

Microglia Kv1.3 Channels Contribute to Their Ability to Kill Neurons

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Many CNS disorders involve an inflammatory response that is orchestrated by cells of the innate immune system: macrophages, neutrophils, and microglia (the endogenous CNS immune cell). Hence, there is considerable interest in anti-inflammatory strategies that target these cells. Microglia express Kv1.3 (KCNA3) channels, which we showed previously are important for their proliferation and the NADPH-mediated respiratory burst. Here, we demonstrate the potential for targeting Kv1.3 channels to control CNS inflammation. Rat microglia express Kv1.2, Kv1.3, and Kv1.5 transcripts and protein, but only a Kv1.3 current was detected. When microglia were activated with lipopolysaccharide or a phorbol ester, only the Kv1.3 transcript (but not protein) expression changed. Using a Transwell cell-culture system that allows separate drug treatment of microglia or neurons, we found that activated microglia killed postnatal hippocampal neurons through a process that requires Kv1.3 channel activity in microglia but not in neurons. A major neurotoxic molecule in this model was peroxynitrite, which is formed from superoxide and nitric oxide; thus, it is significant that Kv1.3 channel blockers reduced the respiratory burst, but not nitric oxide production, by the activated microglia. In addressing the biochemical pathway affected by Kv1.3 channel activity, we found that Kv1.3 acts via a different cellular mechanism from the broad-spectrum drug minocycline, which is often used in animal models of neuroinflammation. That is, the dose-dependent reduction in neuron killing by minocycline corresponded with a reduction in p38 mitogen-activated protein kinase activation in microglia; however, none of the Kv1.3 blockers affected p38 activation.

Key words: microglia activation; neurotoxicity; neuroinflammation; Kv channel roles; Kv expression; anti-inflammatory treatments

Introduction

Microglia, astrocytes, and oligodendrocytes are the main non-neuronal cells of the CNS. Microglia, the endogenous immune cells, are promising cellular targets for controlling CNS inflammation. They extend long processes that are in close proximity to most CNS cells. Thus, they are ideally positioned for immune surveillance and as immediate sensors of acute CNS injuries. In the healthy adult brain, microglia release neurotrophic molecules and growth factors, condition the extracellular environment through pinocytotic and endocytotic mechanisms, and phagocytose dead cells and debris (Polazzi and Contestabile, 2002; Streit, 2002). However, as for other immune cells, the defensive role of microglia can be subverted if they are overactive, present at the wrong time or place, or are directed against self, rather than foreign antigens. Indeed, there is growing evidence that microglia contribute to both acute and chronic CNS disorders (Kreutzberg, 1996; Gonzalez-Scarano and Baltuch, 1999; Stollg and Jander, 1999; Aloisi, 2001; Nelson et al., 2002; Polazzi and Contestabile, 2002). After almost all CNS insults, microglia undergo dramatic

phenotypic, immunochemical, and functional changes, collectively referred to as “activation.” Some cellular functions of activated microglia include proliferation, migration to the injured site, recruitment of blood-borne immune cells, and synthesis of major histocompatibility complex class II molecules, with subsequent antigen presentation and phagocytosis of cells and debris (Gehrmann et al., 1995; Gebicke-Haerter et al., 1996; Streit et al., 1999; Aloisi, 2001; Schlichter and Khanna, 2002). Activated microglia can also produce neurotoxic products, and inflammation can exacerbate or even trigger damage in CNS diseases; thus, it is important to develop therapeutic strategies to control microglia activation in the CNS. Here, we investigated the potential for targeting specific K⁺ channels.

Some ion channels are valuable drug targets for disorders that involve excitable cells (i.e., cardiac, skeletal, and smooth muscle, neurons), wherein specific K⁺ channels regulate the membrane potential and Ca²⁺ influx. Although numerous studies have identified K⁺ channels or currents in immune cells, their roles in cell functions and inflammatory disorders are poorly understood. Best characterized are the roles of voltage-gated Kv1.3 (KCNA3) and Ca²⁺/calmodulin-activated (SK4/IKCa1/KCNN4) K⁺ channels in activation of lymphocytes (Cahalan et al., 2001; Schlichter and Khanna, 2002; Chandy et al., 2004). Indeed, blockers of these two channels are being tested in animal models of T-cell-mediated inflammatory diseases, including delayed-type hypersensitivity, multiple sclerosis, and restenosis of vascular smooth muscle cells (for review, see Cahalan et al.,

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2001; Wulff et al., 2003; Chandy et al., 2004). The same two K^+ channels are expressed in microglia (for review, see Eder, 1998; Schlichter and Khanna, 2002). We showed previously that Kv1.3 regulates microglia proliferation (Kotecha and Schlichter, 1999) and that both channels are involved in the respiratory burst (Khanna et al., 2001). In seeking new ways of controlling microglia activation, the present study examines roles of voltage-gated K^+ channels in their cytotoxic functions and killing of neurons. Our results demonstrate the potential for targeting Kv1.3 channels in microglia to reduce the neurotoxic outcomes of microglia activation.

Materials and Methods

Isolation and activation of microglia. Highly purified cultures of microglia (Khanna et al., 2001) were prepared from brains of postnatal day 2 or 3 Wistar rat pups (Charles River Laboratories, Saint-Constant, Quebec, Canada) as follows. The brain cortex was minced in cold Minimal Essential Medium (MEM; University Health Network, Toronto, Ontario, Canada), passed through a 100-mesh screen, and seeded into 75 cm^2 flasks in 30 ml of MEM containing 5% horse serum (v/v), 5% fetal bovine serum, and 0.05 mg/ml gentamycin (all from Invitrogen, San Diego, CA). Cells were fed on day 2, and after 7–12 d in culture (DIC), flasks were shaken (8–12 h, 100 rpm), and the floating cells were harvested and plated for 1 h at 37°C and then washed to remove residual glial cells. The resulting culture was 98–100% microglia, as judged by staining with isolectin-B₄, which binds to α -D-galactose residues or with OX-42 antibody (anti-CD11b/c), which recognizes the surface complement receptor 3. Subsequently, two types of serum-free media were used to reduce the spontaneous activation of microglia that usually occurs in culture. (1) For neuron-killing assays, microglia were washed, pelleted (10 min, 200 \times g), and resuspended in serum-free Neurobasal A medium with 2% B27 supplement (v/v), 0.5 mM L-glutamine, and 0.05 mg/ml gentamycin (all from Invitrogen) (Neurobasal A/B27 medium). These microglia (1.5×10^6 per well) were plated on porous Transwell inserts (pore diameter, 3 μ m) in 12-well plates (BD Biosciences, Franklin Lakes, NJ). (2) For all other experiments, microglia were cultured in MEM containing astrocyte-conditioned medium (Eder et al., 1997). Specific activated functions of microglia can be stimulated by proinflammatory agents, including the bacterial endotoxin, lipopolysaccharide (LPS), tumor necrosis factor- α , interferon- γ , or phorbol esters (Banati et al., 1991; Boje and Arora, 1992; Colton et al., 1994; Gebicke-Haerter et al., 1996; Smith et al., 1998; Khanna et al., 2001; Bal-Price et al., 2002; Ajmone-Cat et al., 2003; Lieb et al., 2003). Thus, we treated some microglia with LPS or phorbol 12-myristate 13-acetate (PMA), as indicated.

Postnatal rat neuron cultures. Neurons were prepared according to Brewer (1997) with minor modifications. Hippocampi were dissected from 2- to 3-d-old rats, incubated in 2 mg/ml papain (Worthington Biochemical, Lakewood, NJ) (30°C, 30 min), and then triturated in Neurobasal A/B27 medium (see above). Neurobasal A/B27 medium is enriched in essential fatty acids, vitamins, and hormones and supports growth of postnatal and adult neurons (Brewer, 1997; Evans et al., 1998). Brewer's protocol uses the protease papain to gently separate neuronal connections and an Optiprep density gradient (Invitrogen) to increase the proportion of neurons. Next, the cells were transferred to Neurobasal A/B27 medium and plated onto poly-D-lysine (Sigma, St. Louis, MO)-coated coverslips (Bellco Glass, Vineland, NJ) at 4×10^5 cells per coverslip. The cells were fed at 4 DIC by replacing one-half of the medium with fresh Neurobasal A/B27 and used at 7–10 DIC, at which time neuron purity was >80%, as assessed with a neuron-specific anti-mitogen-activated protein (MAP-2) antibody (Sigma). Almost all of the remaining cells were astrocytes, as indicated by staining with an antibody against glial fibrillary acidic protein; <0.1% were microglia (OX-42 positive).

