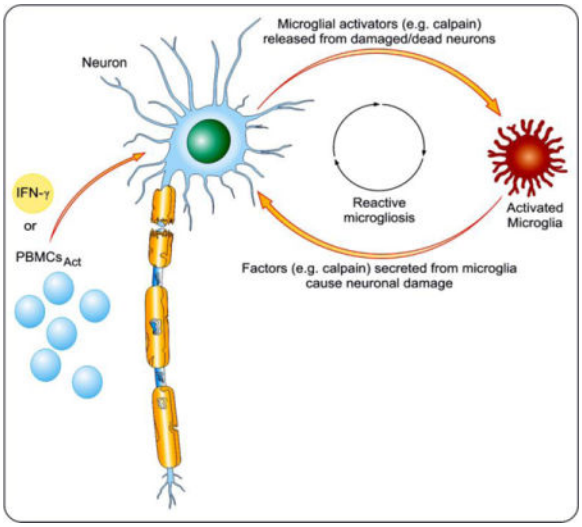
The authors have no conflicting financial interests. Calpain inhibitor SNJ 1945 was obtained from Senju Pharmaceuticals, Kobe, Japan.

neurotoxic factors, including Cox-2, ROS, and calpain, thus activating microglia, which in turn released neurotoxic factors as well. This repeated microglial activation leads to persistent inflammation and neurodegeneration. The released calpain from neurons and microglia was confirmed by calpain inhibitors calpeptin or SNJ-1945 as well as μ and mcalpain knock down using siRNA technology. Our second model using activated PBMCs, a source of pro-inflammatory Th1/Th17 cytokines and calpain released from auto-reactive T cells corroborated results in human primary cell cultures and confirmed calpain to be involved in progressive MS.

These insights into reciprocal paracrine regulation of cell injury and calpain activation in the progressive phase of MS, PD, and other neurodegenerative diseases suggest potentially beneficial preventive and therapeutic strategies, including calpain inhibition

Graphical abstract

Inflammation, neuronal damage, and microglia activation, i.e. bi-directional interaction between neurons and microglia, may be involved in neurodegenerative diseases mechanisms. This study used two in vitro models to advance our understanding of the pathogenesis: (1) the effects of soluble factors from damaged primary cortical neurons upon primary rat neuron-microglia and (2) soluble factors released from CD3/CD28 activated peripheral blood mononuclear cells (PBMCs) of multiple sclerosis (MS) patients on primary human neurons and microglia. Our results indicate that neurons injured with pro-inflammatory agents (IFN-γ) release soluble neurotoxic factors, including Cox-2, reactive oxygen species (ROS), and calpain, that activate microglia, which in turn release neurotoxic factors.



Keywords

calpain; microglia; microgliosis; multiple sclerosis; neurodegeneration; neurons

Introduction

A large body of experimental evidence suggests that inflammation is the most common factor and plays a critical role in many neurodegenerative diseases. The pathology of

degenerative diseases such as Alzheimer's (AD), multiple sclerosis (MS), and Parkinson's disease (PD) is associated with neuronal death and axonal loss leading to progression of neurodegeneration culminating in impaired function. The dynamics of microglia-neuron communication in the neurodegenerative disease have gained attention in recent years. Our evidence indicates that the communication between neurons and microglia is bi-directional and involves several modulatory factors, including calpain. Yet many unanswered questions remain, including the neuron-microglial damage and the immediate consequence.

To this end, renewed interest in axonal and neuronal injury in multiple sclerosis (MS) has significantly shifted the focus of research into this disease toward degeneration (Bjartmar & Trapp 2001, Bitsch *et al.* 2000). The underlying mechanisms and nature of this degenerative process at different phases of disease progression in MS is still a mystery. Although the mechanism of persistent inflammation is accompanied by chronic neurodegeneration, the processes that contribute to neuronal damage in PD and/or cause autoimmune demyelination in progressive MS (Block & Hong 2005, McDowell *et al.* 2011, Samantaray *et al.* 2015, Smith *et al.* 2011) are largely unknown. No drugs have thus far been developed to significantly attenuate inflammation and progression of degeneration and resultant disability in these diseases. A major therapeutic goal, particularly in MS treatment, is to prevent the axonal loss that causes permanent neurologic disability. Because different mechanisms may contribute to axonal/neuronal damage during different stages of disease, an important goal of current and future MS research is to clarify the pathophysiology of axonal and neuronal loss in MS and other diseases.

Microglia, the resident immune effector of the CNS, respond to neuronal damage following their activation by releasing such cytotoxic mediators as pro-inflammatory cytokines (Balasubramaniam *et al.* 2009, Dheen *et al.* 2005), reactive oxygen species (ROS) (Colton & Gilbert 1987), nitric oxide (NO) (Liu *et al.* 2002, Moss & Bates 2001), and proteases (Kim *et al.* 2005, Levesque *et al.* 2010); these in turn can intensify the CNS inflammatory state (Dheen *et al.* 2007, Whitney *et al.* 2009). Numerous *in vitro* and *in vivo* studies have found that inhibition of microglia activation attenuates neurotoxic events and improves neuronal survival. In many neurodegenerative disorders, including MS (Banati *et al.* 2000, Peterson *et al.* 2001, Takeuchi *et al.* 2006a, Takeuchi *et al.* 2006b), Alzheimer's disease (Cagnin *et al.* 2001, Meda *et al.* 1995), and Parkinsonism (Imamura *et al.* 2003, Long-Smith *et al.* 2009), over-activation of microglia is thought to be a key causative factor in the neuropathology. The regulation of microglia is multi-factorial, and their activation is suggested to be regulated by use of 'on' and 'off' signals from neurons influencing microglial activation (Biber *et al.* 2007). Activation of microglia as a consequence of neuronal injury augments their neurotoxicity (Polazzi & Contestabile 2002), and this microglial response to neuronal damage can be long-lived and self-propelling (Lull & Block 2010). Damaged or dying neurons themselves are potential triggers of microglial activation, and repeating cycle of neurotoxic microglial activation in response to neuron injury is a common feature of reactive microgliosis (Block *et al.* 2007, Streit *et al.* 1999).

Activation of microglia following brain insult is associated with the onset or progress of neurological disorders, either alone or in conjunction with other factors (e.g., calpain activation). Calpain, a Ca^{2+}-dependent cysteine protease, has been implicated in the

pathophysiology of many neurological disorders, including PD and MS. Ubiquitous calpain isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calpain) that differ by their sensitivity to Ca^{2+}, are abundantly expressed in the CNS (Ray & Banik 2003), and they are localized in cytosol and membranes (Banik *et al.* 1985). Calpain is critical for cell function and its over-activation causes cell death and degeneration. In addition, activation of calpain may profoundly affect glial function by stimulation of gliosis, infiltration of inflammatory cells, and induction of cell death. Therefore, tight regulation of calpain activity is essential for the preservation of cellular function. Dysregulation of calpain has been associated with a number of diseases, including MS (Guyton *et al.* 2005), optic neuritis (Shields & Banik 1998), Alzheimer's disease (Zatz & Starling 2005), and Parkinson's disease (Crocker *et al.* 2003, Levesque et al. 2010, Samantaray et al. 2015). The pathologic mechanisms underlying and consequences of calpain activation in MS are distinct from the other neurodegenerative diseases (Vosler *et al.* 2008).

IFN-γ, a Th1 pro-inflammatory cytokine, was recently shown to induce apoptotic cell death in VSC4.1 motoneuron and retinal ganglion cells (RGCs), which are known to be affected in MS and optic neuritis. Their exposure to IFN-γ initiated calpain-dependent apoptosis and modulation of calpain activation, which may have clinical significance by reducing neuronal death and improving clinical outcomes (Das *et al.* 2006b). To further support the hypothesis that IFN-γ could be one of the precipitating insults involved in the pathogenesis of MS, we have shown that exposure of VSC4.1 motoneurons to supernatant derived from IFN-γ-activated rat primary microglial cells also resulted in apoptotic cell death (McDowell et al. 2011). To further the argument that calpain is involved in the pathogenesis of MS, pro-inflammatory Th1/Th17 cytokines released from activated MS PBMCs (Smith et al. 2011, Imam *et al.* 2007), a source of auto-reactive T cells that contribute to the diseases initiation and perpetuation, was shown to correlate with the increased calpain activity and inhibition of calpain resulted in an abrogation of activated T cells cytokines (Das et al. 2006b, Trager *et al.* 2014). These studies suggest that calpain inhibition *in vivo* may abrogate both the direct neuronal degeneration and the indirect action of pro-inflammatory cytokine(s) (e.g., IFN-γ) mediated cell death. Thus, the potential of calpain released into the extracellular milieu to damage surrounding neurons formed the subject for further investigation.

The current study was designed to further elucidate the role of pro-inflammatory cytokine(s) (e.g., IFN-γ) initiated neuronal injury and established the mechanism contributing to progressive neuronal degeneration by focusing on neuron-microglia interaction.

Three fundamental questions were addressed: (i) why does the microglial response to neuron damage persist, (ii) why is this response toxic, and (iii) can calpain inhibition suppress neuron death and attenuate degeneration?

Data from two primary culture models – rat and human – demonstrated that extracellular calpain released from both damaged neurons and activated microglia is a key signal driving reactive microgliosis. Furthermore, this calcium-dependent cysteine protease released upon neuron/microglia damage activates microglia and damages naïve neurons to produce ROS and NO followed by increased activity of calpain substrates cyclooxygenase-2 (COX-2) and caspase 3, culminating in apoptosis, and calpain inhibition prevents damage. These findings

indicate that the damaged neurons themselves are culpable in propagating neurotoxicity via pro-inflammatory signals to microglia.

