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# Microglia Induce Neurotoxicity via Intraneuronal $Zn^{2+}$ Release and a $K^+$ Current Surge

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## Abstract

Microglial cells are critical components of the injurious cascade in a large number of neurodegenerative diseases. However, the precise molecular mechanisms by which microglia mediate neuronal cell death have not been fully delineated. We report here that reactive species released from activated microglia induce the liberation of $Zn^{2+}$ from intracellular stores in cultured cortical neurons, with a subsequent enhancement in neuronal voltage-gated $K^+$ currents, two events that have been intimately linked to apoptosis. Both the intraneuronal $Zn^{2+}$ release and the $K^+$ current surge could be prevented by the NADPH oxidase inhibitor apocynin, the free radical scavenging mixture of superoxide dismutase and catalase, as well as by 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) chloride. The enhancement of $K^+$ currents was prevented by neuronal overexpression of metallothionein III or by expression of a dominant negative (DN) vector for the upstream mitogen-activated protein kinase apoptosis signal regulating kinase-1 (ASK-1). Importantly, neurons overexpressing metallothionein-III or transfected with DN vectors for ASK-1 or Kv2.1-encoded $K^+$ channels were resistant to microglial-induced toxicity. These results establish a direct link between microglial-generated oxygen and nitrogen reactive products and neuronal cell death mediated by intracellular $Zn^{2+}$ release and a surge in $K^+$ currents.

### Keywords

zinc; oxidation; nitration; Kv2.1; potassium channel; apoptosis signaling kinase-1

## INTRODUCTION

Microglia, rapidly activated in response to neuronal injury, are critically important in promoting neurodegeneration in many CNS disorders (Bessis et al., 2007; Block et al., 2007; Colton et al., 2000, 2005; Wojtera et al., 2005). In addition to cytokines, activated microglia can release neurotoxic levels of nitric oxide (·NO) and superoxide ($O_2^-$), respectively, generated by inducible nitric oxide synthase (iNOS) and NADPH oxidase (Colton and Gilbert, 1987; Li et al., 2005; Mander and Brown, 2005). Peroxynitrite ($ONOO^-$), rapidly and favorably formed by the cogeneration of · NO and $O_2^-$ (Espey et al., 2002), can trigger or exacerbate neuropathological injury (Bal Price et al., 2002; Beckman et al., 1990; Keynes and Garthwaite, 2004). At physiological pH, $ONOO^-$ becomes protonated and undergoeshomolytic cleavage to generate the protein tyrosine nitrating species nitrogen dioxide ($\cdot NO_2$) as well the highly oxidizing hydroxyl radical (·OH; Schopfer et al., 2003). In addition, peroxynitrite can react

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with $CO_2$ to generate $ONOOCO_2$, leading to $\cdot NO_2$ and $\cdot CO_3$ radicals, also via homolytic cleavage (Schopfer et al., 2003). However, it has been demonstrated that $ONOO^-$ can readily traverse membrane lipids at rates faster than its decomposition pathways (Marla et al., 1997). Thus, unlike $O^-_2\cdot$ or ·OH, $ONOO^-$ can diffuse cellular dimension distances, providing the reactive species it generates widespread access to internal cellular structures (Lee et al., 1998; Marla et al., 1997).

In neurons, exogenous $ONOO^-$ applied under physiological conditions induces the liberation of $Zn^{2+}$ from intracellular binding sites, which, in turn, initiates a cell death cascade mediated by the activation of the mitogen-activated protein kinase (MAPK) p38 (Zhang et al., 2004). In addition, $ONOO^-$ application also initiates a p38-dependent enhancement of voltage-dependent $K^+$ currents (Bossy-Wetzel et al., 2004; Pal et al., 2004), a process that has been tightly linked to neuronal apoptosis (McLaughlin et al., 2001; Redman et al., 2007; Yu et al., 1997). Enhanced $K^+$ currents enable cytosolic $K^+$ efflux, necessary for the completion of the cell death cascade (Bortner and Cidlowski, 2004; Yu, 2003). Consistent with this, blocking $K^+$ efflux following injury can halt neuronal apoptosis in a large number of experimental models (Yu, 2003; Yu et al., 1997). However, despite a pronounced $K^+$ current surge in cortical neurons following exposure to ONOO donors (Pal et al., 2004), neurotoxicity could not be averted by preventing $K^+$ channel function (Zhang et al., 2004). This observation raises the possibility that $ONOO^-$ donors cannot mimic potential physiological sources of the reactive species (Espey et al., 2002) and, consequently, overwhelm the affected neurons, such that $K^+$ efflux is not necessary for the cell death process. Here, we used a co-culture model to investigate whether activated microglia, under conditions more akin to a pathophysiological insult in the brain, could initiate a cell death pathway in cortical neurons that was characterized by intraneuronal $Zn^{2+}$ release and, especially, by a $K^+$ current surge. Our results do indicate that reactive oxygen and nitrogen species originating from activated microglia induces neuronal death via a process that is fully dependent on $Zn^{2+}$ release and an enhancement of $K^+$ currents.