Reverse transcriptase-PCR. Total mRNA was extracted using the TRIZol method (Invitrogen, Burlington, Ontario, Canada). To eliminate genomic contamination, the cell suspension was subjected to DNase I (Amersham Biosciences, Dorval, Quebec, Canada) digestion (0.1 U/ml, 15 min, 37°C). Total mRNA (2 μ g) was reverse transcribed at 42°C for 50 min in 20 μ l of 1 \times reverse transcriptase (RT) buffer [containing the

following (in mM): 75 KCl, 50 Tris, pH 8.3, 3 MgCl₂, 10 dithiothreitol] with 0.5 mM dNTPs, 25 μ g/ml pd(N)₆ (random hexamers), and 40 U SuperScript II reverse transcriptase (all from Invitrogen). After first-strand cDNA synthesis, PCR amplification was conducted using a GeneAmp PCR 2400 system (PerkinElmer, Toronto, Ontario, Canada) with 1 \times PCR buffer (60 mM Tris-SO₄, pH 8.9, 18 mM ammonium sulfate), 2 mM MgSO₄, 0.2 mM dNTPs, and 1 U Platinum TaqDNA polymerase (Invitrogen) using gene-specific primers and conditions (see Table 1). The PCR mixture was incubated for 5 min at 95°C, subjected to 35 cycles consisting of a 20 s denaturing phase at 94°C, a 30 s annealing phase, a 30 s extension phase at 72°C, and a final extension phase of 7 min at 72°C. The resulting DNA products were resolved on 1–2% agarose gels containing 0.5 mg/ml ethidium bromide, and product identities were confirmed by dideoxy sequencing (ACGT Laboratories, Toronto, Ontario, Canada). Band densities were measured using the Northern Eclipse 6.0 software (Empix Imaging, Mississauga, Ontario, Canada) and normalized to each internal control [β actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)].

Western analysis. Microglia (7.5×10^5 per well) in a six-well dish were rinsed in cold PBS and lysed in buffer containing the following: 50 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 2 mM Na₃VO₄, 1 mM NaF, and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (1 μ g/ml), and leupeptin (1 μ g/ml). The protein content of each lysate was measured using the Bradford assay (Bio-Rad, Mississauga, Ontario, Canada), then 50 μ g of each sample was boiled for 3 min in sample buffer [i.e., a 1:3 dilution of 3 \times sample buffer containing 6% SDS, 187.5 mM Tris, pH 6.8, 138 mM DTT, 30% glycerol, 0.03% phenol red (sodium salt)]. Proteins were separated by electrophoresis on an 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose polyvinylidene difluoride (PVDF) membranes (Bio-Rad), blocked with 5% nonfat milk in tris(hydroxymethyl) aminoethane-buffered saline (TBS) with 0.1% Tween 20 (TBST) or in PBS, and incubated with primary antibodies in blocking buffer for 2 h at room temperature or overnight at 4°C. The membranes were washed (twice with TBST and twice with TBS or three times with PBS) and incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibody (1:3000; Cedarlane Labs, Hornby, Ontario, Canada) in blocking buffer. After another wash cycle, labeled proteins were visualized by enhanced chemiluminescence on high-performance film (Hyperfilm TM; Amersham Biosciences). The primary antibodies and dilutions used were the following: polyclonal anti-Kv1.2 (1:100; Alomone Labs, Jerusalem, Israel), polyclonal anti-Kv1.3 (AU13; 1:1000; gift from Dr. Debra Fadool, Florida State University, Tallahassee, FL), polyclonal anti-Kv1.5 (UBI; 1:100), and monoclonal anti-GAPDH (1:10,000; Advanced Immunochemicals, Long Beach, CA). Band densities were normalized to each GAPDH internal control.

For p38 phosphorylation (activation) assays, microglia were lysed in 50 μ l of 3 \times gel-loading buffer containing the following: 210 mM Tris, pH 6.8, 6% SDS, 1 M NaCl, 120 mM dithiothreitol, 0.03% phenol red, and 30% glycerol and then scraped from the wells and immediately transferred to microfuge tubes on ice. Extracts were sonicated for 5 s to shear the DNA and reduce sample viscosity. The lysates were heated to 95–100°C for 5 min, centrifuged for 30 s, and 20 μ l of each sample was electrophoresed on an 8% SDS-polyacrylamide gel. Proteins were transferred onto a PVDF membrane, washed in TBST and then in TBS (two times, 10 min each), and incubated in a primary antibody diluted in blocking solution. First, phospho-p38 MAP kinase (MAPK) was measured, then the membrane was stripped with 0.1 M NaOH and reprobed with p38 MAPK antibody (both polyclonal antibodies were used at 1:750; Cell Signaling Technology, Beverly, MA). Membranes were washed and incubated with an HRP-conjugated anti-rabbit secondary antibody (1:2000–1:3000; Cedarlane Labs). Band densities were measured, and phospho-p38 was normalized to total p38 in each sample.

Other chemicals. LPS (Sigma) was dissolved in PBS and stored at 4°C; PMA was dissolved in DMSO and stored at –20°C. The peroxyxynitrite scavenger 5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron (III) chloride (FeTmPyP; Calbiochem, La Jolla, CA) was stored at –20°C and dissolved in distilled water before use. Five K channel blockers were used at sufficiently high concentrations to block the main channel of

interest (Table 2), as follows. The broad-spectrum Kv1-family blocker 4-aminopyridine (4-AP; Sigma) was stored at 4–6°C in PBS. The more potent and selective peptide toxins agitoxin-2 (AgTx-2), charybdotoxin (ChTx), and α -dendrotoxin (α -DTx) were obtained from Alomone Labs and stored lyophilized at –20°C. Before use, each peptide toxin was dissolved in PBS with 0.1% BSA to reduce its adsorption onto glass or plastic.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling. Microglia alone, growing in 12-well plates on porous Transwell inserts, were left untreated or exposed to LPS for 18–24 h (95% O₂, 5% CO₂, 37°C), with or without a K channel blocker, as indicated. Next, each microglia-bearing Transwell insert was rinsed and placed in a well of a 12-well plate in which postnatal hippocampal neurons were growing on glass coverslips. After 48 h of cocubation (95% O₂, 5% CO₂, 37°C), neuronal death was determined by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) (Roche Applied Science, Laval, Quebec, Canada) as follows. Neurons were washed in PBS (5 min, two times), permeabilized in Triton X-100 (0.01%) and sodium citrate (1%) on ice, washed again in PBS (5 min, two times), and fixed in 4% paraformaldehyde in PBS for 1 h at 20–25°C. After washing again in PBS (10 min, three times), coverslips were incubated with the reaction mixture, consisting of terminal deoxynucleotide transferase and fluorescein-labeled nucleotides for incorporation into DNA strand breaks. After a final washing in PBS (5 min, three times), coverslips were mounted in Slowfade (Molecular Probes, Eugene, OR) with 4',6'-diamidino-2-phenylindole (DAPI), a fluorescent nuclear-intercalating dye that was used to count total numbers of cells. Cells were viewed using a Zeiss (Oberkochen, Germany) Axioplan 2 Imaging microscope equipped with a Zeiss Axiocam digital camera and a 60× oil-immersion objective.

Respiratory burst. Production of reactive oxygen species by microglia was monitored according to Banati et al. (1991), with some modifications. Microglia (1.5 × 10⁵ cells per well) were plated in 12-well plates and incubated overnight in culture medium (MEM containing astrocyte-conditioned medium, as above). Just before use, the cells were washed and incubated for 5 min (95% O₂, 5% CO₂, 37°C) in 500 μ l of this medium, containing 20 μ M dihydrorhodamine-123 (DHR-123; Molecular Probes). DHR-123 readily diffuses into cells in which it is oxidized by cytochrome C, Fe²⁺, or hydrogen peroxide and peroxidase to form the green fluorescent product rhodamine-123 (Banati et al., 1991). Next, 500 μ l of medium was added, with or without 300 nM PMA and a K channel blocker, as indicated. PMA stimulates an NADPH-mediated respiratory burst in microglia (Smith et al., 1998). The cells were incubated for an additional 30 min (95% O₂, 5% CO₂, 37°C), washed several times with PBS, detached using 0.05% trypsin and 0.53 mM EDTA (5 min at 37°C, then 10 min on ice), and resuspended in 600 μ l of FACS buffer (0.1% BSA and 0.5 mM EDTA in PBS). DHR-123-loaded microglia were subjected to flow cytometry on a FACScan analyzer (BD Biosciences), reading the fluorescence intensity with an FL1 emission filter at 515–545 nm. Sorting windows were set to exclude dead cells and debris, and 5000 microglia were counted for each experiment.