Materials and Methods

Ethics Statement

Human fetal tissues were obtained following written approval from adult female patients undergoing therapeutic abortion at 10–14 weeks gestational age at the University of Washington, WA, USA (IRB approval #11449). The use of human fetal tissue was approved by the University of South Carolina, SC, USA (USCeRA#:HSA4636), and is IRB-exempt [45 CFR 46.102(d)]. All primary human fetal neuron cell culture studies were done according to university guidelines in a biocontainment facility approved by the Institutional Biosafety Committee (IBC) of the University of South Carolina.

PBMCs were collected from human subjects enrolled according to protocols approved by the Medical University of South Carolina Institutional Review Board (IRB #9481). Microglial cells were isolated from human adult brain tissues collected from the Medical University of South Carolina operating room during surgical procedures on non-affected/not-damaged brain according to protocols approved by the Medical University of South Carolina, SC, USA (IRB #19380). All experimental protocols were reviewed and approved by the Institutional Review Board of the Medical University of South Carolina, and experiments were undertaken with the understanding and written consent of each subject. The study conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964).

Rat Cell Cultures

Rat cortical cultures (neurons and microglia) were established from two-day-old Spraque-Dawley pups from dams (Harlan) as previously reported (Banker & Goslin 1988, Jana *et al.* 2007). Briefly, cortices were dissected and dissociated by trituration and trypsinization. The dissociated cells were re-suspended in B-27-supplemented neurobasal media (Invitrogen) and plated onto a poly-D-lysine-coated T-flask. Neurons (adherent cells) were treated with 5 μM of the anti-mitotic inhibitor arabinose-C and cultured for 7 days prior to the experiment. Non-adherent cells contained mixed glial cells and were mechanically shaken for 30 min at 200 rpm on a gyratory shaker to isolate microglia (Bhat *et al.* 1992). The purity of rat cortical neurons and microglia cultures was confirmed by immunochemistry with 90–95% positive staining for neuron-specific nuclear protein (NeuN) and CD11b, respectively. Ventral spinal cord 4.1 (VSC4.1) motoneuron cell line, a generous gift from Dr. S. Appel (Baylor College of Medicine, TX, USA), was grown under previously described conditions (Das *et al.* 2005).

Primary Human Brain Cell Cultures

Primary human brains at 8 to 12 weeks gestational age obtained from the University of Washington, Seattle, were cultured as described earlier (Chauhan *et al.* 2003, Mehla & Chauhan 2015). Briefly, meninges and blood vessels were removed from the cortex. The tissue was washed twice with opti-MEM containing 1% penicillin-streptomycin and

amphotericin-B (GibcoBRL). Washed tissue was mechanically disrupted by one passage through a 20-ml syringe without a needle. Disrupted cortex tissue was centrifuged at 1,200 rpm for 20 minutes at 4°C. For differentiation to neurons, pellets were resuspended in opti-MEM medium and cultured in Opti-MEM supplemented with 5% FCS, 0.2% N2 supplement (Gibco BRL), and antibiotics. The purity of neuron and astrocyte cultures was verified by immunostaining using antibody against microtubule-associated protein-2 (Sigma) or glial fibrillary acidic protein (GFAP), respectively. The purity of the neuron cultures were 90–95%. Primary human fetal neuron cultures were maintained for at least a month before use in experiments. To reduce the chance of mycoplasma contamination in these cultures, plasmocin, an anti-mycoplasma agent (Invitrogen), was used during the initial culture passages. Human primary neurons were isolated from 12- to 14-week-old fetal brains, as previously described (Chauhan et al. 2003, Mehla & Chauhan 2015). Microglial cells were isolated from human adult brain tissues collected from the Medical University of South Carolina operating room during surgical procedures on non-affected/not-damaged brain according to the previously published method (De Groot *et al.* 2000).

Isolation and Stimulation of PBMCs from MS Patients

PBMCs from MS patients were isolated as described previously (Smith et al. 2011). Cells were stimulated with 10 μg/ml of anti-CD3 and 5 μg/ml of anti-CD28 (BD, Franklin Lakes, NJ) following pre-treatment with the calpain inhibitor calpeptin (CP, 5 μM) in DMSO for 72 h. After treatment, supernatants were collected and stored at −80°C until use.

Treatment of Rat Primary Cortical Neurons

Primary rat neurons (RN) from cortex were stimulated with IFN-γ (100 units/ml) in the absence or presence of 1 μM CP. After 24 h of incubation, conditioned media (CM) as well as cell pellets were collected (Supplemental Fig. 1A) and analyzed (Fig. 1). CM-RN were collected, speed-vacuum concentrated (10×), and used for treatment of microglia as well as VSC4.1 motoneurons.

To determine if damaged neurons could activate microglia, primary rat microglia (RM) from cortex were pre-treated for one hour with CM-RN±CP followed by collection of CM and cells (Supplemental Fig. 1B) for subsequent analysis (Fig. 2). CM-RM were concentrated and used for further treatment.

Treatment of VSC4.1 Cells

VSC4.1 motoneurons were exposed to CM-RM, CM-RM±CP (1 h pre-treatment), CM-RM ±SNJ-1945 (1 h pre-treatment), or CM-RM + siRNA for calpain 1 or CM-RM + siRNA for calpain 2 (1 h pre-treatment). After 24 hr of treatment, CM as well as cells were collected (Supplemental Fig. 1C) and analyzed (Fig. 3). The delivery of siRNA for calpain 1 (μcalpain) and calpain 2 (mcalpain) into VSC4.1 motoneurons was performed using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions, as described earlier (Das *et al.* 2013b). Cell transfection was carried out 4–6 h, and the medium was then replaced with normal growth medium. Control cells were mock-transfected, and all experimental results were confirmed using scrambled siRNA. After 24 h cells were exposed to CM-RM.

To determine whether conditioned medium (CM) from damaged neurons (CM-RN) could affect fresh naïve neuron viability and whether calpain inhibitor could prevent such damage, VSC4.1 motoneurons were treated with CM-RN±CP (1 h pre-treatment). Two types of negative controls were included: inactivation of IFN-γ by heating at 55°C for 30 min before primary rat culture stimulation and neutralization of IFN-γ by anti-IFN-γ antibody. VSC4.1 cells were either challenged with heat-inactivated IFN-γ (HI–IFN-γ) or with neutralizing antibody to IFN-γ in the absence or presence of 1 μM CP. CM and cells (Supplemental Fig. 1D) were collected and subjected to appropriate analysis (Fig. 4).

Treatment of Human Primary Cells

Fetal human neurons (HN) were exposed to 50 μl of PBMCs, $PBMCs_{Act}$, or $PBMCs_{Act}$ plus 1 μM or 5 μM CP ($PBMCs_{Act}$+CP; 1 h pre-treatment) for 24 hr. After incubation, CM as well as cell pellets were collected (Supplemental Fig. 2A) and subjected to appropriate analysis (Fig. 5). $PBMCs_{Act}$ were concentrated and used for human microglia (HM) treatment.

Primary HM isolated from undamaged cortex from unaffected brains of patients were treated with CM-$HN_{PBMCsAct}$±CP. The CM from incubated microglia as well as the cell pellet were collected (Supplemental Fig. 2B) a (Fig. 6). CM-HM were concentrated and used for fresh or naïve HN treatment.

To determine the effect of calpain upon microglia-mediated damage, fresh naive fetal HN were treated with CM-HM in the presence or absence of CP. CM and cells were collected after treatments (Supplemental Fig. 2C) and subjected to further analysis (Fig. 7). Following treatments, the mixture of attached and detached cell populations was used for estimation of the cell viability by the Trypan blue dye exclusion test (Das *et al.* 2004).

Calpain Activity Assay

Calpain activity in total cell lysates was determined based on the reaction with a calpain substrate, fluorogenic peptide (Ac-LLY-AFC), using a calpain activity assay kit (Abcam, Cambridge, MA). Briefly, cells were lysed then incubated with the substrate and reaction buffer for 1 h at 37°C in the dark. Proteolysis of the fluorescent probe (AFC) was monitored using a fluorimeter with filter settings of 400 nm for excitation and 505 nm for emission.

Measurement of Nitrite and ROS

The production of cytoactive molecules was measured as reported previously (Das *et al.* 2007, Davidge *et al.* 1995). Briefly, NO production was calculated from the amount of nitrite detected by Griess reaction. Absorbance was determined at 550 nm using a microplate reader. Net intracellular accumulation of ROS was detected using fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCF-DA), and the fluorescence was measured at 530 nm after excitation at 480 nm.

Assay for Cyclooxygenase-2 Activity

COX-2 activity was determined using a COX-2 activity assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol, as previously described (Sribnick *et al.*

2010). Briefly, cells (1×10^6) were homogenized in cold buffer 0.1 M Tris-HCl, pH 7.8 containing 1 mM EDTA, mixed with assay buffer, heme, and COX-1 inhibitor. Colorimetric substrate and arachidonic acid were added sequentially and incubated at 37°C for 10 min, and the absorbance was read at 590 nm.

