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Table I. Mutants used, and their phenotype.

Protein name	Phenotype	Rac1 binding	PAK1 binding	Activity
PAK1-wt	Wild type protein	Yes	-	Regulated kinase activity
PAK1-T423E	Dominant positive	Yes	-	Constitutive kinase activity, high
PAK1-H83,86L	Dominant positive	No	-	Constitutive kinase activity, moderate
PAK1-K299A	Dominant negative	Yes	-	Kinase dead
PBD (aa 83-149)	Dominant negative	Yes	-	No kinase domain
PBD-H83L	Dominant negative	No	-	No kinase domain
Rac1-N17	Dominant negative	-	No	GEF binding (negative titration)
Rac1-V12	Dominant positive	-	Yes	GTP-bound, no hydrolysis
Rac1- V12/Y40C	Dominant positive	-	No	GTP-bound, no hydrolysis
VAV1-L213A	Dominant negative	-	-	No GEF activity

Figure Legends

Figure 1. *Ra2 microglia conditionally express myc-tagged PAK1 wild type and mutant protein following lentivirus gene transfer.*

A) TX-100 detergent cell extracts of human promyelocytic HL60 cells and murine microglia cell line Ra2 were Western blotted with an antibody recognizing PAK-1, -2, and -3. In HL60 cells PAK1, and either PAK2 or PAK3, are detected but Ra2 microglia express only PAK1 (see C). B) PAK1 wild type and mutant protein expression was induced overnight in Ra2 microglia transduced with doxycycline-responsive lentivectors, and transgene levels determined by western blotting with anti-myc antibodies. Control cells Ra2 045 express only the doxycycline-binding rtTA-transactivator protein. C) TX-100 extracts of Ra2 045 control cells and Ra2 PAK-WT cells were Western blotted with a polyclonal rabbit anti-PAK1 antibody to indicate the degree of overexpression of PAK-WT (upper band in PAK WT lane) in relation to endogenous PAK1 (lower band). The western blots are representative of two (panel A) or three independent experiments.

Figure 2. *Effect of PAK1 wild type and mutant protein on the actin cytoskeleton in Ra2 microglia.*

Transduced PAK1 protein in the different Ra2 cell populations was visualized by immunofluorescence using α-myc antibodies (red) to localize transgene PAK and Alexa 488-conjugated phalloidin to stain filamentous actin (green). Neither PAK1 wild type, K299A, nor PBD-H83L caused major alterations of the fibrillary actin cytoskeleton. On the other hand dominant positive PAK1-T423E induced "blebbing" of the plasma membrane (arrows) in a high proportion of cells, while PAK1-H83,86L promoted extension of broad lammellipodia (arrows). For comparison, expression of dominant negative Rac1-N17, visualized with α-HA tag antibodies (red), caused complete cell rounding with loss of ruffles, filopodia, and lamellipodia. Bars in all panels 10 μm. The shown images are representative of at least three independent experiments.

Figure 3. *PAK1 regulates superoxide production following FMLP stimulation.*

A) FMLP-induced superoxide release in Ra2 cells expressing PAK1 wild type or mutant protein was measured by luminol E-CL. The ordinate shows chemiluminescent counts per second normalized to control cells, which were chosen as PAK1 WT expressing cells. Arrow marks time point of FMLP injection. Results are presented as mean and SEM of an individual experiment performed in quadruplicate wells. B) Mean and SEM of peak response of four

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separate experiments performed as above. C) Ra2 cells transduced with increasing concentrations of PBD-H83L lentivector (50, 75, and 400 µl) were inhibited in FMLP-stimulated superoxide production in a dose-dependent manner. The graph shows mean and SEM from a single experiment performed in triplicate, and is representative of a total of two independent experiments.

Figure 4. *VAV1 is required for FMLP-stimulated NADPH oxidase activity in Ra2 microglia.* A) TX-100 extracts of Ra2 cells expressing VAV1-L213A or Rac1-N17 were Western blotted with antibodies to myc- or HA-tags, and with antibodies against VAV1 or Rac1, respectively. Arrow in lower, right panel indicates transgene Rac1-N17. B) FMLP-induced superoxide production was measured by luminol E-CL in Ra2 045 control cells or cells transduced with increasing concentrations of lentivector conditionally expressing dominant negative VAV1-L213A. Arrow points to time point of FMLP injection. Note the titer-dependent inhibition of superoxide production. For comparison, expression of dominant negative Rac1-N17 entirely abrogated superoxide production. Mean and SEM of three independent experiments each performed in triplicate is shown.