Nitric oxide production. Microglia were plated in 12-well plates in 1 ml of culture medium at 5 × 10⁵ cells per well. To measure nitric oxide (NO) production, the Griess reagent kit (Molecular Probes) was used to monitor accumulated nitrite and nitrate in the supernatants above microglia (Green et al., 1982), as follows. Supernatant (150 μ l) from each well was mixed with 130 μ l of distilled water and 20 μ l of equal parts 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1% sulfanilic acid. The mixture was incubated for 30 min at room temperature, and then the optical density was measured at 540 nm using an ELISA plate reader (model EL311SX; Bio-Tek Instruments, Winooski, VT). For each experiment, a standard curve was constructed using known amounts of nitrite in culture medium. The supernatant was assessed immediately or frozen at –80°C and assayed later.

Statistical analysis. All data are presented as the mean of each treatment group \pm the SEM, and statistical analyses were performed using Origin 7.0 software (Microcal Software, Northampton, MA). Unless otherwise specified, a one-way ANOVA with the Bonferroni's correction was used

Table 1. Primers used for RT-PCR

| Gene | Accession number | Primers |
|----------------|------------------|--|
| Kv1.2 | J04731 | FP: CAC CGG GAG ACA GAG GGA RP: TCA GAC ATC AGT TAA CAT |
| Kv1.3 | M30312 | FP: AAT GAG TAC TTC TTC GAC CGC AAC AGA CCC AGC TTC GA RP: CCA ATG AAA AGG AAA ATG AGC AGC CCC AG |
| Kv1.5 | M27158 | FP: TGC AGA TCC TGG GTA AGA CC RP: AGA GGG CAT ACA GGG ACC TC |
| β -Actin | GI191581 | FP: TGG AAT CCT GTG GCA TCC ATG AAA C RP: TAA AAC GCA GCT CAG TAA CAG TCC G |
| GAPDH | M17701 | FP: GGT CAG TGC CGG CCT CGT CTC AT RP: CGC GCT CCT GGA AGA TGG TGA TG |

FP, Forward primer; RP, reverse primer.

to determine whether there were statistically significant differences between groups.

Results

Kv channel expression

To assess whether Kv channel expression changes with activation, rat microglia were grown in astrocyte-conditioned medium to reduce their spontaneous activation (Eder et al., 1997) and then activated with the gram-negative bacterial endotoxin LPS. LPS is commonly used *in vitro* (Boje and Arora, 1992; Jeohn et al., 2002; Xie et al., 2002) and *in vivo* (Morimoto et al., 2002). The effect of overnight treatment with the protein kinase C activator PMA was also assessed. Using gene-specific primers (Table 1), mRNA expression was monitored for three Kv channels that we found previously in cultured rat microglia: Kv1.2, Kv1.3, and Kv1.5 (Kotecha and Schlichter, 1999) and standardized to a housekeeping gene in each sample (Fig. 1). Although we previously found low levels of Kv1.6 mRNA in cultured microglia (Kotecha and Schlichter, 1999), its expression was not examined because it was not involved in killing of neurons (see below). Only Kv1.3 transcript levels changed, with a 258% increase after LPS and a 237% increase after PMA treatment. Because transcript levels do not always correspond with protein expression, Western analysis was conducted on a large number of independent protein samples from microglia (Fig. 2). Kv1.2 and Kv1.5 protein levels were not affected, and, although there was a trend toward increased Kv1.3 protein after microglia activation with LPS or phorbol ester, it was not statistically significant. In the absence of stimuli, we previously observed no change in Kv1.3 or Kv1.5 protein levels when rat microglia were cultured for ~3 weeks (Khanna et al., 2001).

The Kv current amplitude was compared after treating microglia with a phorbol ester (PMA) for 1 h (Fig. 3) or overnight with 10 ng/ml LPS. In all microglia examined, the current activated in a time- and voltage-dependent manner at about –40 mV, the activation rate increased with depolarization, and inactivation occurred over hundreds of milliseconds during positive voltage steps. Despite the presence of Kv1.2 and Kv1.5 channel protein, the only voltage-gated current detected appeared to be Kv1.3, because it was fully blocked by 5 nM agitoxin-2 (Fig. 3A,B), which potently blocks Kv1.3 (K_d , ~200 pM in T lymphocytes) (Grissmer et al., 1994; Garcia et al., 1997; Koo et al., 1997) but not Kv1.2 or Kv1.5 (Table 2). The current was not inhibited by α -dendrotoxin (data not shown). To quantify the Kv1.3 current, the AgTx-2-sensitive portion (which was at least 75% of the total current) was isolated by subtracting the current remaining in the presence of 5 nM AgTx-2. Under all conditions, the remaining current was not time dependent or voltage activated, it indicated an input resistance of 1–3 G Ω and was most likely leak or the anion current

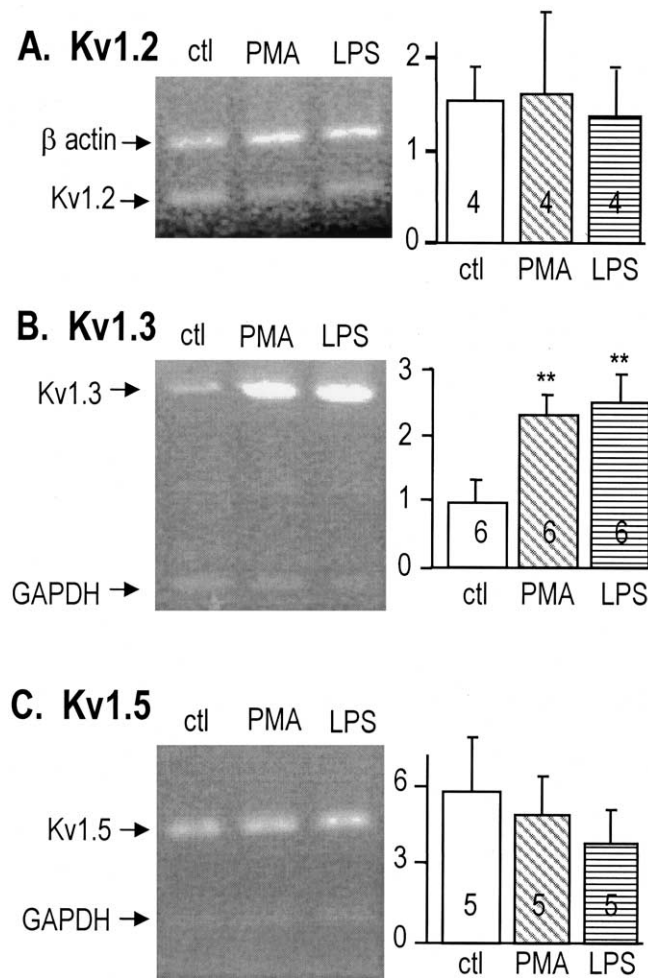


Figure 1. Kv channel transcript expression after microglia activation. **A–C**, Essentially pure rat microglia were left untreated [control (ctl)] or treated for 16 h with PMA (100 nm) or LPS (10 ng/ml). Left panels, Representative gels showing RT-PCR products for each Kv channel (**A**, Kv1.2; **B**, Kv1.3; **C**, Kv1.5) and an internal control in the same sample (β -actin or GAPDH). Right, Each band density (in arbitrary units) was normalized to its internal control, and the mean \pm SEM is shown for the number of rat litters indicated on each bar. ** $p < 0.01$.

described previously in these cells (Schlichter et al., 1996). The Kv1.3 current (AgTx-2-sensitive component) was then normalized to the cell capacitance to account for differences in cell size. Whereas short-term treatment with PMA had no effect, overnight LPS treatment increased the current at +40 mV ($p < 0.05$; $n = 23$) (Fig. 3C). The $\sim 180\%$ increase in Kv1.3 current could have resulted from increased surface expression of the channels (Kotecha and Schlichter, 1999) and/or increased channel activity, because LPS did not significantly increase the total Kv1.3 protein expression.