Colorimetric Assays for the Measurement of Caspase-8, Caspase-9, and Caspase-3 Activities

Measurements of caspase activities in cells were performed using commercially available caspase-8, caspase-9, and caspase-3 assay kits (Sigma), as previously reported (Das *et al.* 2006a). Briefly, activities were assayed based on hydrolysis of the Ac-IETD *p*-nitroaniline (*p*NA) by caspase-8, Ac-LEHD *p*NA by caspase-9, and Ac-DEVD *p*NA by caspase-3. The absorbance read at 405 nm. Caspase activities in μmol of *p*NA released from the substrate per min per ml of cell lysate were calculated based on the formula: $\mathbf{OD \times d/\varepsilon^{mM} \times t \times v}$, where d was dilution factor, t was reaction time, v was volume of sample and the constant value ε^{mM}=10.5. Data were expressed as % of change compared to control ± SEM, where control was set as 100%.

Statistical Analysis

Results of at least 3 different experiments were expressed as the percentage of control where control values were set to 100%. Significant differences between groups were determined by one-way analysis of variance ANOVA followed by Tukey's multiple comparison test using GraphPad PRISM 5.0. Significant differences were indicated by *p<0.05, **p<0.01, or ***p<0.001.

RESULTS

IFN-γ Induced Cell Death in Primary Rat Neurons

Primary RN culture was used as an *in vitro* model to investigate the cytotoxic action of the Th1 pro-inflammatory cytokine IFN-γ, a potent autoimmune inducer known to be upregulated in MS. As expected, neurons were affected by IFN-γ treatment, as indicated by a 2.6 fold reduction of viability after 24 h of treatment (Fig. 1A) and 3-fold increased (p<0.001) calpain activity (Fig. 1B). The increased vulnerability of neurons to IFN-γ injury was almost completely abolished by the calpain inhibitor CP.

Next, the mechanisms involved in cell death were investigated, and whether calpain inhibition could block neuron injury was determined. Neurons exposed to IFN-γ demonstrated a significant increase in ROS production (3.6 fold, p<0.01) at 24 h post-exposure (Fig. 1C). Activation of caspase-3, considered a central regulator of apoptosis, consistently increased by 3.1 fold (p<0.01), confirming that cells were already committed to apoptosis (Fig. 1D). Treatment with CP attenuated both ROS generation and caspase-3 activity following Th1 cytokine insult (Fig. 1, C–D). This suggests that calpain acts to increase the vulnerability of neurons to IFN-γ by affecting both ROS generation and caspase-3 activity.

Injured Primary Rat Neurons Secrete Factors that Activated Rat Microglia In Vitro

Deleterious microglial activation may be triggered by mediators released from damaged neurons causing a self-propelling cycle, a mechanism for chronic neuronal loss proposed in diverse neurodegenerative diseases (Block & Hong 2005, Gao *et al.* 2003, Levesque et al. 2010). In line with this, CM-RN stimulated with IFN-γ was used to activate primary RM. Microglial cells were vulnerable to the toxic mediators that were released into the CM after neuronal damage, as indicated by reduced cell viability (2.9 fold, $p<0.05$, Fig. 2A). The significant increases were observed in a variety of pro-inflammatory and apoptotic mediators such as calpain activity (3.3 fold, $p<0.01$, Fig. 2B), ROS generation (2.3 fold, $p<0.05$, Fig. 2C), NO production (2.1 fold, $p<0.05$, Fig. 2D), COX-2 activity (3.2 fold, $p<0.001$, Fig. 2E), and caspase-3 activity (4.2 fold, $p<0.001$, Fig. 2F). Calpain inhibition suppressed the effects of these mediators with caspase 3 activity being considered the most significant (Fig. 2F). This suggests that secretory factors, especially calpain, from damaged RNs activated microglial cells *in vitro*.

Calpain Inhibition/Knockdown Prevented VSC 4.1 Cell Death Stimulated by Secreted Factors Released From Damaged Primary Neurons

In parallel studies using VSC4.1 motoneurons, treatment with CM-RM was shown to decrease cell viability (Fig. 3A) while calpain activity (Fig. 3B), ROS production (Fig. 3C), and caspase-3 activity (Fig. 3D) were significantly increased ($p<0.001$). Calpain inhibition by cell-permeable inhibitor CP or the novel water-soluble calpain inhibitor SNJ-1945 significantly suppressed these effects ($p<0.001$; Fig. 3, B–D).

A similar effect was observed when a specific siRNA-oligonucleotide for calpain 1 or calpain 2 was transfected into VSC4.1 cells as revealed by decreased calpain activity (Fig. 3B). The microglia-induced damage indicated by ROS production (Fig. 3C), and caspase 3-activity (Fig. 3D) was significantly reduced ($p<0.001$) whereas non-specific siRNA had no inhibitory effect (data not shown). This is indicative that inhibition of calpain secreted from damaged primary neurons may prevent cell death.

Secreted Factors Released From IFN-γ Damaged Primary Neurons Induced VSC 4.1 Motoneuron Cell Death

To investigate whether damaged neurons could affect fresh naïve neuron viability, VSC4.1 cells were treated with CM-RN±CP (1 h pre-treatment). CM-RN were incubated CP and SNJ for 1 h prior to incubation with VSC4.1 cells. In a parallel experiment, primary microglia were also challenged with heat inactivated IFN-γ or neutralizing antibody treated CM-RN±CP. Treatment of CM-RN with CP was done 1 h prior to incubation with VSC4.1. As expected, VSC 4.1 cells showed increased sensitivity to CM-RN (Fig. 4A). No significant change in viability was seen after treatment with CM-RN+CP or comparable sets of cells treated with heat inactivated and neutralizing antibody treated CM-RN±CP (Fig. 4A). As demonstrated, the CM-RN treatment not only decreased cell viability but also increased calpain activity (Fig. 4, B–C) and elevated oxidative stress response, as revealed by ROS production (Fig. 4D). CP treatment inhibited calpain activity (Fig. 4, panels B–C) as well as production of ROS (Fig. 4D), and this inhibitory effect was closely linked to IFN-γ activity being dramatically decreased after heat inactivation of IFN-γ (Fig. 4, B–D). Calpain

activity and production of ROS were also evaluated after treatment of CM-RN with anti-IFN-γ neutralizing antibody (Fig. 4, B–D), and they were significantly decreased. Therefore, secretory factors from damaged primary neuron both increased the activity of cell death markers and decreased cell viability.

Activated PBMCs from MS Patients Secreted Factors that Induced Damage to Human Neurons in vitro and Calpain Inhibition Prevented Damage

The second model used activated PBMCs as insulting stimulus in human primary cultures. Previously, activated PBMCs were shown to be a source of auto-reactive T cells releasing Th1/Th17 pro-inflammatory cytokines (Imam et al. 2007, Smith et al. 2011). This model was chosen based on the general consensus supporting a role of T cells subsets and their contribution to the disease initiation and perpetuation in EAE and MS. First, the major player and the mechanism that contributed to IFN-γ–initiated activation of neuron-microglia associated with calpain-mediated bi-directional cytotoxicity in rat cultures were determined. Secondly, whether the same or a similar mechanism contributing to bi-directional cytotoxicity is elicited by activated PBMCs from MS patients was explored.

Neurons treated with $PBMCs_{Act}$ showed reduced viability that was reversed by CP treatment (Fig. 5A). Calpain activity of neurons treated with PBMCs vs. $PBMCs_{Act}$ was also monitored. As shown in Fig. 5B, calpain activity was increased by 86.77 ± 15.26% and 183.1 ± 27.89% in neurons treated with PBMCs and $PBMCs_{Act}$, respectively, suggesting that PBMCs were already secreting calpain even without CD3/CD28 *in vitro* activation. Calpain activity was decreased when HN were treated with $PBMCs_{Act}$ +CP (2.1 fold, $p<0.01$, Fig. 5B).

Our results on HN indicate that treatment with $PBMCs_{Act}$ significantly increased ROS production (2.8 fold, $p<0.001$) while CP treatment significantly decreased ROS generation (1.9 fold, $p<0.001$, Fig. 5C). As expected, COX-2 activity was greatly increased (6.3 fold, $p<0.001$), and CP treatment reduced COX-2 activity (3 fold, $p<0.001$) in exposed neurons (Fig. 5D).

Whether $PBMCs_{Act}$ could stimulate caspase-8, an apoptotic initiator in the extrinsic pathway, was also determined. While a marked increase in caspase-8 activity occurred after 24 h of stimulation (5.7 fold, $p<0.001$), this effect was abolished by 1.6 fold following CP treatment (Fig. 5E). Our data demonstrate that the loss of HN viability was associated with the consequent activation of the intrinsic pathway, caspase-9 (6.2 fold, $p<0.001$, Fig. 5F), and the final executioner, caspase-3. Caspase-3 was increased by 86.8% and 136.3% in HN treated with PBMCs and $PBMCs_{Act}$, respectively (Fig. 5G). In contrast, treatment of HN with $PBMCs_{Act}$+CP decreased caspase-3 activity by 1.9 fold ($p<0.001$), as shown in Fig. 5G. Thus, treatment with activated PBMCs increased calpain activation and HN cell death via the intrinsic pathway *in vitro*.

Calpain Inhibition Prevented Human Microglia Damage *in vitro*

Whether toxic mediators released from damaged HN could cause microglia injury was also investigated. Our results indicate that treatment with CM-HN reduced microglia viability ($p<0.001$, Fig. 6A) and demonstrated increases in calpain activity (2.9 fold, $p<0.01$, Fig.