## MATERIALS AND METHODS

### Cell Culture

Cortical cultures were prepared from embryonic, day 16 Sprague-Dawley rats as previously described (Hartnett et al., 1997). Briefly, cortices were dissociated, and the resultant cell suspension was adjusted to 600,000 cells/well (six-well tissue culture plates containing five, 11-mm poly-L-ornithine-treated coverslips/well) in a growth medium composed of a volume-to-volume mixture of 80% Dulbecco's modified minimal essential medium (MEM), 10% Ham's F12-nutrients, 10% bovine calf serum (heat-inactivated, iron-supplemented; Hyclone, Logan, UT) with 25 mM HEPES, 24 U/mL penicillin, 24 μg/mL streptomycin, and 2 mM L-glutamine. Cultures were maintained at 37°C, 5% $CO_2$, and the media was partially replaced on a monday–wednesday–friday schedule. Glial cell proliferation was inhibited after 2 weeks in culture with 1–2 μM cytosine arabinoside, after which the cultures were maintained in growth medium containing 2% serum and without F12-nutrients. Cultures were utilized at 18–22 days *in vitro* (DIV) and contained ~20% neurons (Rosenberg and Aizenman, 1989). In these cultures, neuronal somata and proximal processes are phase bright and protrude above a phase dark, flat astrocyte layer that covers distal processes (Harris and Rosenberg, 1993). Immortalized rat brain microglial cells (Cheepsunthorn et al., 2001) were generously supplied by J. Connor (Pennsylvania St. University, Hershey, PA). Microglia were maintained in Dulbecco's modified MEM supplemented with 10% heat-inactivated fetal bovine serum, and plated in trans-well inserts (Corning, Corning, NY) at a density of $0.5 \times 10^6$ cells/well for 24 h prior to activation (Li et al., 2005). For the $Zn^{2+}$ imaging and electrophysiological studies, trans-well inserts containing microglia were placed on top of neuron-containing coverslips in

24-well plates immediately prior to microglial activation. To ensure a robust and consistent activation of microglia, 10 U/mL interferon-γ (IFN-γ, Chemicon, Temecula, CA) and 1 μg/mL lipopolysaccharide were added directly into the well inserts (Duport and Garthwaite, 2005). Chemical activation of this microglial cell line leads to activation of both iNOS and NADPH oxidase in less than 30–60 min (Cheepsunthorn et al., 2001; Qian et al., 2007). The insert-containing co-cultures were then immediately transferred to the incubator and maintained in the dark at 37°C and 5% $CO_2$ for the duration of the exposure (see later) to minimize the light-dependent consumption of microglial-generated NO by cell culture media components (Keynes et al., 2003).

## Intracellular Zinc Measurements

After a 60-min exposure to activated microglia, trans-well inserts were removed and cortical cultures, in the absence of microglia, were thoroughly rinsed and loaded with the $Zn^{2+}$-sensitive fluorescent reporter Fluozin-3 AM (5 μM, Molecular Probes, Carlsbad, CA) for 30 min in buffered solution (144 mM NaCl, 3 mM KCl, 10 mM HEPES, 5.5 mM glucose, with 5 mg/mL bovine serum albumin; pH 7.3). Coverslips were then transferred to a recording chamber (Warner, Hamden, CT) mounted on an inverted epifluorescence microscope and superfused with MEM, supplemented with 25 mM HEPES and 0.01% BSA. Images were acquired with 490 nm excitation light using a computer-controlled monochromator (Polychrome II, TILL Photonics, Martinsried, Germany) and a CCD camera (IMAGO, TILL Photonics). After baseline images were acquired, free intracellular $Zn^{2+}$ was chelated with 20 μM tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN). The relative $Zn^{2+}$ fluorescence for all neuronal cell bodies in a single field ($n$ = 5–30) was determined by subtracting the fluorescence signal after TPEN application from the baseline signal ($\Delta F_{TPEN}$). $Zn^{2+}$ signals emanating from supporting astrocytes are minimal under these conditions, as these cells appear to buffer intracellular $Zn^{2+}$ more effectively than neurons (Dineley et al., 2000).

## Electrophysiology

After a 60-min exposure to activated microglia, trans-well inserts were removed and cortical cultures were returned to the incubator for an additional 3 h, a time-point by which we have observed pronounced $K^+$ current surges in neurons undergoing apoptosis (McLaughlin et al., 2001). Following this time, cultures were rinsed thoroughly and transferred to a recording chamber. All recordings were performed from neurons at room temperature using whole cell recordings, with 2–3 MΩ patch electrodes as previously described (McLaughlin et al., 2001). The extracellular recording solution consisted of 115 mM NaCl, 2.5 mM KCl, 2.0 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, pH 7.2; 0.25 μM TTX was added. The electrode solution consisted of (in mM) 120 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 0.22 ATP; pH 7.2. Currents were amplified with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 2 kHz, and digitized at 10 kHz. Potassium currents were evoked with 80-ms voltage steps from −70 mV in 10-mV increments. For analysis, steady-state current amplitudes were measured at + 10 mV and normalized to cell capacitance. Series resistance was compensated in all cases (~80%).

## Neuronal Transfection

Neurons were transfected with either apoptosis signal-regulating kinase-1 dominant negative (DN) vector (ASK1 DN; gift from H. Ichijo, Tokyo Medical University, Japan), metallothionein III (MT-III), or a DN truncated mutation of Kv2.1 (gift from J. Nerbonne, Washington University, St. Louis, MO) in 18–22 DIV neurons using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described by Pal et al. (2003). Transfection rates for neurons in this type of culture are normally around 1% (Santos and Aizenman, 2002), but can be as high as 5% (unpublished). For electrophysiological recordings, neurons were also transfected with

a plasmid encoding enhanced green fluorescent protein (eGFP). For toxicity assays, neurons were co-transfected with a luciferase-expressing vector (Boeckman and Aizenman, 1996; Pal et al., 2003). The MT-III plasmid was constructed by amplifying full-length cDNA from rat brain. The primers used were as follows: sense, agaagcttgccaccatggaccctgagacctgccc; antisense, gaggatcctggcagcagctgcatttct. The MT-III cDNA obtained was inserted into an eGFP-expressing vector. Transfections were performed 24 h prior to activated microglial exposure.