Figure 5. *FMLP activation of PAK1 depends on VAV1 and Rac1 signaling.* ·
A) Untransduced Ra2 cells were stimulated with 2,5 µM FMLP in suspension, and at intervals cell aliquots were withdrawn for TX-100 extraction, immunoprecipitation of endogenous PAK1, electrophoresis, and Western blotting with antibodies detecting PAK1-T423 auto-phosphorylation. One western blot representative of three is shown. B) Mean and SEM band intensity of three individual experiments performed as in A is shown. C) Ra2 cell lines expressing PAK1 wild type protein alone, or together with VAV-L213A, or Rac1-N17, were established and stimulated with FMLP. Subsequently, level of activated PAK1 in PAK1-immunoprecipitates was determined by western blotting with phospho-PAK1-T423 specific antibodies. Total PAK1 was also detected with anti-myc antibodies as load control. The western blot shown is representative of three individual experiments. D) Densitometric analysis of three experiments performed as above. Mean and SEM of phospho-PAK1/ total-PAK1 ratios is shown.

Figure 6. *PAK1 modulates PMA-stimulated superoxide production in microglia.*
A) Superoxide production of PMA-stimulated Ra2 microglia expressing wild type or mutant PAK1 protein or Rac1-N17 was measured by luminol E-CL. A single representative replicate from one out of four independent experiments is shown. B) Mean and SEM of superoxide production peak response as measured above. Results are presented as chemiluminescent

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signal normalized to control. Data were derived from four independent experiments performed in triplicate or quadruplicate.

Figure 7. *Constitutively active mutants of Rac1 that differentially activate PAK1 have different effects on PMA-stimulated NADPH oxidase activation.* FMLP (A,B) or PMA (C) stimulated superoxide production of Ra2 045 control, Rac1-V12, or Rac1-V12/Y40C-expressing cells was measured by luminol E-CL. Traces from a single well is shown in A, the traces in C and the bar graph B represent in arbitrary units the mean and SEM of superoxide generation of three independent experiments, each performed as duplicate wells in triplicate runs.

Figure 8. *Dominant positive mutants of PAK1 cause phosphorylation of p47phox, but not RhoGDI, in Ra2 microglia cells.* A) Unstimulated Ra2 cells expressing the indicated PAK1 mutants were lysed in 2D lysis buffer and isoelectric focusing performed with IPG strips covering the range of pH 3-10 (left column) or pH 6-11 (right column). After 2D gel electrophoresis and semidry transfer to PVDF membranes, Western blotting was performed with polyclonal rabbit antibodies to p47phox. Note that expression of PAK1-H8386L (and to a lesser degree PAK1-T423E) causes the appearance or intensification of anodic (more negative) p47phox species, marked by arrows, indicative of phosphorylation. B) Ra2 PAK1 cell lysates were separated by 2D-gel electrophoresis and transferred as above, and membranes subsequently Western blotted with anti-RhoGDI antibodies. The arrow points to a single phosphorylated species of RhoGDI, which did not seem affected by expression of PAK1 mutants. Western blots in A and B are representative of 3-5 individual experiments.