6B), ROS production ($p<0.01$, Fig. 6C), COX-2 activity ($p<0.01$, Fig. 6D), and caspase-3 activity ($p<0.01$, Fig. 6D). The increase in ROS generation was accompanied by a significant increase in NO production (1.8 fold, $p<0.001$, Fig. 6F). Furthermore, calpain inhibition suppressed the effects of these toxic mediators and restored microglial viability (Fig. 6, A–F), which suggests that calpain secreted by HNs induced microglial damage.

Activated Microglia Released Factors that Induced Apoptosis in Naïve Human Neurons: the Effect Reversed by Calpain Inhibition

To explore the role of activated human microglia on cell viability, the effect of CM-HM on neurons was examined. Treatment of HN with CM-HM produced significant injury, causing decreased cell viability to 61.5% ($p<0.01$, Fig. 7A). The neuronal toxicity of CM-HM was attenuated by pretreatment with CP ($p<0.01$, Fig. 7A). Our study using human primary cultures confirmed the role of calpain as a key signal molecule released from activated neurons, as increased extracellular calpain activity was observed upon exposure of naïve HN to CM-HM (3.1 fold, $p<0.01$, Fig. 7B). To further reinforce the role of calpain, released by activated microglial cells into CM, parallel neuronal cultures were co-incubated with CP. As shown in Fig. 7A, CP reduced percentage of apoptotic cells and neuronal viability was almost completely restored.

Decreased cell viability was also associated with a concomitant increase in ROS production (4.2 fold, $p<0.01$, Fig. 7C), suggesting the involvement of oxidative damage in this process. Consistent with this result, activity of caspase-3, a critical player in apoptosis, was also increased (3.1 fold, $p<0.01$, Fig. 7D). In contrast to these apoptotic features, pretreatment of HN with CP followed by treatment with CM-HM suppressed the effects of these neurotoxic mediators (Fig. 7, panels B–D). Therefore, calpain secretion by microglia induces apoptosis in HN and the viability of HN could be restored following treatment with CP.

Discussion

Over the years many ideas have been proposed to explain the pathophysiology of neurodegenerative processes in progressive AD, MS, and PD (Correale 2014, Frischer *et al.* 2009, Kassmann *et al.* 2007, Meuth *et al.* 2008). The triggering mechanisms by which this progression occurs remain unclear. Since neuronal dysfunction and injury are thought to be present at the onset of these disease, damaged neurons may release factors that can not only bring their own demise, but also are likely to promote microglial and PBMC activation, contributing to progression of the degenerative process (Block & Hong 2005, Kim et al. 2005, Peterson et al. 2001). In MS, cortical lesions exhibit neuronal injury, including neuritic swellings and axonal transection (Peterson et al. 2001). Studies have confirmed the concept that axonal transection begins at the disease onset, and the cumulative axonal loss provides the pathologic substrate for the progressive disability experienced by most long-term MS patients. The transition from relapsing-remitting MS subtype to its secondary progressive stage and the subsequent development of progressive permanent motor disability occurs when a threshold of neuronal or axonal loss is reached or when adaptive immune response of the central nervous system (CNS) is exhausted (Reddy *et al.* 2000). Current therapeutic options for progressive MS are disappointing and remain challenging, reflecting a lack of

understanding of pathogenic microglial mechanisms driving this MS subtype. Microglial activation correlates well with the presence of cortical lesions, alteration of synaptic function, and axonal transport, each indicative of neuronal dysfunction (Rasmussen *et al.* 2007). T cell infiltrates into CNS were found in mice with experimental autoimmune encephalomyelitis (EAE) at disease onset and through the acute phase whereas microglial activation was sustained during the chronic phases (Murphy *et al.* 2010, Rasmussen et al. 2007, Vainchtein *et al.* 2014). However, at present, the mode of persistent microglial activation in MS/EAE is unknown. Using both rat and human primary cultures as *in vitro* models, the mechanism of chronic neuronal cell death was studied following exposure to two different insulting stimuli: IFN-γ and $PBMCs_{Act}$ from MS patients. The exposure of RN to IFN-γ, which mimics that occurring in MS inflammation, caused their injury and functional impairment (Fig. 1).

One of the most important cell death initiators in this process was the increased intracellular Ca^{2+} concentration that activated calpain and implicated in related disturbances. Whether treatment of retinal ganglion cells (RGCs) with IFN-γ could increase Ca^{2+} and trigger calpain activation, which might lead to cell death, was previously examined (Das et al. 2006b), and degeneration of RGCs has been implicated in loss of visual function in demyelinating and other diseases (Das *et al.* 2013a, Shields & Banik 1998, Smith et al. 2011). In addition, elevated production of ROS, NO, and COX-2 are also factors involved in chronic neuronal loss (McTigue & Tripathi 2008). Another player involved in neuronal injury by IFN-γ in this *in vitro* model, as demonstrated here, was caspase-3. It should be emphasized that increased calpain activity is an important factor required to cleave inactive caspase-3 to its active form, as caspase-3 is one of the calpain substrates (Blomgren *et al.* 2001). Prompt activation of this caspase was also observed in microglia treated with CM-RN (Fig. 2), indicating that caspase-3 activation is a characteristic hallmark of IFN-γ–induced apoptotic machinery.

While intracellular calpain is involved in physiological function, over-activation of calpain is found in pathologic conditions and has been implicated in cell injury and degeneration in many neurodegenerative diseases, including MS (Crocker et al. 2003, Ray & Banik 2003, Shields *et al.* 1999, Shields *et al.* 1998, Trager et al. 2014, Samantaray et al. 2015). Pathologic calpain has been found to activate microglia and to be involved in activation of T cells and their migration in demyelinating conditions (Guyton *et al.* 2010, Smith et al. 2011, Trager et al. 2014). Degenerated neurons release several signaling molecules, including nucleotides, cytokines, and chemokines, to recruit microglia and enhance their activities (Biber et al. 2007). The death of naïve neurons by CM-RN, as shown, indicates that normal neurons are susceptible to factors that are released from damaged neurons, including calpain, which acts as a signaling protease, thus propelling their own demise and confirming earlier findings (Levesque et al. 2010, Siman *et al.* 2005, Smith *et al.* 2012). In addition, activation of microglia by released calpain and possibly other factors complete the cycle and perpetuate the progressive degeneration process by reactive gliosis (Levesque et al. 2010, Samantaray et al. 2015, Smith et al. 2012) (Fig. 4). In line with this, a recent report demonstrated the signaling protease matrix metalloprotease-3 was released from damaged neurons and activated microglia (Kim et al. 2005). Thus, the bi-directional interaction between neurons and microglia is important for understanding of chronic neuroinflammation

or degeneration and gives us clues for future therapeutic strategy against neurodegenerative disorders. One strategy may be to block neuron damage with the use of calpain inhibitor, which will prevent subsequent microglial activation and disease progression. Calpain inhibition by CP or SNJ-1945 suppressed this process, which was further confirmed by knockdown of calpain using siRNA (Fig. 3). Such inhibition of microglia and T cell activation by calpain inhibitors has been found to block neuron and axon damage with reduction of the degenerative processes in demyelinating and other conditions (Crocker et al. 2003, Guyton et al. 2010, Levesque et al. 2010, Samantaray et al. 2015, Trager et al. 2014).

Our results indicating IFN-γ-induced apoptosis corroborate similar findings in human primary cultures. Neurons treated with $PBMCs_{Act}$+CP reduced the secretion of inflammatory mediators, including ROS production, COX-2 activity, and activity of caspases, which in turn reduced neuronal cell death (Fig. 5). Apoptosis, a fundamental process involved in the death and degeneration of cells that occurs in progressive MS, is triggered in the “execution” phase by activation of caspases – a family of cysteine proteases that cleaves critical intracellular substrates. As reported here, exposure of HN to $PBMCs_{Act}$ induced apoptosis via activation of caspases-8, -9, and -3 (Fig. 6). Activation of caspase-8 indicated involvement of the extrinsic (death receptor-mediated) pathway of apoptosis while activation of caspase-9 followed by activation of caspase-3 suggested participation of the intrinsic (mitochondria-mediated) pathway as well. Treatment with CP partially blocked activation of caspases-8, -9, and-3, supporting the notion that neuroprotection is, in part, due to inhibition of both the receptor-mediated and the mitochondrial apoptotic pathway, and both involve activities of calpain consistent with the action of calpain inhibitors.

Beneficial or destructive outcomes in the CNS may be determined by a dialogue between innate and adoptive immune responses that are in most cases T cells and microglia. Our data support the hypothesis that T cells and microglia are the main drivers of MS pathology, and both cell types have been found to overexpress pathogenic calpain in this disease (Friese & Fugger 2007, Shields & Banik 1998, Shields et al. 1999). Based on our data, we propose a cascade of events that culminates in progressive MS (Fig. 8). Thus, calpain released from damaged neurons not only kills surrounding normal neurons and activates T cells, it also promotes activation of microglia contributing to different functions. Microglia are not likely to be committed to one function (Hu *et al.* 2015, Jeong *et al.* 2015) and conversion between different phenotypes seems now critical next step to clarify the factors that initiate or promote such changes. Thus, inhibition of microglia and T cell activation by calpain inhibitor as a therapeutic agent may block neurons and axon damage and reduce progression of the degenerative process.