### Toxicity Assay

Cell death was measured using a luciferase activity assay (Boeckman and Aizenman, 1996; Pal et al., 2003). Twenty-four hours after transfecting neurons with a luciferase expressing vector plus or minus a vector of interest, microglia (50,000 cells/mL) were plated directly on top on neurons and activated for 60 min as described previously. Trans-well inserts were not utilized under these conditions to minimize expenses, since individual neurons did not have to be identified for recordings (i.e., $Zn^{2+}$ measurements or electrophysiology). Cell viability was assayed 48 h later on transfected neurons as previously described (Pal et al., 2003). An overnight exposure to staurosporine (0.5 μM) or NMDA (200 μM) in the absence of microglia was used as a positive control for maximal neuronal cell death. We also took advantage of the fact that neuroprotective manipulations of neurons could be performed without affecting microglial function. As such, the overexpression of DN vectors for either the p38 upstream MAPK apoptosis signal regulating kinase-1 (ASK-1), or Kv2.1, could be directed to neurons prior to co-culture and activation of microglia. These conditions were important to control since some of the signaling pathways triggered in neurons during cell death are also involved within microglia to promote their activation, including both p38 phosphorylation and changes in $K^+$ channel function (Kaushal et al., 2007; Koistinaho and Koistinaho, 2002).

### Immunocytochemistry

Cortical cultures were examined for the presence of 3-nitrotyrosine under control conditions or after exposure to activated microglia (Mander and Brown, 2005). Ninety minutes after microglia exposure as described earlier, neurons were fixed for 10 min in 4% paraformaldehyde. Following a 5-min wash in phosphate buffered saline (PBS), neurons were treated with 0.3% triton in PBS, washed again, and blocked in 1% bovine serum albumin. Cultures were then incubated overnight with 3.3 μg/mL of antinitrotyrosine monoclonal antibody (Upstate, Lacke Placid, NY) at 4°C. A FITC-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) was used to detect cells labeled with the primary antibody.

## RESULTS

### Activated Microglia Induce an Increase in Intraneuronal $Zn^{2+}$

Neurons previously exposed to microglia activated with LPS/IFN-γ (see Materials and Methods) exhibited a significant increase in TPEN-sensitive $Zn^{2+}$ fluorescence ($\Delta F_{TPEN}$) when compared with untreated control cells (Figs. 1 and 2). This increase in $\Delta F_{TPEN}$ was not evident in neurons treated for 60 min with LPS/IFN-γ in the absence of microglia, or in neurons exposed for 60 min to resting microglia (Fig. 2A). To determine the origin of increased cytosolic $Zn^{2+}$ concentration ($[Zn^{2+}]_i$), we added a cell impermeable $Zn^{2+}$ chelator $Ca^{2+}$-EDTA while neurons were exposed to activated microglia. Under these conditions, we still observed a significant increase in $\Delta F_{TPEN}$, suggesting that influx of extracellular $Zn^{2+}$ is not necessary for the increase in $[Zn^{2+}]_i$ (Fig. 2B). We did note, however, that even in control neurons the overall $Zn^{2+}$ fluorescence in $Ca^{2+}$-EDTA exposed cells was generally lower in intensity compared with neurons that remained in a normal buffer. This may have resulted from the removal of exchangeable $Zn^{2+}$ pools normally available to neurons (Frederickson et al., 2002). Nonetheless, as $[Zn^{2+}]_i$ was still substantially elevated in microglia-exposed cells in the

presence of $Ca^{2+}$-EDTA, we conclude that measurable changes in $Zn^{2+}$ fluorescence are primarily due to $Zn^{2+}$ release from intracellular sources (Aizenman et al., 2000).

To characterize the origin of the reactive species responsible for intraneuronal $Zn^{2+}$ release, selective scavengers and inhibitors of microglial-generated free radicals were used. When the $O^{-}_{2}$ scavenging mixture of superoxide dismutase/catalase was added to the neuronal culture medium during activated microglia exposure, $\Delta F_{TPEN}$ was attenuated compared with neurons exposed to activated microglia alone (Fig. 2C). Similarly, $\Delta F_{TPEN}$ measured after exposure to apocyanin-treated activated microglia was also significantly decreased, suggesting that NADPH oxidase was the primary source of $O^{-}_{2}$ production (Fig. 2C). Because NADPH-derived microglial $O^{-}_{2}$ contributes to $ONOO^{-}$ formation (Li et al., 2005), neurons were also treated with 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) chloride (FeTPPS) during activated microglial exposure. FeTPPS is widely believed to be an $ONOO^{-}$ decomposition catalyst with minimal superoxide dismutase activity, and does not appear to complex with NO (Lee et al., 1998;Misko et al., 1998). Following this treatment, we observed a significant reduction of the liberation of intraneuronal $Zn^{2+}$ caused by activated microglia (Fig. 2C). On the basis of these results, we suggest that activated microglia produce oxygen and nitrogen reactive species, which causes an increase in $[Zn^{2+}]_i$ in co-cultured neurons. Importantly, evidence that the nitrating species $\cdot NO_2$ was generated in our system was confirmed by the presence of 3-nitrotyrosine immunoreactivity in neurons exposed to activated microglia (Fig. 2D).

## Activated Microglia Trigger a Surge in Voltage-Gated Potassium Currents in Neurons

Oxygen and nitrogen-derived reactive species induce intracellular $Zn^{2+}$ release and enhance voltage-gated $K^+$ current during apoptosis (Aizenman et al., 2000; Bossy-Wetzel et al., 2004; McLaughlin et al., 2001; Pal et al., 2004, 2006). Therefore, we investigated whether microglial-generated reactive products would also induce a surge in neuronal $K^+$ currents. $K^+$ current densities in neurons exposed to activated microglia were significantly larger than the current densities recorded from control neurons (Figs. 3 and 4). Furthermore, this current enhancement could be prevented by apocyanin or FeTPPS (Fig. 4A). To determine whether the microglia-mediated increase in $[Zn^{2+}]_i$ caused $K^+$ current enhancement via the p38 upstream MAPK ASK-1 (Aras and Aizenman, 2005), neurons were transfected with metallothionein-III (MT-III), to bind excess intracellular $Zn^{2+}$, or a DN vector for ASK-1. We observed that microglia-exposed neurons expressing either MT-III or ASK-1 DN exhibited $K^+$ current densities similar to control cells (Fig. 4B). Likewise, both empty vector-expressing neurons and untransfected cells behaved similarly when exposed to activated microglia. These results demonstrate that an increase in $[Zn^{2+}]_i$ triggered by activated microglia is capable of inducing a key step in the neuronal cell death process, namely an ASK-1-mediated $K^+$ current surge.