Activated microglia kill neurons

With Brewer's protocol (Brewer, 1997; Evans et al., 1998), the percentage of mature hippocampal neurons increased with time. By 9 d, the time used for neurotoxicity assays, $>80\%$ of the cells expressed high levels of the neuron-specific marker MAP-2 and had substantial voltage-gated Na^+ and K^+ currents (Fig. 4A–C). Importantly, the remaining cells were astrocytes, with a very low incidence ($<0.1\%$) of microglia. A key advantage of the present study is that, with the porous well inserts (Zietlow et al., 1999; Polazzi and Contestabile, 2002; Xie et al., 2002), we could separately treat either microglia or neurons, then wash them before

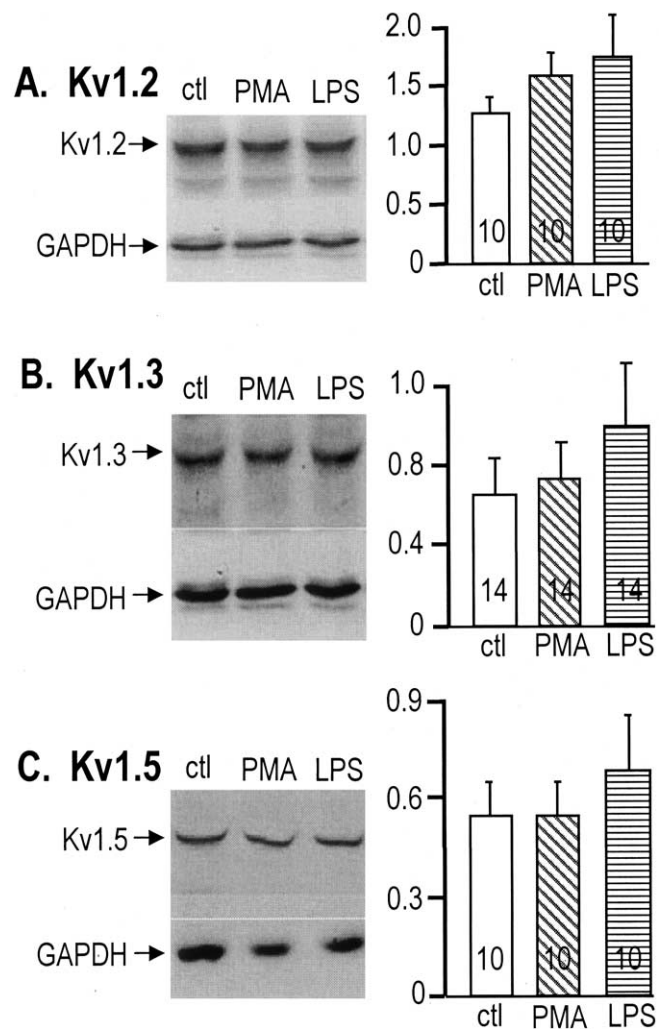


Figure 2. Protein expression of Kv channels after microglia activation. Microglia were treated as in Figure 1. **A–C**, The left panels show representative Western blots (**A**, Kv1.2; **B**, Kv1.3; **C**, Kv1.5). The anti-Kv1.2 antibody recognized a strong band of the expected size (67 kDa) and a weaker lower-molecular-weight band. The anti-Kv1.3 antibody recognized the expected 65 kDa band, and the anti-Kv1.5 antibody recognized the expected 60 kDa band. The blot was simultaneously (Kv1.2) probed for the smaller housekeeping protein GAPDH or was cut and the lower portion separately probed for GAPDH (Kv1.3, Kv1.5). Right, Each band density (in arbitrary units) was normalized to its internal GAPDH control, and the mean \pm SEM is shown for the number of independent samples indicated. ctl, Control.

coincubation. Moreover, after the cells had communicated via diffusible factors in the medium, the neurons and microglia could be separated for further analysis. LPS is a potent microglial activator *in vitro* and triggers an inflammatory response and subsequent neurodegeneration *in vivo* (Boje and Arora, 1992; Jeohn et al., 2002; Morimoto et al., 2002; Xie et al., 2002). LPS-activated microglia have been shown to kill neurons by apoptosis, as judged by both TUNEL and caspase-3 activation (Lee et al., 2004). We assessed the ability of LPS-activated microglia to kill neurons by calculating the percentage of TUNEL-positive cells, in which all cell nuclei were labeled with DAPI (Fig. 4D). In separate aliquots, we colabeled cells with TUNEL and MAP-2 to confirm that they were neurons, not astrocytes.

In control batches, spontaneous death of neurons cultured alone for the 3 d experimental period was $27 \pm 5\%$ ($n = 5$) (Fig. 4E). Importantly, neuron death ($24 \pm 4\%$; $n = 5$) was the same when unstimulated microglia were placed in the upper chamber

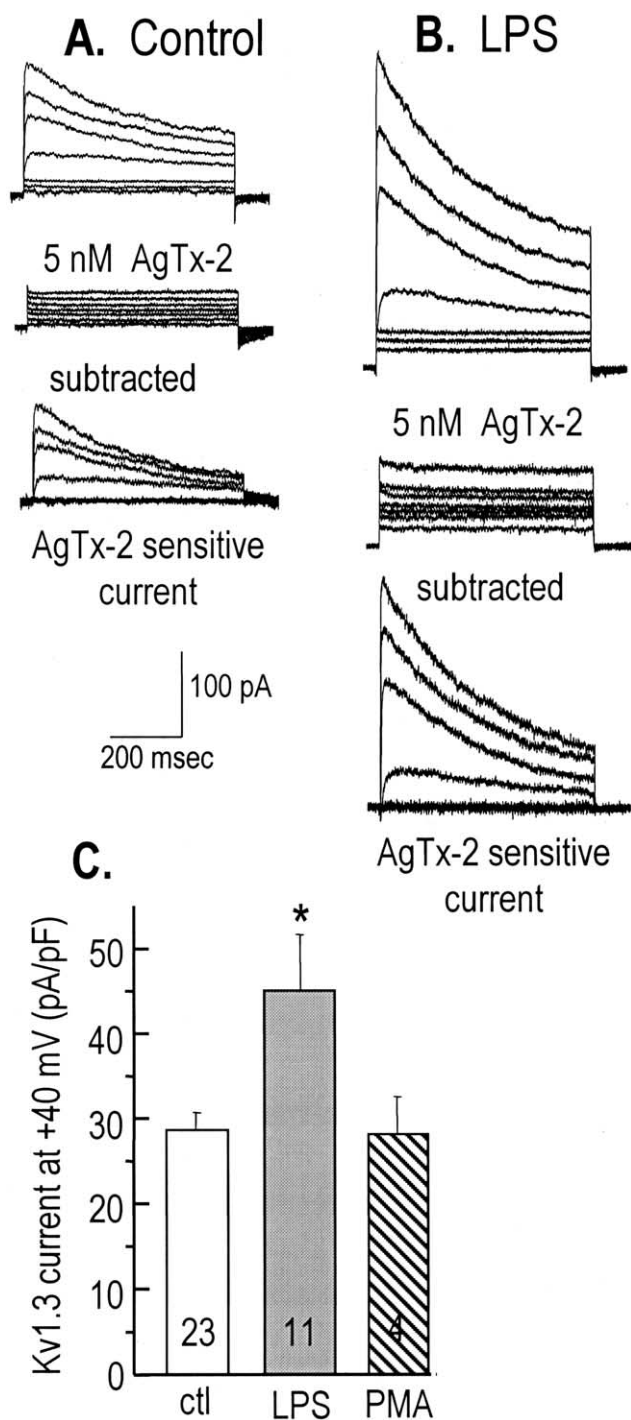


Figure 3. Microglia activation and Kv1.3 current. Conventional whole-cell recordings were made from microglia. The membrane potential was held at -100 mV, and voltage steps were applied between -80 and $+40$ mV in 20 mV increments. Representative currents are shown for an untreated microglia cell (**A**) and one treated overnight with 100 ng/ml LPS (**B**). Top, Recordings in normal bath solution; middle, after addition of 5 nM agitoxin-2; bottom, difference currents to isolate the AgTx-2-sensitive component. **C**, Summary of untreated [control (ctl)] cells or cells treated with LPS or PMA. Mean amplitude (\pm SEM) of AgTx-2-sensitive (Kv1.3) current measured during a step to $+40$ mV and normalized to the cell capacitance (number of cells indicated on each bar). * $p < 0.05$.

above neurons in the Transwell apparatus. In contrast, neuron death increased by 81% over the control level (to $42 \pm 3\%$; $p < 0.05$; $n = 11$) when microglia were preactivated with LPS (100 ng/ml) and then washed and added to the neurons. For subsequent experiments, microglia were treated with 100 ng/ml LPS,

because a higher concentration (1000 ng/ml) produced a similar level of neuron death ($37 \pm 4\%$; $n = 3$). Although the neurons were exposed to little or no LPS, we ensured that 100 ng/ml LPS alone was not directly toxic and had no effect on the survival of neurons. These results show that neurotoxicity required the presence of activated microglia, and none of the test compounds directly affected neuron survival.