Our study should lead to further understanding of the mechanisms underlying the progressive nature of MS and other neurodegenerative diseases and particularly the nature, activation, and role of microglia. Although the goal is to attenuate neuronal damage, our results indicate that communication between neurons and microglia is bi-directional, involving several modulatory factors, including calpain. Modulation of microglial activation for therapeutic purposes – suppressing deleterious actions while simultaneously preserving their protective functions – appears well worth pursuing. Since microglia participation is critical in many neurodegenerative diseases, especially those with an inflammatory

component, ongoing research upon microglia modulation could provide greater insight necessary before any potential strategies for targeting microglia to attenuate inflammation and modify disease course is feasible. Targeting the interaction between neurons, microglia, and T cells is a potential approach to constrain the chronic inflammation and curb the progressive form of MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CM	conditioned media
CNS	central nervous system
COX-2	cyclooxygenase-2
CP	calpeptin
HI-IFN-γ	heat inactivated IFN-γ
HM	human microglia
HN	human neurons
MS	multiple sclerosis
NO	nitric oxide
$PBMCs_{Act}$	activated peripheral blood mononuclear cells
PBMCs	unactivated peripheral blood mononuclear cells
RM	rat microglia
RN	rat neurons
CM-RN	CM from RN stimulated with IFN-γ
CM-RN±CP	CM from RN stimulated with IFN-γ in the presence or absence of CP
CM-RM	CM from RM treated with CM-RN

CM-RM±CP	CM from RM treated with CM-RN in the presence or absence of CP
CM-RN$_{HI\text{-}IFN\text{-}\gamma}$	CM from RN treated with heat inactivated IFN-γ
CM-HN$_{PBMCs}$	CM from HN treated with PBMCs
CM-HN$_{PBMCsAct}$	CM from HN treated with activated PBMCs (PBMCs$_{Act}$)
CM-HN$_{PBMCsAct}$±CP	CM from HN treated with PBMCs$_{Act}$ in the presence or absence of CP
CM-HM±CP	CM from HM treated with CM-HN$_{PBMCsAct}$ in the presence or absence of CP
CM-HN±CP	CM from HN treated with CM-HM in the presence or absence of CP
ROS	reactive oxygen species
siRNA	small interfering RNA
VSC4.1	ventral spinal cord 4.1

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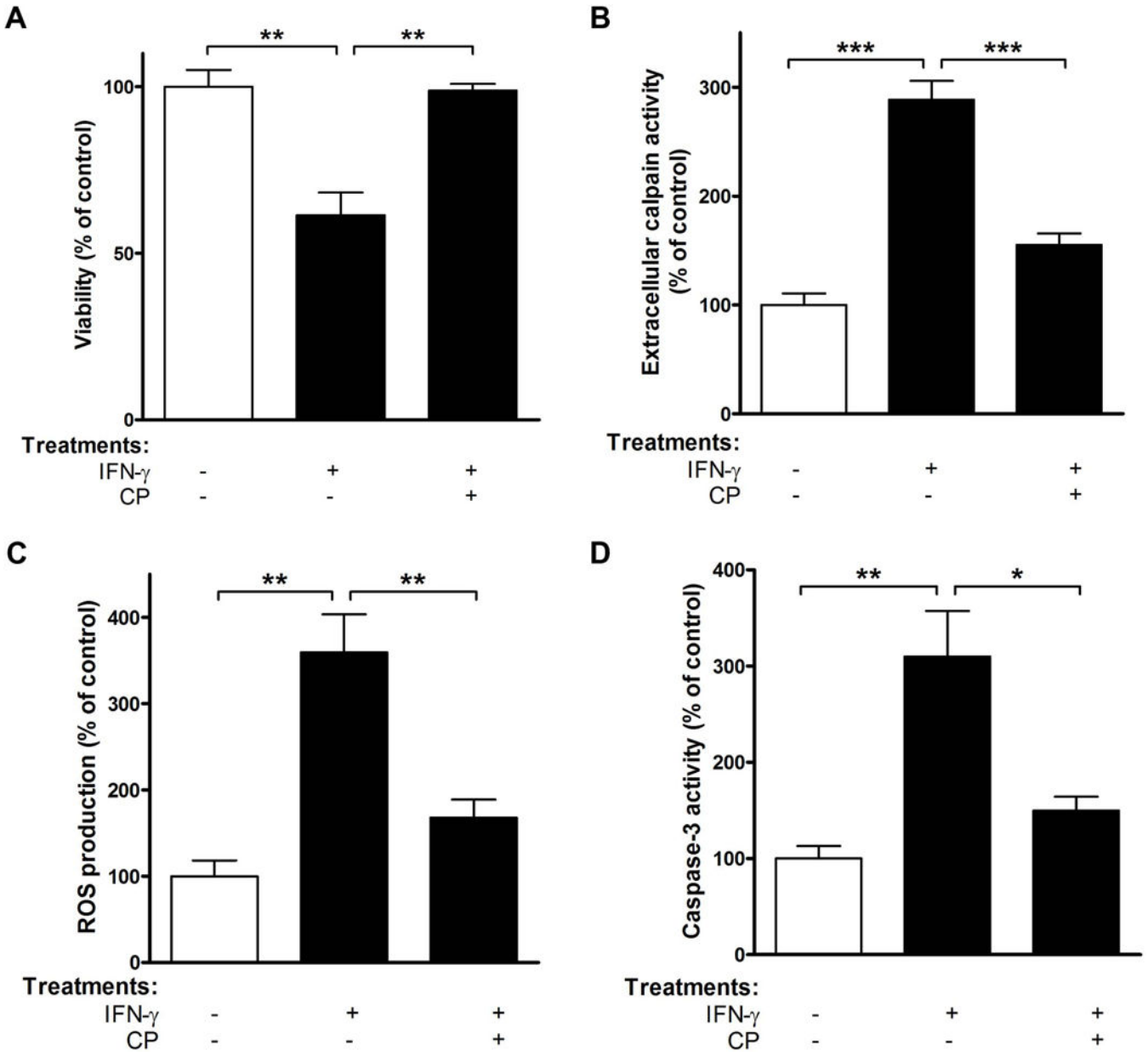

FIGURE 1. Calpain inhibition prevents primary rat neuron death after IFN-γ stimulation

Primary RN from cortex were exposed to IFN-γ in the absence or presence of 1 μM CP for 24 h. CM and cells were then collected and subjected to (**A**) Trypan blue dye exclusion test; (**B**) extracellular calpain activity assay; (**C**) ROS production determination; (**D**) caspase-3 activity assay. *p<0.05; **p<0.01; ***p<0.001; N=3.

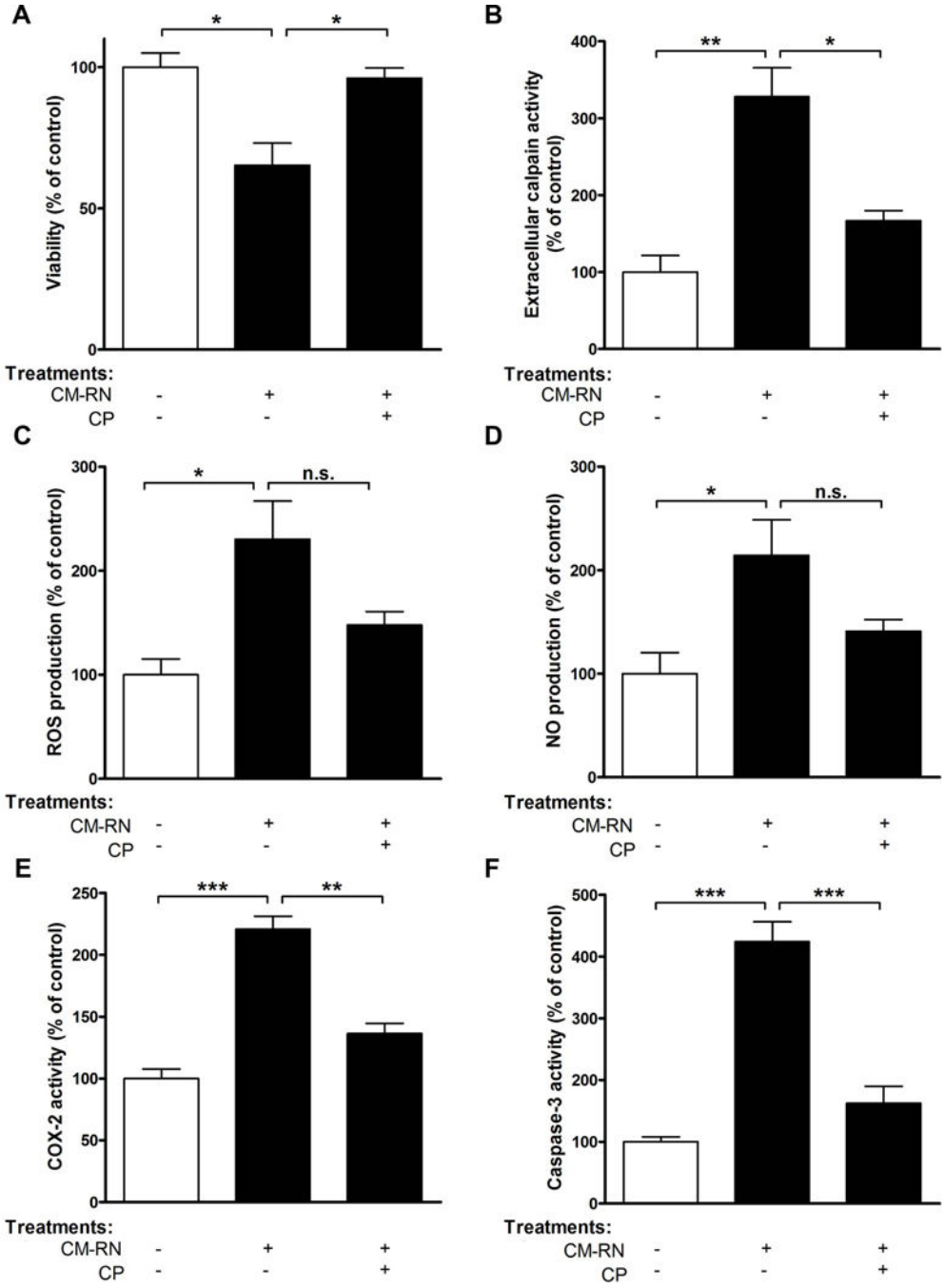

FIGURE 2. Pre-treatment with calpeptin prevents death of rat primary cortical microglia by supernatant from IFN-γ-damaged neurons