## Microglial-Induced Neuronal Cell Death

We hypothesized that reactive species produced by activated microglia, capable of increasing neuronal $[Zn^{2+}]_i$ and producing an ASK1-dependent $K^+$ current surge, would cause neuronal cell death via this pathway (Bossy-Wetzel et al., 2004; McLaughlin et al., 2001). A cell viability assay was thus employed to investigate the mechanism of microglial-mediated neurotoxicity. We first observed that activated microglial-induced neuronal death could be prevented by apocyanin (Fig. 5A), confirming the role for NADPH oxidase in this process (Block et al., 2007). FeTPPS appeared to interfere with the luciferase viability assay. However, treating co-cultures with the iNOS inhibitor 1,400 W effectively attenuated microglia-mediated neurotoxicity (Fig. 5A). The fact that inhibition of either NADPH oxidase or iNOS was sufficient to halt neuronal death strongly suggests that microglial-derived reactive oxygen and nitrogen species are responsible for mediating neuronal damage.

Finally, we investigated the molecular progression of microglial-mediated neuronal death by neuronal overexpression of either MT III, ASK-1 DN, or Kv2.1 DN (Fig. 5B). A nonpharmacological approach was necessary to investigate the progression of this pathway, as inflammatory microglia also exhibit activation of p38 MAP kinase and voltage-gated $K^+$ currents, two of the key signaling components proposed in our neuronal death pathway (Fordyce et al., 2005). Overexpressing metallothionein-III effectively increased neuron viability, indicating that the increased availability of a high affinity $Zn^{2+}$ buffer was sufficient to rescue neurons from the microglial-triggered cell death process. We also observed that interfering with ASK-1 function, required for Kv2.1-mediated $K^+$ current surge (Aras and Aizenman, 2005), attenuated cell death. Finally, overexpression of a DN vector for Kv2.1, the $K^+$ channel responsible for mediating neuronal apoptosis in cortical, midbrain dopaminergic, and cerebellar granule neurons (Jiao et al., 2007;Pal et al., 2003;Redman et al., 2006), was also highly neuroprotective. Taken together, these results demonstrate that microglial-derived reactive species induce neuronal cell death via a process that involves the release of $Zn^{2+}$ from intracellular sources, ASK-1 activation, and a surge in Kv2.1-mediated voltage-gated $K^+$ currents.

## DISCUSSION

We provide evidence that two important components of a previously characterized cell death cascade in neurons are intimately related to microglial-mediated neuronal injury, namely, intracellular $Zn^{2+}$ release and a surge in voltage-dependent $K^+$ currents (Aizenman et al., 2000; McLaughlin et al., 2001). Interfering with these processes proved to be highly effective in blocking neuronal cell death as a result of microglial activation. There are increasing numbers of studies suggesting a close association between microglia activation and neuronal injury in central nervous system disorders (Bessis et al., 2007; Colton et al., 2000, 2005; Duport and Garthwaite, 2005; Wojtera et al., 2005). In acute injury, such as that occurring during stroke, microglial-derived oxygen and nitrogen reactive species may represent critical components for initiating or compounding neuronal damage as it normally precedes but can amplify the production of proinflammatory cytokines (Block et al., 2007; Qin et al., 2004). Naturally, the production of microglial cytokines is a well-established mechanism of neuronal injury and has been implicated with many chronic neurodegenerative disorders (Block et al., 2007). Additionally, the production of reactive species by microglia is likely to impair other cellular components, such as membrane lipids, that may or may not necessarily involve the liberation of intracellular $Zn^{2+}$. Of note however, aldehydes, which are lipid peroxidation products, can react with $Zn^{2+}$-thiolate clusters in proteins and readily liberate the metal (Hao and Maret, 2006). We therefore suggest that targeting intraneuronal $Zn^{2+}$ dysregulation, or the cell death signaling transduction cascades activated by $Zn^{2+}$, could provide effective therapeutic strategies for minimizing neuronal damage in stroke and related disorders.

Since $Zn^{2+}$ can be readily liberated from intracellular metal-binding proteins by oxygen and nitrogen-derived stressors (Maret, 2006; Sidorkina et al., 2003), and can subsequently trigger neuronal cell death (Aizenman et al., 2000; Bossy-Wetzel et al., 2004; McLaughlin et al., 2001), it is also entirely possible that the processes described here will be shown to be widely expressed in many forms of neurodegeneration. In fact, release of intracellular $Zn^{2+}$ during neuronal injury has now been reported to be involved in a number of neurodegenerative conditions, including epileptic seizures (Lavoie et al., 2007; Lee et al., 2000), cerebral ischemia (Calderone et al., 2004), and target deprivation (Land and Aizenman, 2005). Importantly, microglial activation has been shown to be a potential crucial component of the neuropathological changes observed in all of these conditions (Boer et al., 2006; De Simoni et al., 2000; Ekdahl et al., 2003; Milligan et al., 1991b; Rizzi et al., 2003; Wang et al., 2007). Microglia have also been shown to be activated during normal brain development (Milligan et al., 1991a), where they play a critical role in the programmed death of neurons (Marin-Teva

et al., 2004). Interestingly, it has been reported that both intraneuronal $Zn^{2+}$ accumulation and enhanced $K^+$ currents are cellular markers of developmental neuronal cell death (Hribar et al., 2004; Lee et al., 2006). It must be mentioned, however, that microglia activated in a manner similar to that described here were unable to generate sufficient NO to injure neurons in hippocampal slice cultures (Duport and Garthwaite, 2005). This preparation, however, is markedly insensitive to NO-mediated damage (Keynes et al., 2004), and thus other regulatory processes may be at play, including effective scavenging of reactive oxygen species by endogenous substances, thereby possibly limiting the production of $ONOO^-$ and its reactive products. It is also important to consider the possibility that the responses observed here in neurons exposed to activated microglia may also be expressed during nonpathological conditions. As such, an upregulation of delayed rectifier neuronal currents following microglial signaling could lead to pronounced changes in intrinsic excitability (Misonou et al., 2005).