Figure 9. *Arachidonic acid (AA) synergizes with PAK1-H83,86L in NADPH oxidase activation, and p47phox mutant S303/304/328D greatly increases NADPH oxidase activity.* A) Ra2 p47phox-GFP cells expressing wild type or mutant PAK1 protein were incubated with 200 nM BIM-1 (a PKC-inhibitor) and then stimulated with 10 μM arachidonic acid (arrow) for superoxide detection by luminol-E CL. The graph shows in arbitrary units the mean superoxide production (n = 3) of one representative experiment out of three, all performed in triplicate. The higher basal superoxide production of PAK1-H83,86L cells before AA stimulation (<5 min) in the shown graph was not consistently observed. B) The bar graph shows mean and SEM of peak superoxide generation for three independent experiments performed as above (arbitrary units). C) Ra2 cells co-expressing PAK1-H83,86L and p47phox-GFP were stimulated with arachidonic acid for 10 minutes and then fixed for immunofluorescence with anti-myc tag antibodies. PAK1 (red) can be seen to colocalize with p47phox-GFP (green) at cell-cell junctions or on the free plasma membrane (arrows). The
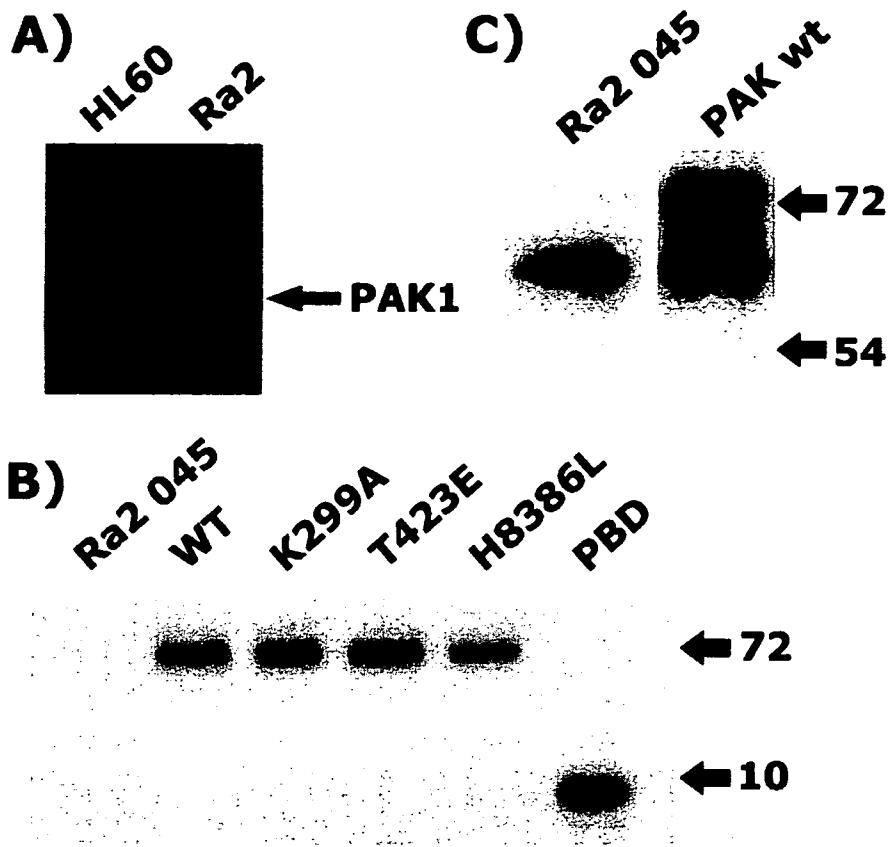
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experiment was performed three times with similar results. Bar, 10 μm. D and E) Ra2 045 control, p47phox wild type or p47phox-S303/304/328D mutant expressing cells were stimulated with FMLP (D) or PMA (E), and superoxide release measured with luminol E-CL. The ordinate represents superoxide generation in arbitrary units normalized to Ra2 045 control cells. Traces in each case represent mean of three wells of a single experiment, and is representative of four experiments performed. Note that expression of the p47phox-S303/304/328D causes superoxide production even under unstimulated conditions (inset in D).