Primary RM from cortex exposed to CM-RN±CP. After 24 h of incubation, (**A**) viability, (**B**) extracellular calpain activity, (**C**) ROS production, (**D**) NO production, (**E**) COX-2 activity, and (**F**) caspase-3 activity were determined. *p<0.05; **p<0.01; ***p<0.001; n.s.-not significant; N=3.

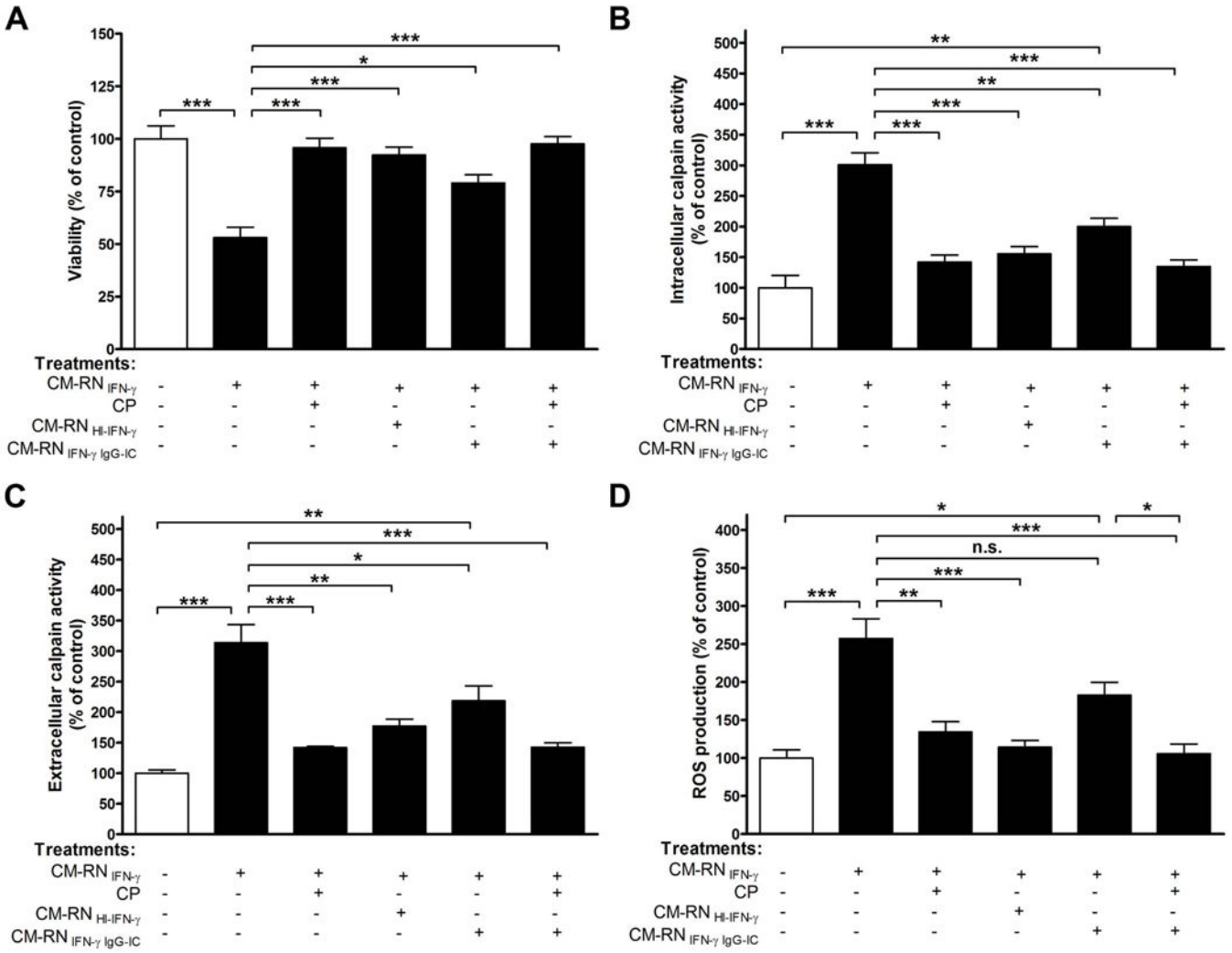

FIGURE 3. Calpain inhibition/knockdown prevents VSC4.1 motoneuron cell death stimulated by condition media released from activated primary microglia

VSC4.1 motoneurons were exposed to CM-RM; CM-RM+CP; CM-RM+SNJ-1945; CM-RM+μcalpain RNAi or CM-RM + mcalpain RNAi. After 24 h of treatment, (**A**) viability, (**B**) extracellular calpain activity, (**C**) ROS production, and (**D**) caspase-3 activity were determined. **p<0.01; ***p<0.001, N=3.

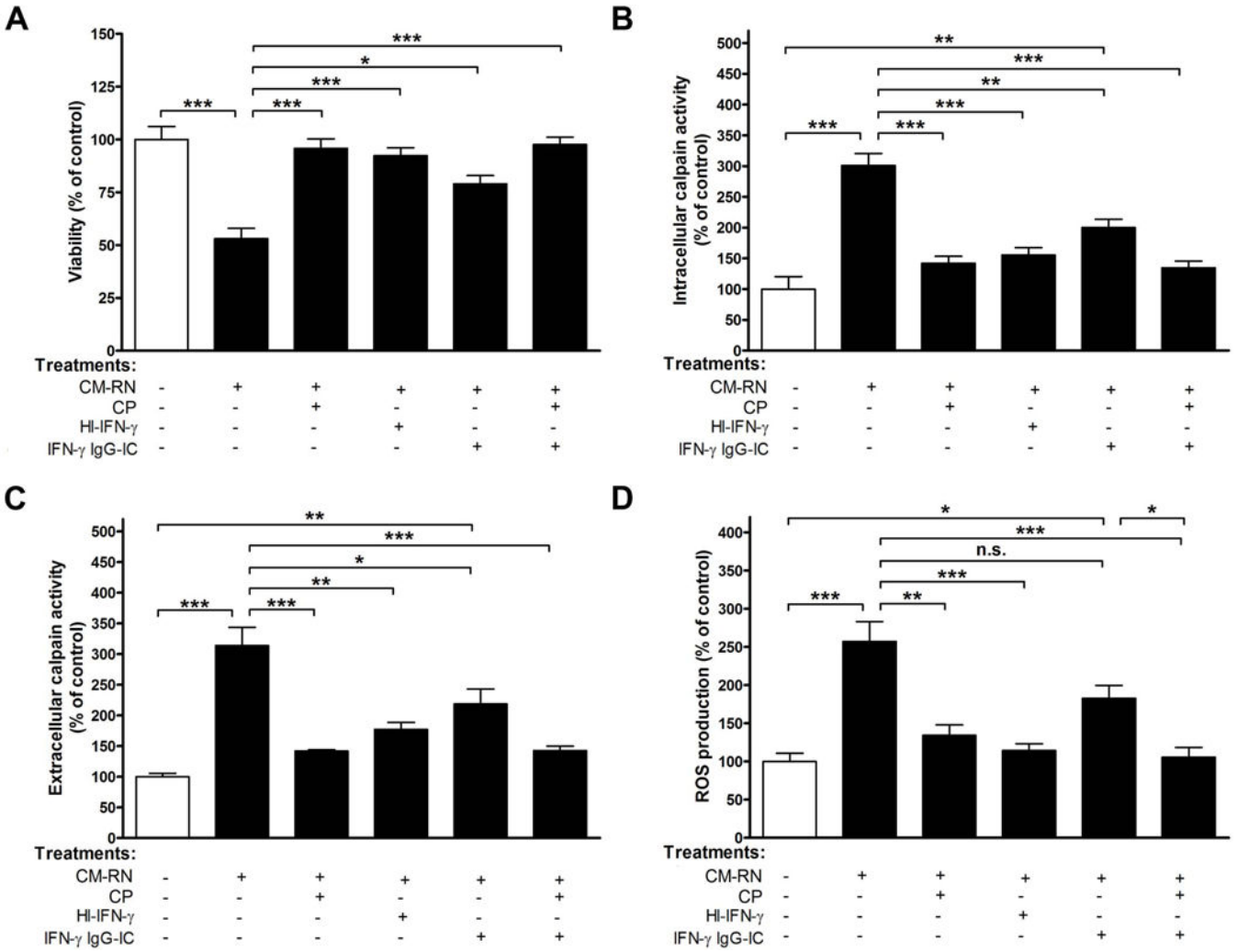

FIGURE 4. Pre-treatment with calpeptin prevents apoptotic death of VSC4.1 motoneurons by supernatant from IFN-γ damaged neurons

VSC 4.1 cells were exposed to CM-RN±CP; heat inactivated CM-RN; CM-RN treated with neutralizing antibody±CP. After specified treatment, (**A**) viability, (**B**) intracellular calpain activity, (**C**) extracellular calpain activity, and (**D**) ROS production were determined. $p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; N=3.