It has yet to be established if $Zn^{2+}$ liberation under all injurious conditions precedes the characteristic $K^+$ current surge that is widely present in neuronal apoptosis. It is also not known whether neuronal cell death can always be averted by preventing cellular $K^+$ efflux. In fact, our own work has shown that following exposure to $ONOO^-$ donors these two processes, $Zn^{2+}$ liberation and $K^+$ current surge, are both not required together for cell death to ensue. Specifically, we were unable to rescue cortical neurons from exposure to the $ONOO^-$ generator 3-morpholino-sydnonimine (SIN-1) by preventing cellular $K^+$ efflux (Zhang et al., 2004), even though a pronounced $K^+$ current surge could be easily detected under similar circumstances (Pal et al., 2004). This suggests that an exogenous $ONOO^-$ neurotoxicity circumvents the $K^+$ efflux requirement for completion of a cell death program. Other studies have shown that the toxic actions of $ONOO^-$ generators such as SIN-1 can be complex and that they can sometimes involve an excitotoxic component (Trackey et al., 2001). This additional injurious component may not necessarily require either $Zn^{2+}$ release or $K^+$ extrusion to be fully expressed. More significantly, however, addition of an exogenous $ONOO^-$ generator or $ONOO^-$ itself may prove to be a stimulus not analogous to the release of reactive species by activated microglia under pathological situations, as suggested by others (Espey et al., 2002).

The results presented in this study demonstrate that under conditions previously shown to be highly toxic to both neurons (Xie et al., 2002) and oligodendrocytes (Li et al., 2005), reactive oxygen and nitrogen species derived from activated microglia induce a molecular cell death cascade that requires both the release of intracellular $Zn^{2+}$ and a MAPK-dependent enhancement of Kv2.1-mediated $K^+$ currents. These two critical steps in neuronal apoptosis thus provide discrete molecular targets for combating neurodegenerative conditions, where activation of microglia could be deleterious to neurons.

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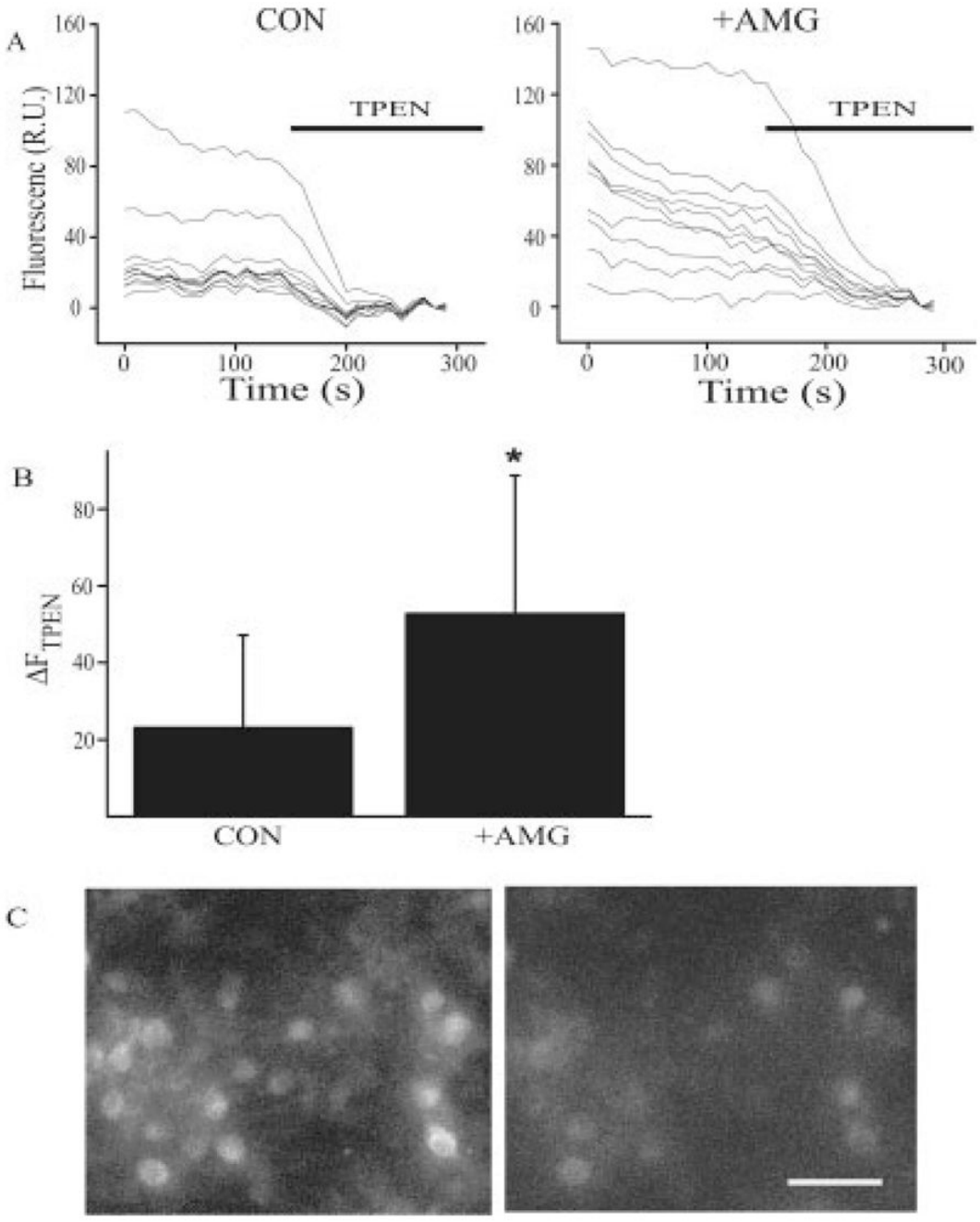