Blocking microglial Kv1.3 channels inhibits neurotoxicity

The next step was to determine whether any of the K^+ channels in microglia affect their activation and ability to produce neurotoxic molecules. Blockers were selected for their ability to inhibit the K^+ channels that have been identified at the level of mRNA, protein, or ionic current in cultured microglia (i.e., Kv1.2, Kv1.3, Kv1.5, Kv1.6, and SK4). Blocker concentrations were chosen to substantially inhibit the current of interest (Fig. 5) while recognizing that none of the blockers is perfectly selective (Table 2). The top chamber containing microglia was incubated in a blocker and LPS and then washed three times before placing the chamber above the neuron-containing lower chamber. Although the naive test neurons were exposed to little if any blocker, as an added control, separate neurons were treated with the same concentration of each K^+ channel blocker, and no toxicity or excess neuron death was seen. Compared with the $81 \pm 6\%$ increase in neuronal death caused by LPS-stimulated microglia, three of the channel blockers significantly reduced the killing. After microglia were treated with 4-aminopyridine, a broad-spectrum blocker of members of the Kv1 channel family, neuron death was reduced to $33 \pm 14\%$ ($p < 0.05$) above the control level. Charybdotoxin, which blocks Kv1.2, Kv1.3, and SK4 (all present in rat microglia) (Figs. 1, 2) (Khanna et al., 2001) reduced killing to $37 \pm 7\%$ ($p < 0.05$), and agitoxin-2, which blocks Kv1.3 and Kv1.6, reduced killing to $32 \pm 10\%$ ($p < 0.01$). Interestingly, α -dendrotoxin, which blocks Kv1.2 and Kv1.6, did not inhibit killing, thus ruling out a role for both channels. Because Kv1.3 is the only channel in microglia that is susceptible to all the neuroprotective blockers, this channel plays a critical role in LPS-induced neuron killing.

Kv1.3-mediated neuron killing involves the respiratory burst and peroxynitrite formation

Peroxyntirite, which is formed from superoxide and nitric oxide, has been found to be neurotoxic in previous studies (Bal-Price and Brown, 2001; Xie et al., 2002). Although the half-life of peroxyntirite itself is short, because generation of nitric oxide, superoxide, and other reactive oxygen species by LPS-stimulated microglia can continue for many hours, even days (Xie et al., 2002), peroxyntirite production can be sustained. To assess whether peroxyntirite was responsible for the microglia-mediated neuron killing, we used FeTmPyP, a specific decomposition catalyst that converts peroxyntirite to nitrate (Misko et al., 1998) without affecting superoxide production (Xie et al., 2002). Adding FeTmPyP ($2 \mu\text{M}$) to a Transwell chamber containing neurons, astrocytes, and LPS-activated microglia (Fig. 6A) reduced neuron death from 81 ± 6 to $13 \pm 9\%$ above the background level ($p < 0.001$; $n = 3$), indicating that peroxyntirite plays a major role in killing neurons in this model. In separate control experiments on neurons and astrocytes, we ensured that the same concentration of FeTmPyP was not toxic and did not directly affect neuron death. We have not identified the cellular source of the nitric oxide and superoxide that produced the peroxyntirite in this model, but our experiments on pure microglia (below) provide additional information.

The neuroprotection by FeTmPyP implies that reducing pro-

Table 2. Expected block of K⁺ channels in microglia

| Drug (concentration used) | Kv1.2 [K_d (% block)] | Kv1.3 [K_d (% block)] | Kv1.5 [K_d (% block)] | Kv1.6 [K_d (% block)] | SK4 [K_d (% block)] |
|-------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|------------------------|
| 4-Aminopyridine (5 mM) | 590 μ M (89%) | 195 μ M (96%) | 270 μ M (95%) | 1500 μ M (77%) | |
| Charybdotoxin (50 nM) | 14 nM (78%) | 2.6 nM (95%) | >7000 nM | >7000 nM | 2 nM (96%) |
| Agitoxin-2 (5 nM) | | 4 pM (>99.9%) | | 40 pM (99%) | |
| α -Dendrotoxin (50 nM) | 17 nM (75%) | | | 20 nM (71%) | |

References: Grissmer et al., 1994; Garcia et al., 1997; Cahalan et al., 2001; Schlichter and Khanna, 2002; Chandy et al., 2004.

duction of either superoxide or nitric oxide should be neuroprotective. Thus, we assessed the ability of the K⁺ channel blockers to inhibit their production by microglia. The respiratory burst and consequent production of superoxide, hydroxyl free radicals, and hydrogen peroxide can be stimulated *in vitro* by phorbol esters through protein kinase C activation (Colton et al., 1994; Smith et al., 1998; Spranger et al., 1998; Bal-Price et al., 2002). We exposed cultured rat microglia to PMA (150 nM), and the respiratory burst (with or without Kv channel blockers) was monitored by flow cytometry after DHR-123 loading (see Materials and Methods). The time course of the fluorescence signal was first monitored for 15–120 min in microglia preloaded with DHR-123 for 5 min and then stimulated with PMA in the continued presence of DHR-123. The fluorescence reached a plateau at 60 min, which was then used for subsequent experiments. To confirm that the fluorescence signal was generated by an NADPH-mediated respiratory burst, we used the NADPH-oxidase inhibitor diphenylene iodonium (DPI; 5 nM) (Khanna et al., 2001). PMA caused a significant increase in respiratory burst (by $164 \pm 2\%$; $p < 0.05$). As a negative control, when 100 nM of the inactive phorbol ester analog 4 α -PMA was added to the bath solution instead of PMA, it had no effect (Fig. 6B). Two Kv channel blockers dramatically inhibited the respiratory burst in pure microglia cultures (Fig. 6C). That is, 4-aminopyridine, at a concentration that blocks all of the Kv1 channel types we have identified in rat microglia, reduced the increase in respiratory burst from 164 ± 2 to $6.1 \pm 8.9\%$ above the control level ($p < 0.01$). Agitoxin-2, at a concentration that should block essentially all of the Kv1.3 and Kv1.6 channels, reduced the increase in respiratory burst to $14.5 \pm 8.2\%$ ($p < 0.05$). In contrast, α -dendrotoxin, which blocks Kv1.2 and Kv1.6, did not inhibit the respiratory burst. Hence, this panel of Kv channel blockers demonstrates an important role for Kv1.3 and rules out the need for Kv1.2, Kv1.5, and Kv1.6.

Peroxyntirite formation requires NO, and inducible nitric oxide synthase and subsequent NO production by activated microglia can be neurotoxic (Boje and Arora, 1992; Chao et al., 1992; Xie et al., 2002; Lieb et al., 2003). Thus, we assessed whether Kv channels contribute to NO production by monitoring nitrite levels (Green et al., 1982) accumulating in the culture medium of microglia exposed to LPS (Bal-Price and Brown, 2001). First, pure microglia cultures were stimulated with a range of LPS concentrations (1–1000 ng/ml) for 3–24 h to determine an appropriate concentration and duration of treatment. Consistent with other studies (Nakamura et al., 1999; Dijkstra et al., 2001), 10 ng/ml LPS was adequate and produced a detectable increase in NO within 3 h. Because there was no additional increase after 12 h, this time point was used for subsequent experiments (Fig. 6D). To control for variable baseline NO production, results were standardized to the control levels from unstimulated microglia in each cell batch. LPS increased NO production by $152 \pm 16\%$ ($p < 0.01$; $n = 4$), and this increase was not affected by any of the Kv channel blockers; thus, none of the Kv channels we detected in microglia (Kv1.2, Kv1.3, Kv1.5, Kv1.6) appear to be needed for nitric oxide release.

p38 MAPK activation and microglia-mediated neuron killing

Activation of p38 MAP kinase in microglia is an important upstream regulator of production of numerous potential neurotoxins, including proinflammatory cytokines and reactive nitrogen species (Jeohn et al., 2002; Koistinaho and Koistinaho, 2002). Hence, we assessed whether the neuroprotective effect of Kv1.3 channel blockers is through interference with p38 MAPK phosphorylation and consequent activation. Because minocycline, which is a strong inhibitor of microglia activation, is known to reduce p38 activation in microglia (Chen et al., 2000; Tikka et al., 2001; Wu et al., 2002), we compared it to the Kv channel blockers. The upper and lower chambers of the Transwell apparatus were separated, and only microglia were treated with LPS with or without varying concentrations of minocycline. Then, the microglia chamber was washed three times before it was placed on top of the neuron chamber (Fig. 7A). The microglia were not in physical contact with neurons, and because minocycline is lipophilic, it is very unlikely that any remaining drug reached the neurons. Minocycline reduced neuron killing by LPS-activated microglia in a dose-dependent manner, with a calculated IC_{50} value of ~ 90 nM.