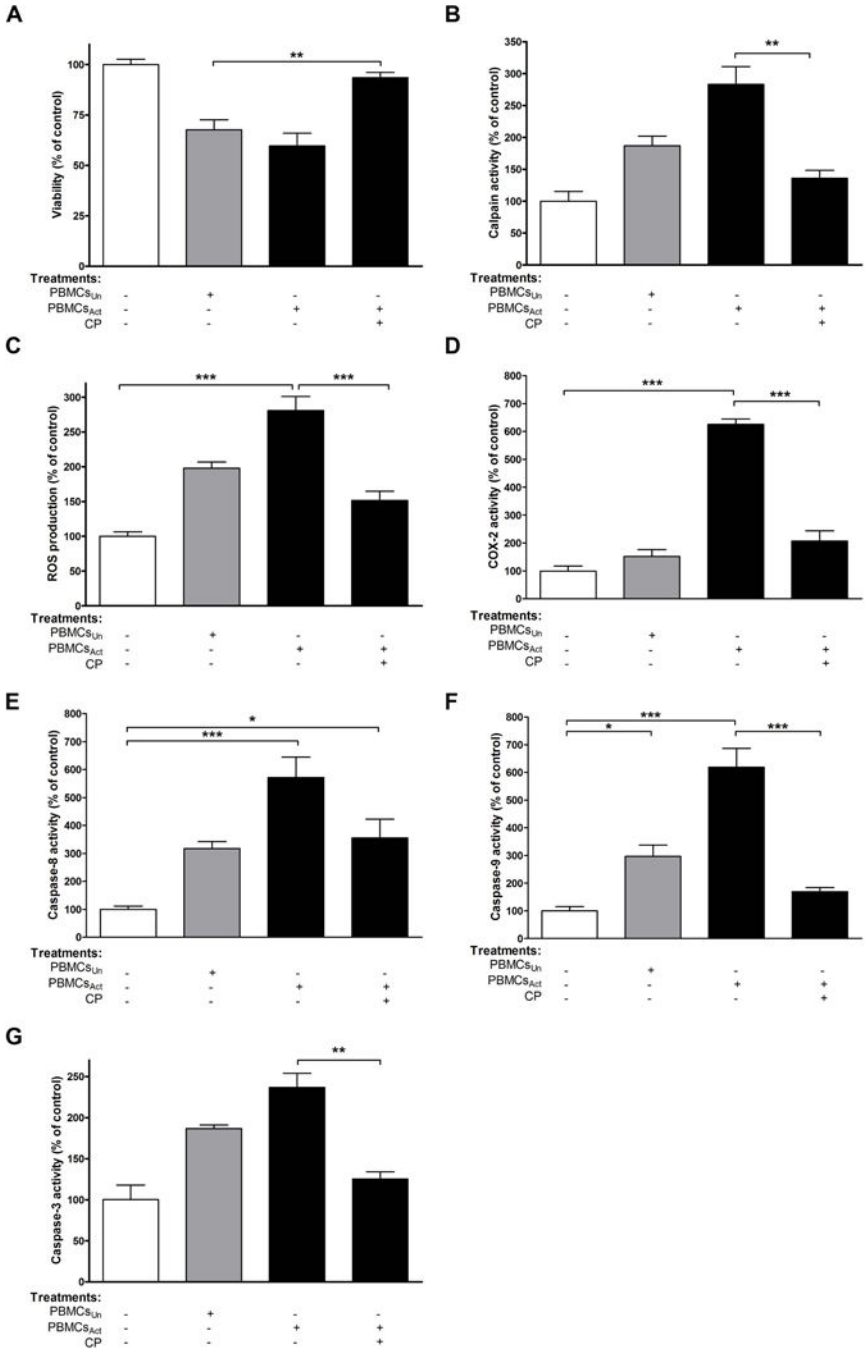

FIGURE 5. Calpain inhibition prevents human neurons degeneration after their stimulation with PBMCs$_{Act}$

Fetal HN were exposed to PBMCs; PBMCs$_{Act}$; or PBMCs$_{Act}$ + CP. After 24 h of incubation, (**A**) viability, (**B**) calpain activity, (**C**) ROS production, (**D**) COX-2 activity, (**E**) caspase-8 activity, (**F**) caspase-9 activity, and (**G**) caspase-3 activity were determined. *p<0.05; **p<0.001; ***p<0.001; N=3.

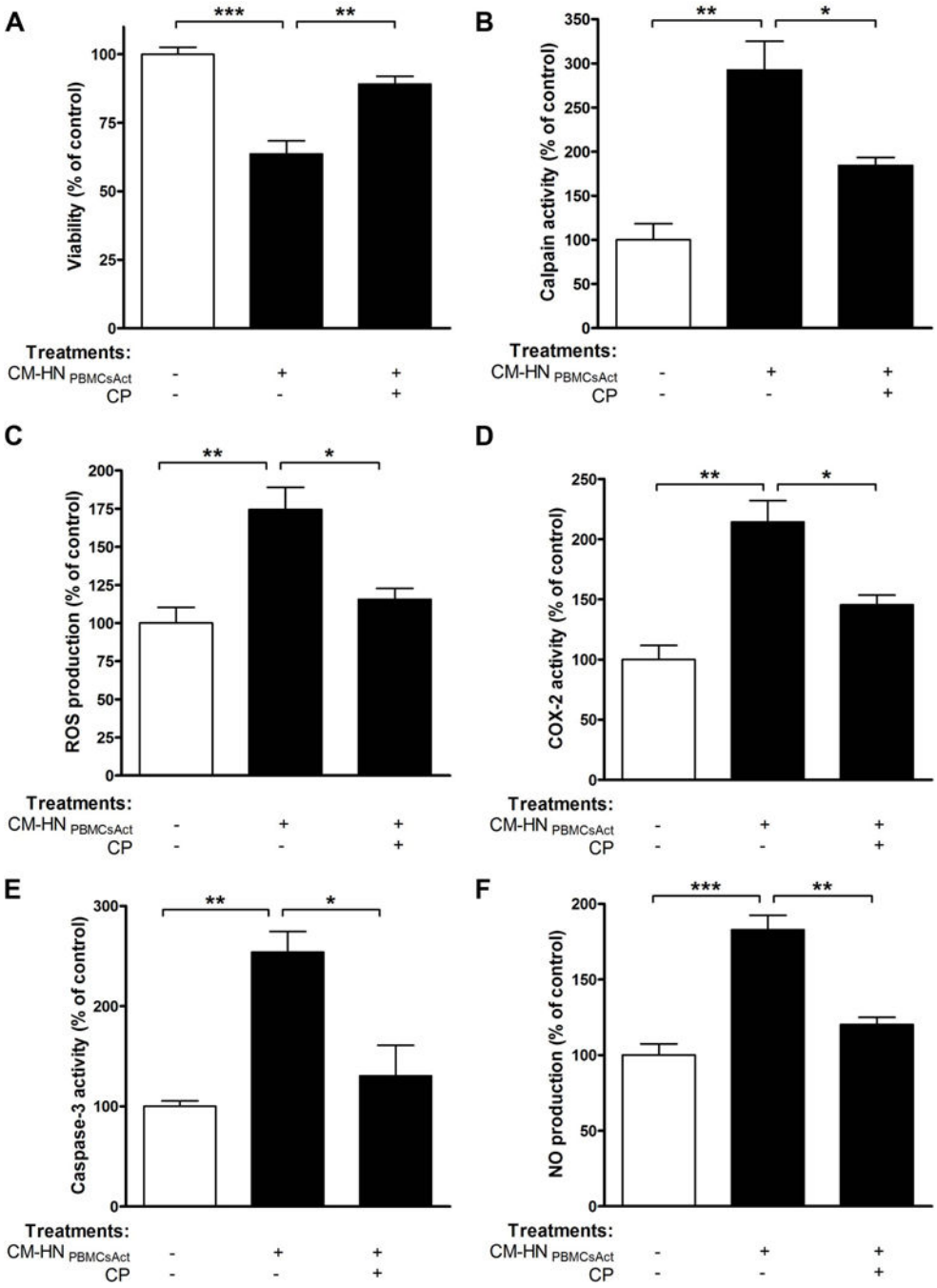

FIGURE 6. Pre-treatment with calpeptin prevents apoptotic death of human primary microglia by supernatant from PBMC-damaged human neurons

Primary HM from undamaged cortex were exposed to CM-$HN_{PBMCsAct}$ or CM-$HN_{PBMCsAct}$+CP. After 24 h of incubation, (**A**) viability, (**B**) calpain activity, (**C**) ROS production, (**D**) COX-2 activity, (**E**) caspase-3 activity, and (**F**) NO production were determined. *p<0.05; **p<0.01; ***p<0.001; N=3.