**Fig. 1.**

Intraneuronal $Zn^{2+}$ accumulation as a result of activated microglia. (**A**) Fluorescence traces from individual neurons before and after addition of the $Zn^{2+}$ chelator TPEN (20 μM). Compared with control cells, neurons previously exposed to activated microglia (AMG) have higher baseline $Zn^{2+}$ fluorescence and greater quenching by TPEN. (**B**) Average (mean ± SD) TPEN-sensitive $Zn^{2+}$ fluorescence ($\Delta F_{TPEN}$) from the control neuron-containing coverslip shown earlier ($n = 9$ cells) and the neuronal culture exposed to activated microglia ($n = 9$ cells; $P < 0.05$; *t*-test). (**C**) Examples of fluorescence images of rat cortical neurons loaded with 5 μM fluozin-3 AM following a 60-min exposure to activated microglia (10 U/mL IFN-γ + 1

μg/mL LPS), before (left) and after (right) treatment with 20 μM TPEN. The picture shown is representative of the range in responses observed in a typical coverslip. Bar: 60 μm.

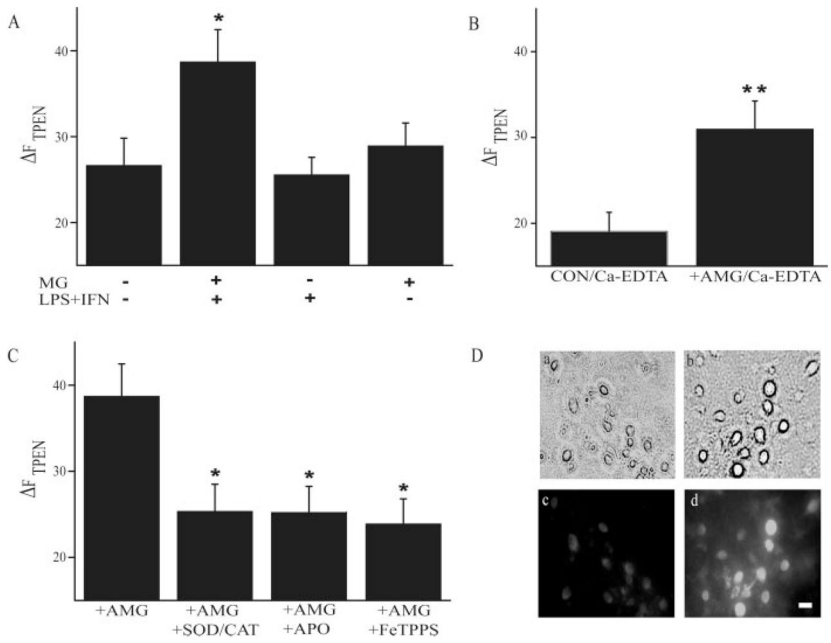


**Fig. 2.**

Properties of microglial-induced intraneuronal $Zn^{2+}$ release. (**A**) Pooled (mean ± SEM) TPEN-sensitive $Zn^{2+}$ fluorescence ($\Delta F_{TPEN}$) from control neurons, neurons exposed to microglia (MG) activated with LPS/IFN-γ, neurons exposed to LPS/IFN-γ alone or to quiescent microglia ($n$ = 13–18 coverslips per group, $P < 0.05$; ANOVA, Dunnett). (**B**) $\Delta F_{TPEN}$ in control and activated microglia-exposed cortical neurons in the presence of 1 mM Ca-EDTA to chelate extracellular $Zn^{2+}$ ($n$ = 8–11 coverslips per group, $P < 0.01$; $t$-test). (**C**) $\Delta F_{TPEN}$ in neurons exposed to activated microglia in the absence ($n$ = 10) or presence of the NADPH oxidase inhibitor apocynin (APO, 1 μM) ($n$ = 10), SOD/CAT (SOD 500 U/mL, catalase 100 U/mL) ($n$ = 9) or FeTPPS (5 μM) ($n$ = 10) ($P < 0.05$; ANOVA, Dunnett). (**D**) Immunostaining of tyrosine nitration in rat cortical neurons. Top panels, phase contrast images of control neurons (a) and neurons exposed to activated microglia (b). Bottom panels, fluorescent images of the same fields shown earlier demonstrating that 3-nitrotyrosine immunoreactivity is virtually nonexistent in control neurons (c) whereas extensive labeling is observed in neurons previously exposed to activated microglia (d). Bar: 20 μm.