LPS induced a transient phosphorylation (activation) of p38 MAPK, with a peak at 30 min (Fig. 7B), a time course that is consistent with previous studies (Ajmone-Cat et al., 2003). As expected, minocycline significantly inhibited phosphorylation of p38 MAPK (Fig. 7C) [i.e., to $72 \pm 6\%$ of the LPS-induced level ($p < 0.05$; $n = 6$) (Fig. 7E)]. Although the inhibition of p38 was not as profound as the inhibition of neuron killing by minocycline-treated microglia, both are consistent with previous reports (Chen et al., 2000; Tikka et al., 2001; Wu et al., 2002) and likely indicate that other pathways are also involved. Most importantly, none of the Kv channel blockers reduced p38 MAPK phosphorylation (Fig. 7D,E), thus ruling out direct or indirect inhibition of this kinase in the neuroprotective effect we observed with the Kv1.3 channel blockers.

Discussion

There are several key findings in this study. (1) Rat brain microglia express Kv1.2, Kv1.3, and Kv1.5 transcripts and protein, but only the Kv1.3 transcript expression increased when cells were activated with LPS or phorbol ester. Consistent with our previous reports (Schlichter et al., 1996; Kotecha and Schlichter, 1999; Khanna et al., 2001; Schlichter and Khanna, 2002), the only Kv current detected in the present study was essentially identical to Kv1.3, and, despite a lack of change in protein expression, the current was increased after LPS treatment. (2) Because almost nothing is known about the roles of K⁺ channels in microglia, we exploited a well insert model that allows separate drug treatment of microglia (not neurons or astrocytes). We found that Kv1.3 channels in LPS-activated microglia contribute strongly to their ability to kill cultured postnatal hippocampal neurons. (3) We confirmed that peroxyntirite (which requires superoxide and nitric oxide production) contributes strongly to the observed neuron killing and that Kv1.3 channels are needed for the respiratory burst but not for nitric oxide production by microglia. (4) We found that the signal transduction pathway affected by Kv1.3

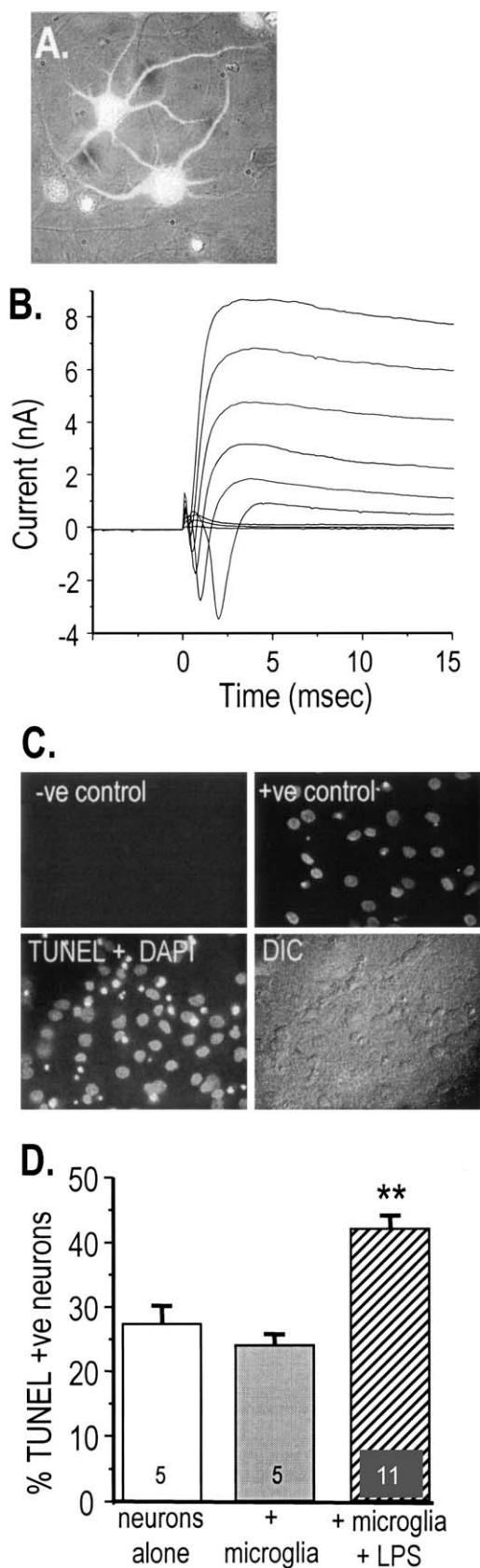


Figure 4. Activated microglia kill neurons. **A–C**, Properties of postnatal hippocampal neurons grown in Neurobasal A/B27 medium. Immunofluorescence image with differential interference contrast image overlaid (**A**) of fixed and permeabilized postnatal neurons after 5 DIC in serum-free Neurobasal A/B27 medium. All cell nuclei were labeled with DAPI, and the somato-dendritic compartments of mature neurons were labeled with an anti-MAP-2 monoclonal

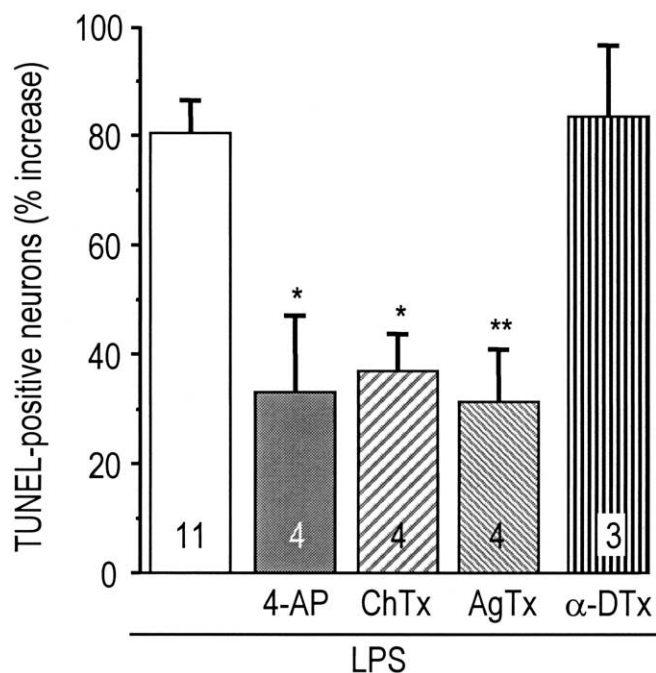


Figure 5. Kv1.3 blockers reduce neuron killing by microglia. Microglia were grown on membranes in the upper Transwell chamber, then treated with LPS (as in Fig. 4), with or without a K^+ channel blocker, and then washed three times before placing them above neurons in the lower Transwell chamber. The percentage channel block was calculated as $1/[1 + (K_d/[D])]$, where $[D]$ is the concentration of blocker used in the experiment. From the K_d values in Table 2, ChTx (50 nM) blocks 78% of Kv1.2, 95% of Kv1.3, and 96% of SK4 channels; AgTx (5 nM) blocks ~100% of Kv1.3 and Kv1.6 channels; α -DTx (50 nM) blocks 75% of Kv1.2 and 71% of Kv1.6 channels. Neuron killing was assessed by TUNEL as in Figure 4 and is expressed as the mean \pm SEM for the number of independent experiments indicated on each bar. * $p < 0.05$; ** $p < 0.01$.

channel blockade in LPS-activated microglia is independent of p38 MAP kinase activation, the inhibition of which is seen with the commonly used, broad-spectrum neuroprotectant minocycline. By affecting a different pathway, Kv1.3 channels offer a new mechanism for reducing neurotoxicity. Although no similar studies have been reported, some specific aspects of the current work can be compared with previous studies.

Most studies of cultured microglia describe a K^+ current that is essentially identical to Kv1.3 in its voltage and time dependence and block by 4-aminopyridine, charybdotoxin, margatoxin, and agitoxin-2 (Norenberg et al., 1994; Schlichter et al., 1996; Visentin and Levi, 1997; Eder, 1998; Schlichter and Khanna, 2002); however, discrepancies exist concerning changes in K^+ channel expression under various conditions. There is some evidence that the Kv current in microglia can be increased by proinflammatory stimuli (e.g., LPS, interferon- γ), but large currents have also been

←

antibody. Scale bar, 20 μm . Representative whole-cell voltage-clamp recordings (**B**) from a neuron after 9 DIC in Neurobasal A/B27. From a holding potential of -100 mV, voltage steps were applied in 20 mV increments from -80 to $+80$ mV. **C, D**, Diffusible factors from activated microglia kill neurons. **C**, A representative experiment. Top, Postnatal hippocampal neurons growing in the bottom chamber of the Transwell apparatus, showing negative (no terminal transferase added) and positive controls (DNase I added) for the TUNEL assay. Bottom left, An upper chamber containing a porous insert with microglia was pretreated with LPS (100 ng/ml; 18–24 h), washed three times, and then placed above the lower chamber containing neurons. After 48 h, cells in the lower chamber were labeled with DAPI and TUNEL. Bottom right, Differential interference contrast image of same field of cells. **D**, Summary of neuron death (percentage TUNEL-positive cells); mean \pm SEM, number of independent experiments indicated on each bar. ** $p < 0.01$. -ve, Negative; +ve, positive.