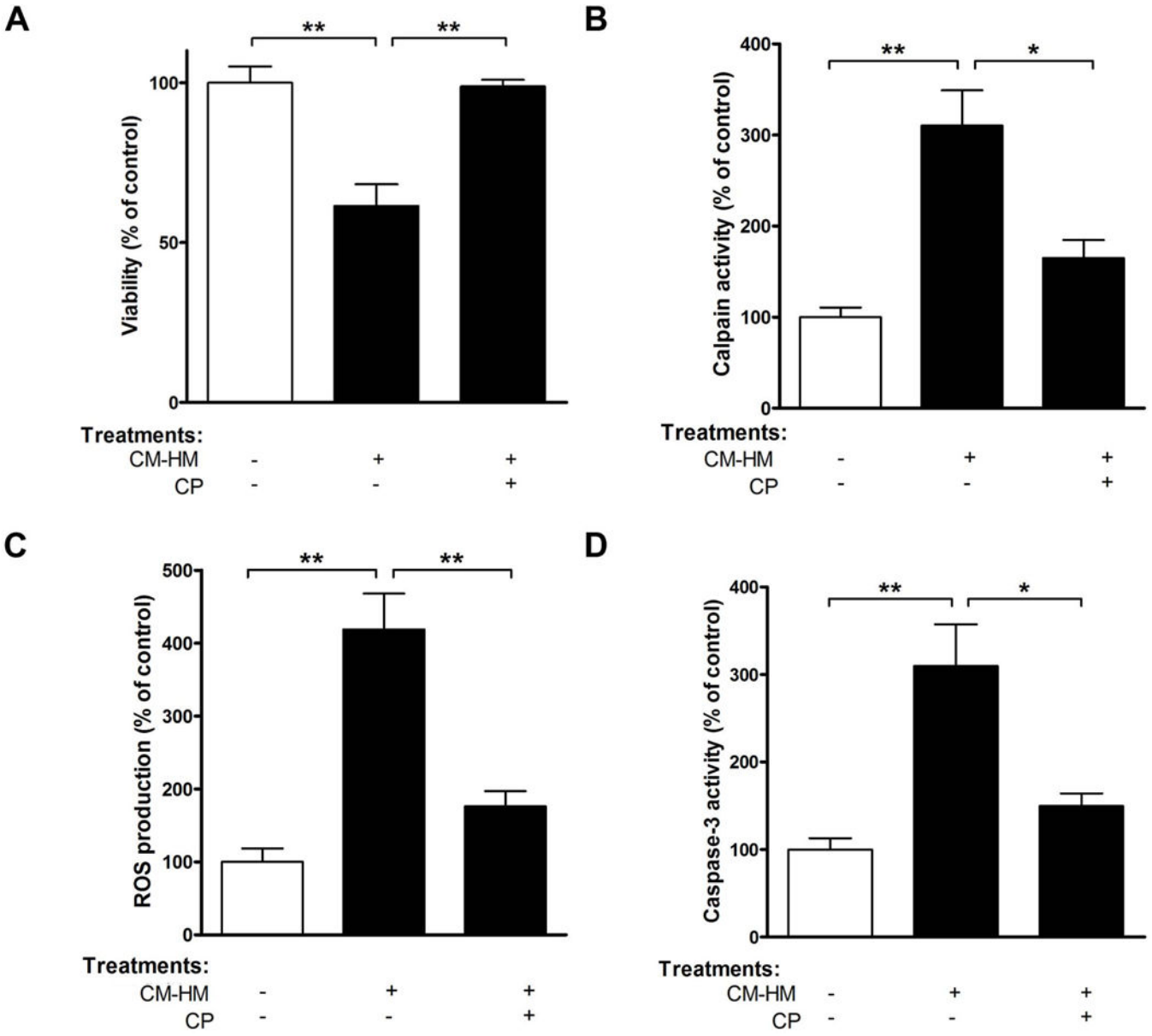

FIGURE 7. Calpain inhibition prevents microglia-mediated human neurons degeneration *in vitro*

Fetal HN were exposed to CM-HM or CM-HM+CP. After 24 h of incubation, (**A**) viability, (**B**) calpain activity, (**C**) ROS production, and (**D**) caspase-3 activity were determined. *p<0.05; **p<0.01; ***p<0.001; N=3.

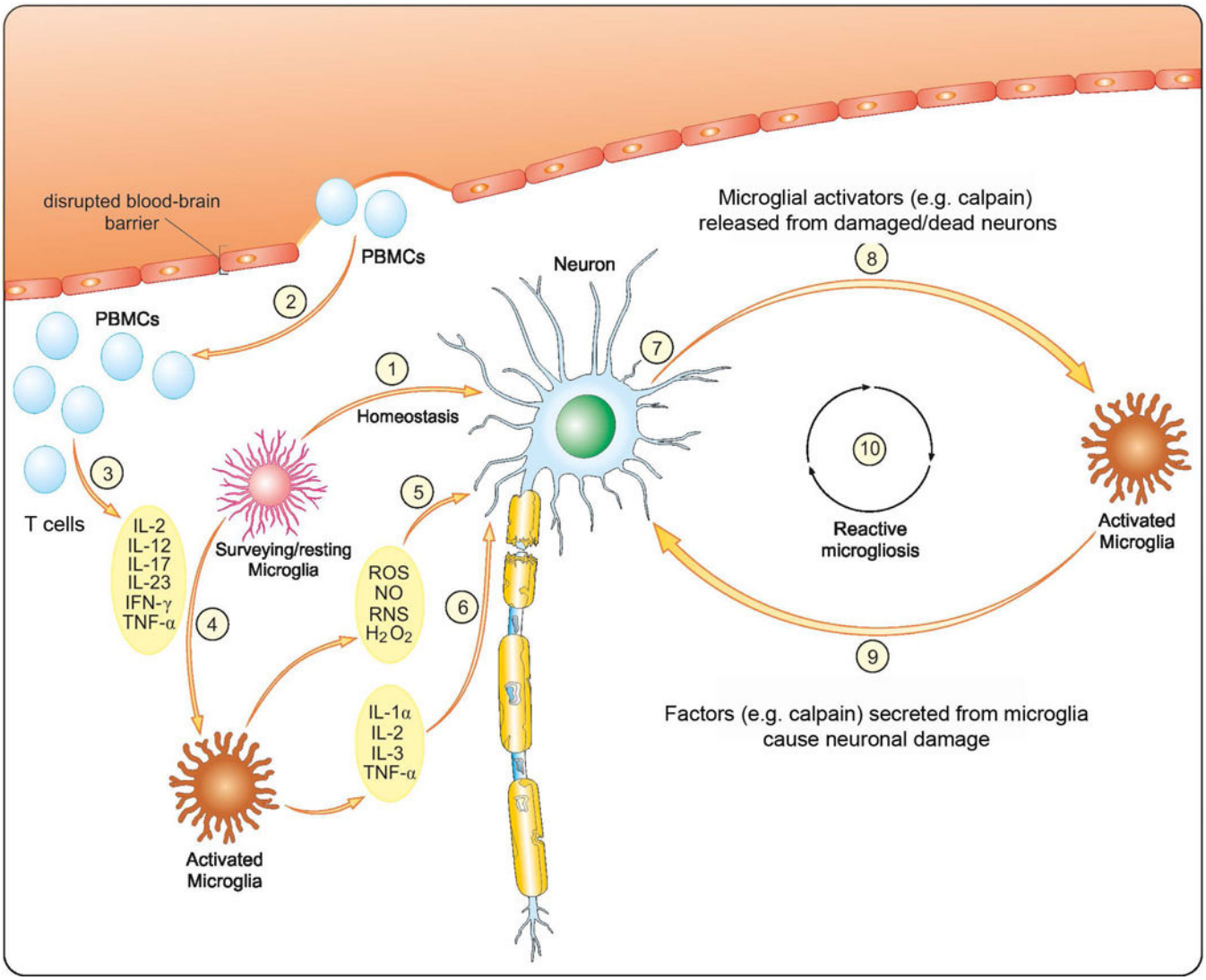

FIGURE 8. Main mechanisms involved in progressive nature of MS driven by neuron-microglia interaction

(1) In the surveillance state microglia monitor brain parenchyma and detect danger signals. **(2)** As a consequence of brain injury an activated peripheral blood mononuclear cells (PBMCs) infiltrate CNS through disrupted the blood-brain barrier. **(3)** The activated PBMCs release pro-inflammatory cytokines Th1 and Th17 (Imam et al. 2007, Smith et al. 2011). **(4)** Homeostasis is lost and resident microglia change phenotype to an activated state. Activation of microglia is associated with production of pro-inflammatory cytokines **(5)** as well as toxic metabolites **(6)** (McDowell et al. 2011). Supernatant from activated microglia containing released cytokines causes neurons injury **(7)** (McDowell et al. 2011). Increased production of different cytotoxic substances, including calpain and cytokines, induces important alterations in cortical neurons and axons. Released from dying/damaged neurons calpain-containing conditioned media activates microglial cells **(8)** while conditioned media from activated microglia causes injury to surrounding naïve neurons **(9)**. Activation of microglia and neurons induce calpain-mediated bi-directional cytotoxicity to initiate a self-

propelling cycle of neurons and microglia damage/death with pathology of reactive microgliosis **(10)**. Calpain inhibitors (e.g., calpeptin) modulate calpain-mediated bi-directional cytotoxicity and could halt progressive MS.