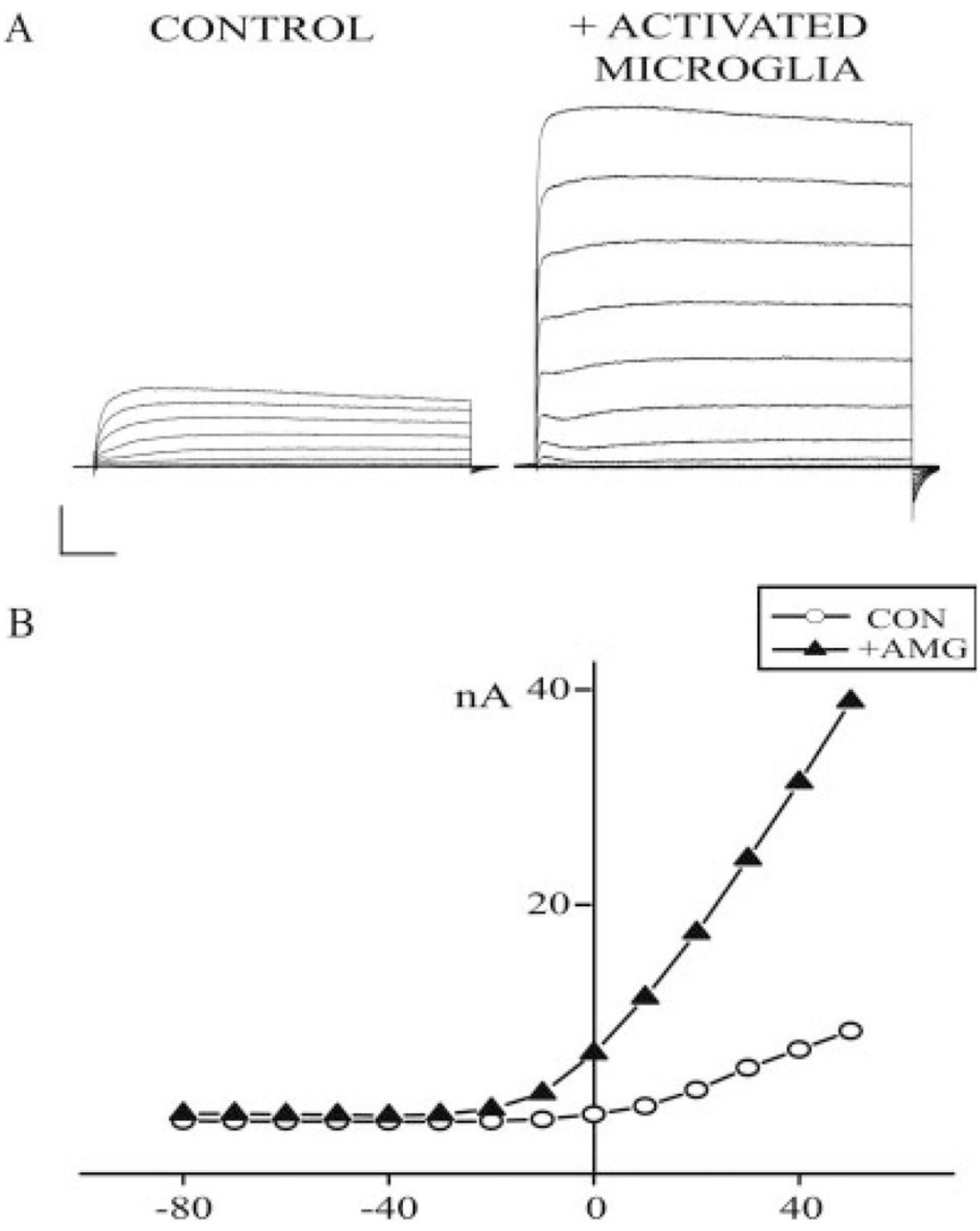


**Fig. 3.**

Exposure to activated microglia induces a voltage-gated $K^+$ current surge in neurons. (**A**) Representative $K^+$ currents evoked by a series of 10-mV incremental voltage steps to + 70 mV, from a holding potential of −70 mV in a control neuron (left) and a neuron previously exposed to activated microglia (right). Calibration: 25 ms, 5 nA. (**B**) Steady-state current–voltage relationship from traces shown in A.