recorded under conditions thought to suppress microglia activation (e.g., after culture in astrocyte-conditioned medium, granulocyte-macrophage colony-stimulating factor, or TGF- β) (for review, see Eder, 1998; Schlichter and Khanna, 2002). In order for a channel to be an amenable target for drug therapy, it must be present on the cell surface. It is significant that we observed an increase in Kv1.3 current amplitude without a change in channel protein expression after LPS treatment. The two most likely explanations are an increase in channel activity by posttranslational modulation or an increase in channel expression on the membrane. Although the present study does not address the mechanism, we previously reported a marked change in membrane expression of Kv1.3 and Kv1.5 channels in microglia within tissue prints from rat hippocampal slices (Kotecha and Schlichter, 1999). The cells initially had robust Kv1.5 currents as a result of abundant Kv1.5 protein expression on the cell surface. Then, as the tissue prints were cultured, the current in microglia changed to Kv1.3, coincident with a switch in membrane localization of Kv1.3 and Kv1.5 protein. Kv1.3 channel activity is known to be affected by post-translational modulation by serine/threonine or tyrosine phosphorylation in immune cells (Chung and Schlichter 1997a,b; Cayabyab et al., 2000; Schlichter and Khanna, 2002).

We found that cultured postnatal neurons were killed by microglia that had been activated with LPS. Activation of the neurotoxic functions of rat microglia was inhibited by all the drugs that block Kv1.3 channels (4-aminopyridine, charybdotoxin, agitoxin-2) but not by the Kv1.2 blocker α -dendrotoxin. The extent of neuron killing was similar to a previous study (Xie et al., 2002); however, in that study, effects of LPS on microglia could not be distinguished from those on neurons because both cell types were exposed simultaneously to LPS. Another study (Wei et al., 2003) added the nonselective K⁺ channel blocker tetraethylammonium to mixed cortical cultures in the presence of inhibitors of excitatory amino acid receptors. Although neuron death in response to oxygen-glucose deprivation was reduced, it is unclear which cell type or specific K⁺ channels were involved. Thus, a key result from the present study is that the neurotoxicity of activated microglia was reduced by exposing the microglia (not the neurons) to peptide toxins that block Kv1.3 channels, and a contribution from the other Kv channels we identified in microglia was ruled out.

Despite numerous papers on ion currents in microglia (for review, see Eder, 1998; Schlichter and Khanna, 2002), the challenge of determining their roles in microglia functions has been sorely neglected. Some previous studies show that neurotoxicity of microglia can involve peroxynitrite (van der Veen et al., 1997;

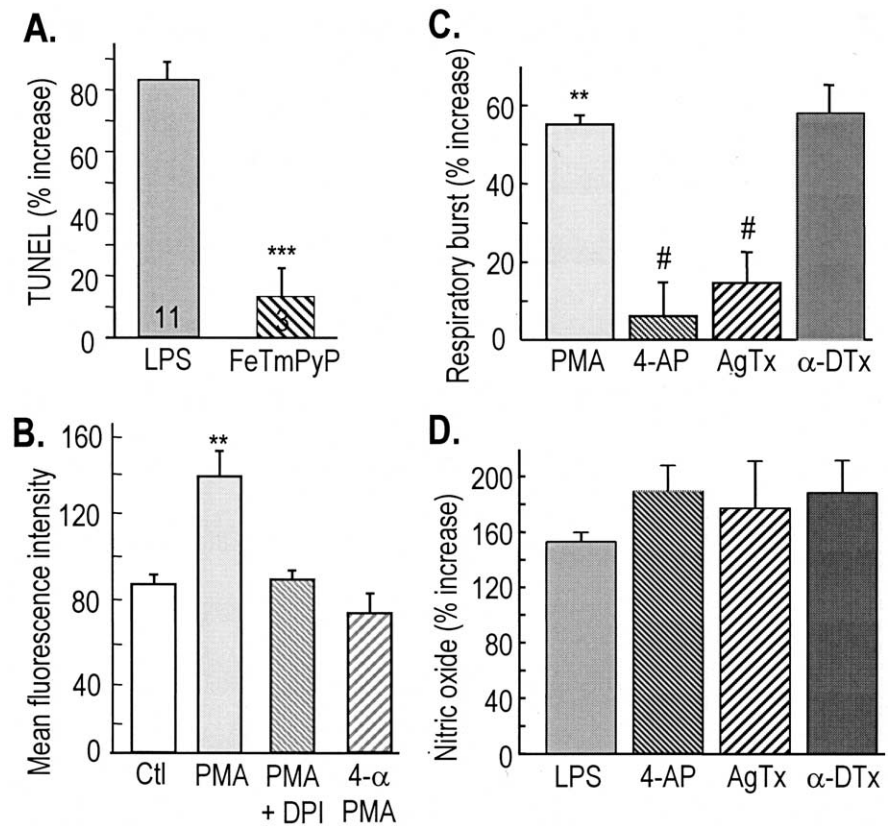


Figure 6. Mechanism of Kv1.3-mediated neuron killing. **A**, Microglia-dependent killing is caused by peroxynitrite. Microglia growing in porous upper chambers were treated with LPS, washed three times, and then placed above neurons in the lower Transwell chambers, as in Figures 4 and 5. The peroxynitrite scavenger FeTmPyP (2 μ M) when added to neuron cocultures at the same time as LPS-stimulated microglia, dramatically reduced the neuron killing assessed at 48 h ($***p < 0.001$). **B**, The respiratory burst in microglia. In separate wells, microglia alone were untreated [control (ctl)] or treated with PMA (150 nM), an inactive analog (4- α -PMA; 150 nM), or the NADPH-oxidase inhibitor DPI (1 μ M). Each compound was added to cells in medium containing 2 μ M DHR-123, and the fluorescence was monitored by flow cytometry after 60 min. For each experiment, 5000 cells were counted, with three replicates from each microglia culture isolated from four different rat litters. The summarized data are presented as mean fluorescence intensity (\pm SEM) in arbitrary units ($**p < 0.01$; $n = 4$). **C**, Kv1.3 blockade inhibits the respiratory burst by microglia. Microglia, with or without a Kv channel blocker, were subjected to flow cytometry after PMA treatment as in **B**. Data are presented as the percentage increase in respiratory burst (mean \pm SEM; $n = 4$ separate experiments) compared with unstimulated microglia. The PMA-induced respiratory burst was inhibited by both compounds that block Kv1.3 (5 nM 4-aminopyridine, 5 nM agitoxin-2; $^{\#}p < 0.05$) but not by the Kv1.2 and Kv1.6 blocker (50 nM α -dendrotoxin). **D**, Nitric oxide production is not inhibited by the Kv channel blockers. Microglia were untreated (ctl) or treated with 10 ng/ml LPS for 12 h, with or without a Kv blocker. Then, the concentration of nitrite (micromolar) released from 50,000 cells per well was determined using the Griess assay. Four replicates for each blocker were used from each of three microglia cultures. Data are presented as the relative percentage increase compared with unstimulated microglia (\pm SEM; $n = 4$ separate experiments).

Xie et al., 2002), a membrane-permeant molecule that causes DNA strand breaks and lipid peroxidation, inactivates metalloenzymes, and triggers release of proapoptotic factors from mitochondria. Because superoxide combines with NO to form peroxynitrite (Gehrmann et al., 1995; van der Veen et al., 1997; Gonzalez-Scarano and Baltuch, 1999; Xie et al., 2002), agents that inhibit either the respiratory burst or NO formation should be neuroprotective. It is well known that activated microglia can produce superoxide (Colton et al., 1994; Bal-Price et al., 2002) and nitric oxide (Boje and Arora, 1992; Chao et al., 1992; Xie et al., 2002; Lieb et al., 2003). Consistent with some previous studies (Bal-Price and Brown, 2001; Xie et al., 2002), in our model of neuron killing by LPS-activated microglia, FeTmPyP (a specific peroxynitrite decomposition catalyst) (Misko et al., 1998; Xie et al., 2002) dramatically reduced neuron death. Because it was added to the Transwell cocultures, we cannot rule out a contribution of microglia, astrocytes, and neurons to peroxynitrite pro-

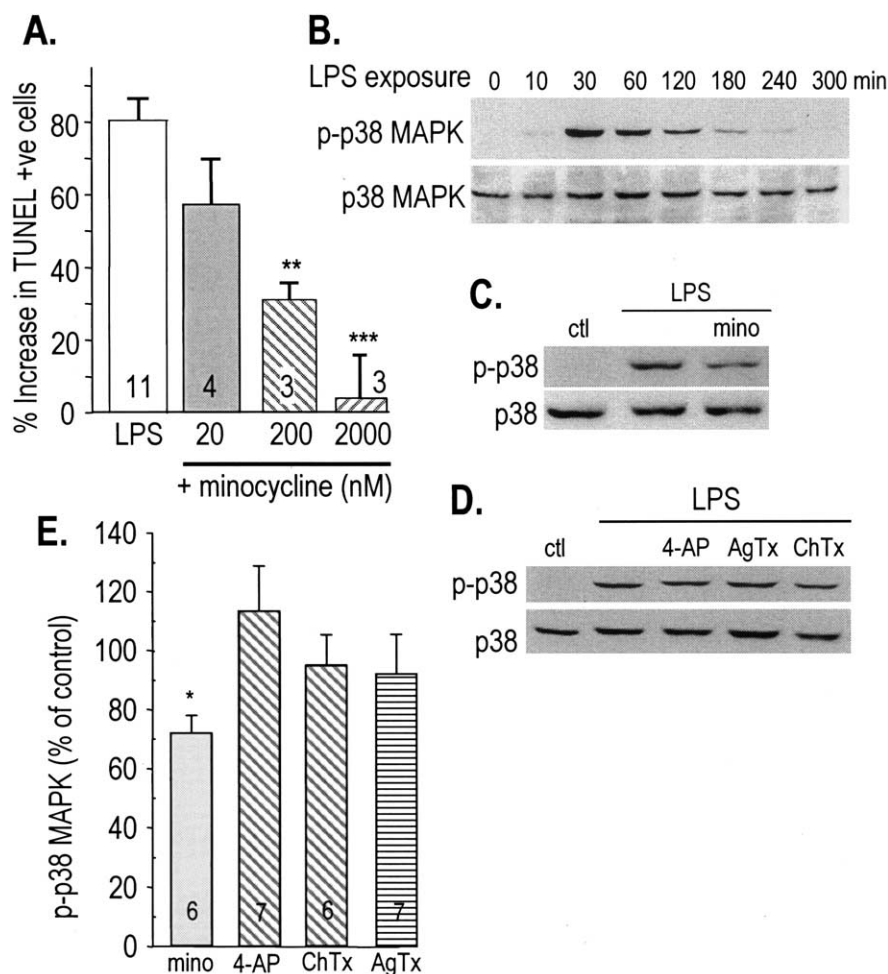


Figure 7. p38 MAPK activation. Effect of Kv blockers compared with minocycline. **A**, Minocycline inhibits neuron killing by activated microglia. As in Figures 4 and 5, microglia growing in porous upper chambers of the Transwell apparatus were treated with LPS, with or without minocycline. The microglia were washed three times and then placed above postnatal hippocampal neurons growing in the bottom Transwell chamber. Neuron death was assessed by TUNEL after 48 h, and expressed as the percentage increase in TUNEL-positive (+ve) cells beyond that with unstimulated microglia (mean \pm SEM; number of independent cell cultures tested). ** $p < 0.01$, *** $p < 0.001$. **B**, LPS induces time-dependent activation of p38 MAPK. Cultured pure microglia were treated with LPS (100 ng/ml) and then lysed at the indicated times and subjected to Western blotting using an antibody that recognizes the activated, phosphorylated form of p38 MAPK. Then, the gel was stripped and re probed with an antibody that recognizes total p38 MAPK. This gel, which is representative of three independent experiments, shows a transient p38 MAPK phosphorylation with a peak at 30 min. **C–E**, Minocycline (mino), but not Kv channel blockers, reduces LPS-induced p38 MAPK activation. Cultured pure microglia were treated with LPS (100 ng/ml) for 30 min with or without 200 nM minocycline (**C**) or one of the Kv channels blockers (**D**) that was found to be neuroprotective (see Fig. 5): 4-AP (5 mM), AgTx (5 nM), ChTx (50 nM). ctl, Control. Densitometric analysis (**E**) was used to assess p38 MAPK activation as in **B**.

duction. However, an important finding in the present study is that the Kv1.3 channel blockers inhibited the respiratory burst (but not nitric oxide production) by microglia. Inhibition of the respiratory burst has been reported when either poorly selective K⁺ channel blockers (Colton et al., 1994; Spranger et al., 1998) or more selective toxins were used (Khanna et al., 2001); however, only the present panel of toxins is sufficient to pinpoint Kv1.3 as the essential channel. The lack of effect on nitric oxide production is in accord with a previous study that used the broad-spectrum Kv inhibitor 4-aminopyridine on LPS-treated microglia and macrophage cell lines (Brown et al., 1998). A report (Pyo et al., 1997) of reduced nitric oxide production after several days treatment of microglia with 4-aminopyridine failed to consider the potential cytotoxicity of this weak base (Kim et al., 2000).

In microglia, many proinflammatory stimuli cause phosphor-

ylation (activation) of the mitogen-activated protein kinase p38 MAPK and consequent production of cytotoxic molecules, including nitric oxide (Jeohn et al., 2002; Koistinaho and Koistinaho, 2002). Similar p38 MAPK activation occurs in numerous acute and chronic brain disorders, including stroke, spinal cord injury, Alzheimer’s disease, and Parkinson’s disease (Koistinaho and Koistinaho, 2002), and there is increasing evidence that p38 MAPK inhibitors can protect against microglia-induced neurotoxicity *in vivo* (Barone et al., 2001; Koistinaho and Koistinaho, 2002). Thus, we asked whether blocking microglia Kv1.3 channels might reduce their neurotoxic functions by inhibiting activation of p38 MAPK. For comparison, we assessed minocycline, a second-generation tetracycline derivative, which can be neuroprotective *in vitro* and *in vivo* (Yong et al., 2004). Minocycline inhibits p38 MAPK activation, inducible nitric oxide synthase induction, and nitric oxide production in microglia and activity of caspases 1 and 3 in neurons (Chen et al., 2000; Tikka et al., 2001; Wu et al., 2002; Lee et al., 2004; Yong et al., 2004).

Our key finding is consistent with the lack of effect of Kv blockers on NO production; that is, (unlike minocycline) the Kv1.3 blockers did not inhibit LPS-induced p38 MAPK phosphorylation, thus pointing to a separate intracellular pathway mediating the neurotoxic effects of activated microglia. Although it will be interesting to determine the downstream events affected by blocking Kv1.3 channels in microglia, such signal-transduction pathways might not be as amenable to therapeutic interventions as the channels themselves.

The present results may have broad implications for controlling neuroinflammation, because microglia are often the first cells to respond to brain injury or disease (Gehrmann et al., 1995; Aloisi, 2001; Streit, 2002). Furthermore, microglial activation is associated with pathogenesis in

both acute and chronic disease states, including bacterial infection, human immunodeficiency virus, amyotrophic lateral sclerosis, multiple sclerosis, prion disease, retinal degeneration, brain trauma, and ischemic stroke (Kreutzberg, 1996; Gonzalez-Scarano and Baltuch, 1999; Stollg and Jander, 1999; Nelson et al., 2002; Polazzi and Contestabile, 2002). The present study provides the first evidence that a specific K⁺ channel can regulate the neurotoxic properties of microglia. Moreover, the restricted cellular expression and roles of Kv1.3 in the CNS mean that selective Kv1.3 blockers might provide a novel anti-inflammatory strategy in CNS injury and disease. Kv1.3 is predominantly expressed in cells of the immune system, including microglia, lymphocytes, and macrophages, and many studies show that this channel is important for activation of T lymphocytes (for review, see Cahalan et al., 2001; Schlichter and Khanna 2002; Chandy et al.,

2004). Some peptide inhibitors of Kv1.3 have already been tested *in vivo* in animal models of delayed-type hypersensitivity (Koo et al., 1997) and the experimental autoimmune encephalomyelitis model of multiple sclerosis (for review, see Cahalan et al., 2001; Chandy et al., 2004). Potential side effects might be minimal despite the presence of small amounts of Kv1.3 in some neurons in the CNS (i.e., in a small subset of neurons in the hippocampus, pyriform cortex, and olfactory bulb) (Kues and Wunder, 1992). For instance, the only CNS deficit observed in Kv1.3-deficient mice was hypersensitivity to odorants caused by defects in the development of the olfactory bulb (Fadool et al., 2004). Thus, the potential for developing Kv1.3 blockers to inhibit neuroinflammation is good, particularly because new classes of nonpeptidyl blockers with high specificity and better pharmacodynamics are being developed as immunosuppressants for organ and tissue transplant (for review, see Cahalan et al., 2001; Chandy et al., 2004).

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