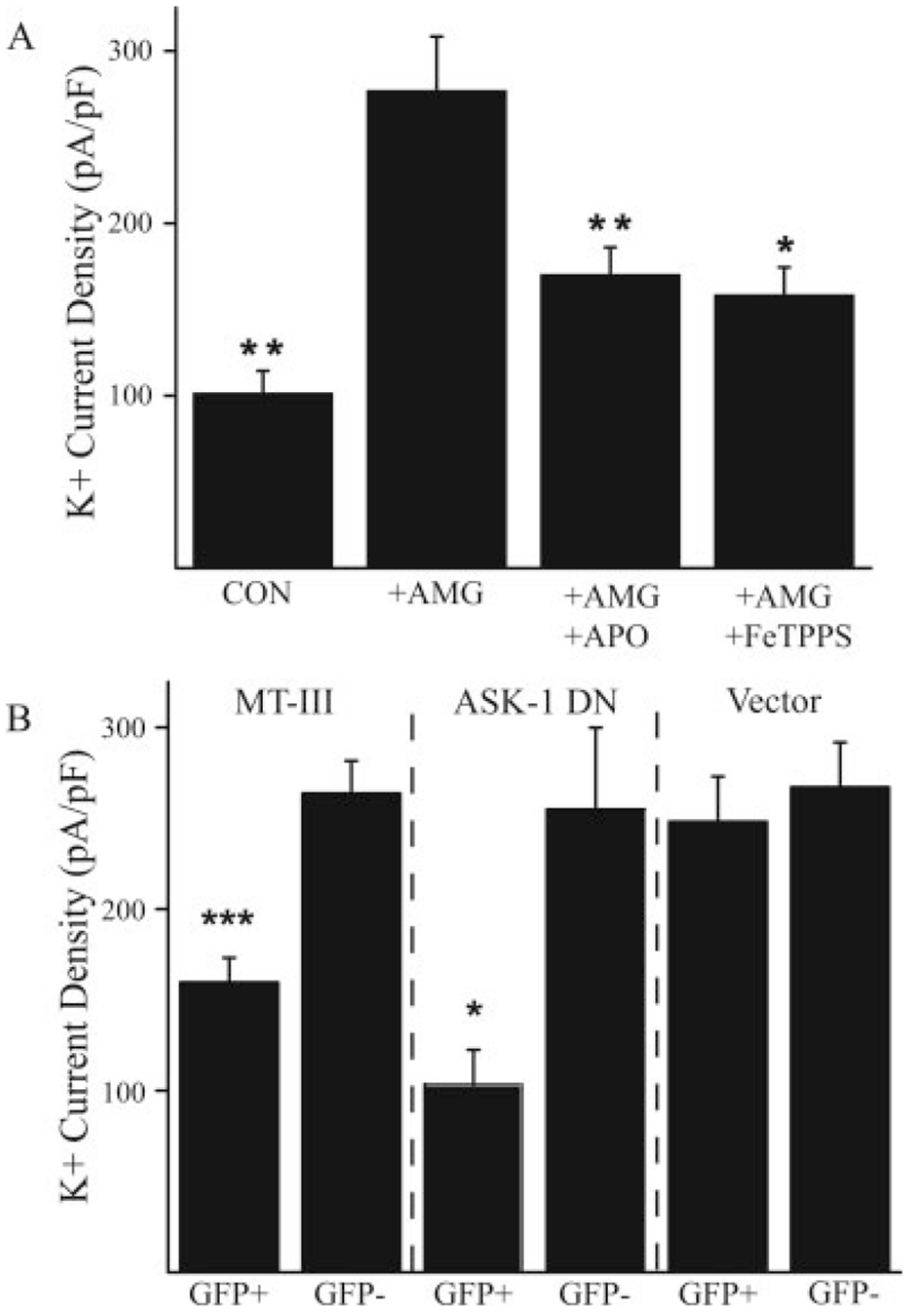


**Fig. 4.**

Molecular components of the $K^+$ current surge mediated by microglia. (**A**) Mean (±SEM) current densities (single voltage step to 10 mV) in control neurons ($n = 29$, $P < 0.01$), and in neurons exposed to activated microglia in the absence ($n = 37$) or presence of apocyanin (APO, 1 μM) ($n = 22$, $P < 0.01$) or FeTPPS (50 μM, $n = 13$, $P < 0.05$); $^{**}P < 0.01$, ANOVA, Dunnett. (**B**) Mean (±SEM) current densities (single voltage step to 10 mV) comparing neurons overexpressing either MT-III ($n = 13$, $P < 0.001$), ASK-1 DN($n = 9$, $P < 0.05$) or an empty eGFP-expressing vector ($n = 10$) to untransfected neurons on the same coverslip ($n = 9$ for each group); $^{***}P < 0.001$; *t*-test.

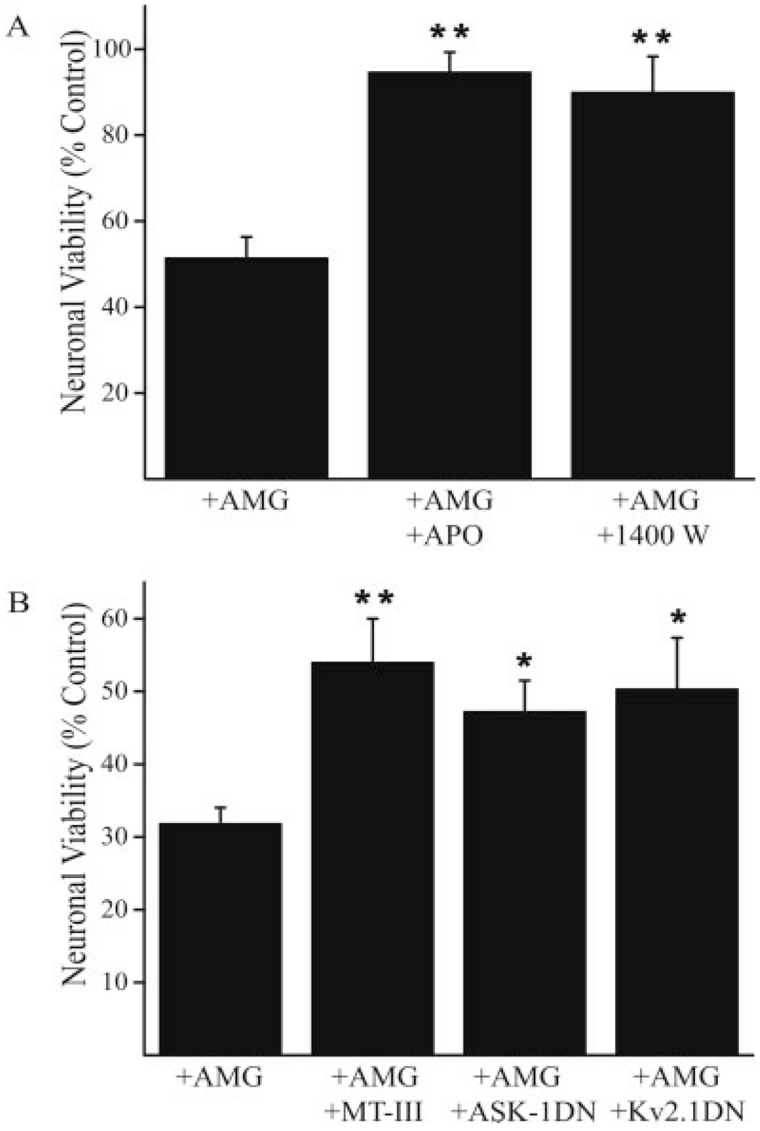


**Fig. 5.**

Molecular components of microglial neurotoxicity. (**A**) Mean (±SEM) viability (expressed as % control) in neurons exposed to activated microglia in the absence ($n = 9$) or presence of apocyanin (1 μM, $n = 3$) or 1,400 W (25 μM, $n = 6$) ($P < 0.01$; ANOVA, Dunnett). (**B**) Mean (±SEM) viability (expressed as % control) in control neurons ($n = 10$) and in neurons overexpressing metallothionein-III (MT-III) ($n = 3$, $P < 0.01$), ASK-1 DN ($n = 4$, $P < 0.05$) or Kv2.1 DN ($n = 3$, $P < 0.05$); *,**$P < 0.05$, 0.01, ANOVA, Dunnett.