Figure 1

Figure 2

29

Figure 3

30

Figure 4

A

FMLP 0 20" 1' 2' 3' 5' 10' Cont 5'

C

PAK wt	PAKwt Rac1-N17	PAK wt VAV1-L213A	
- +	- +	- +	FMLP

pT423

myc-tag

D

☐ PAK wt
■ PAK wt + Rac1-N17
■ PAKwt + VAV1-L213A

Figure 5

32

33

Figure 6

Figure 7

34

Figure 8

35
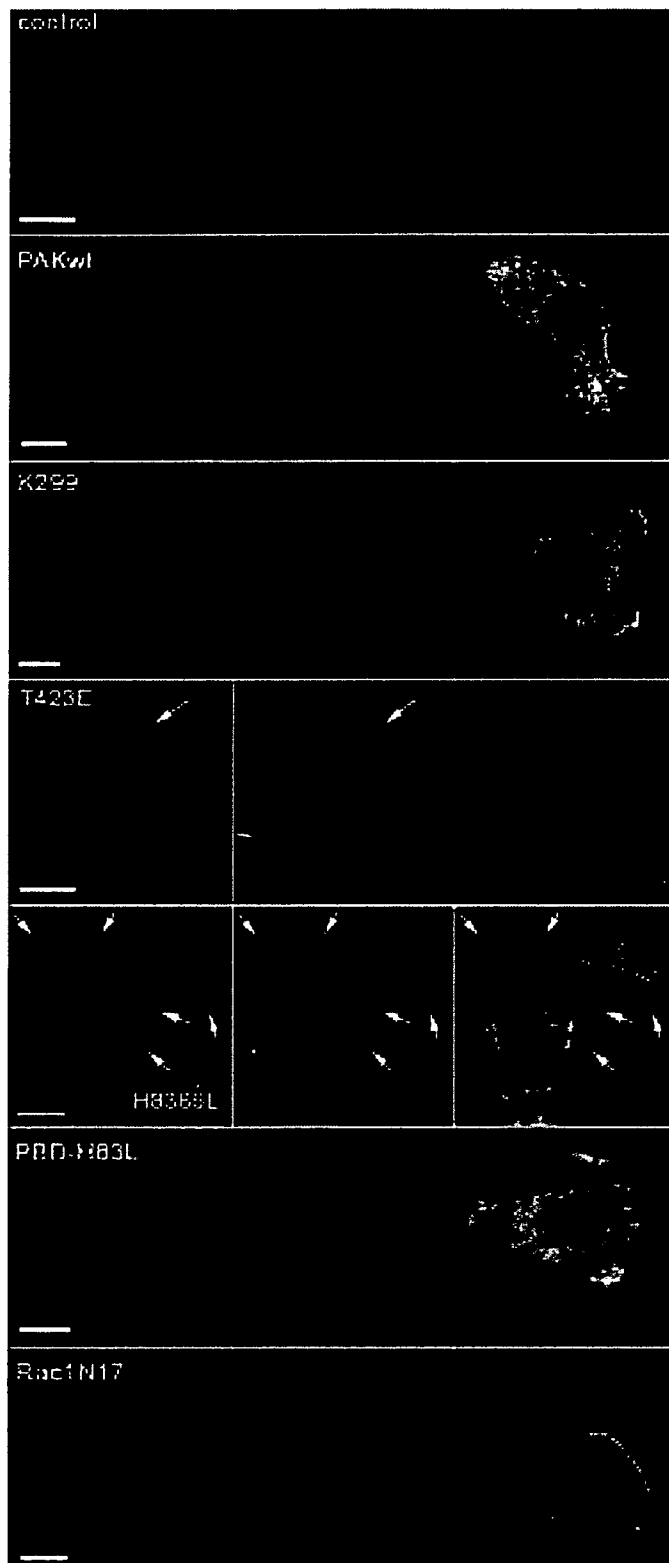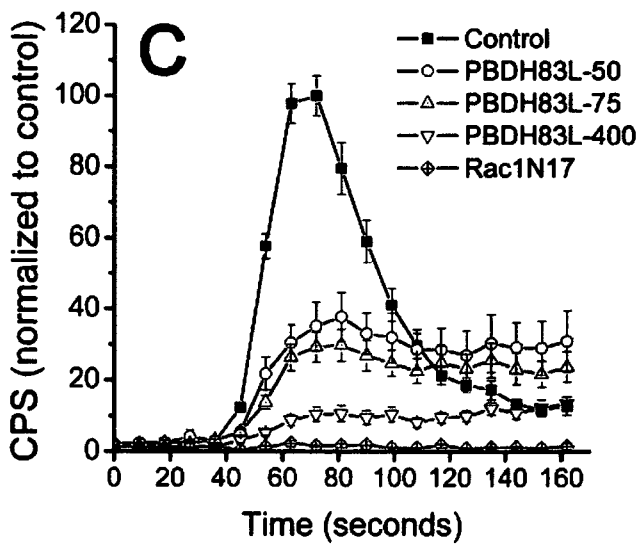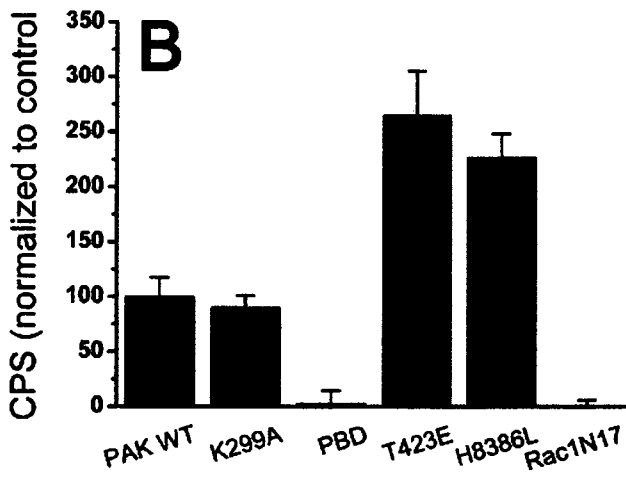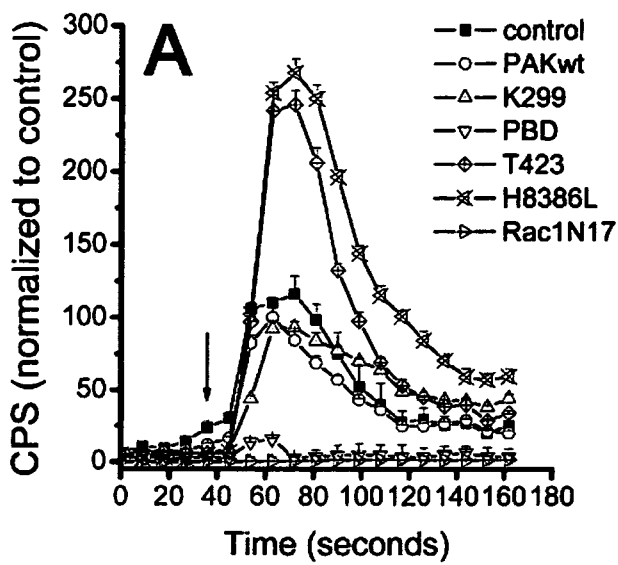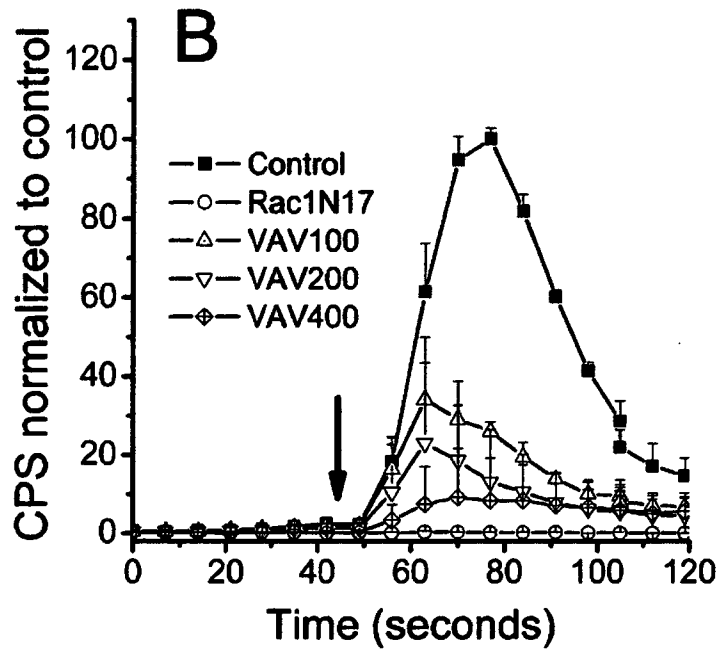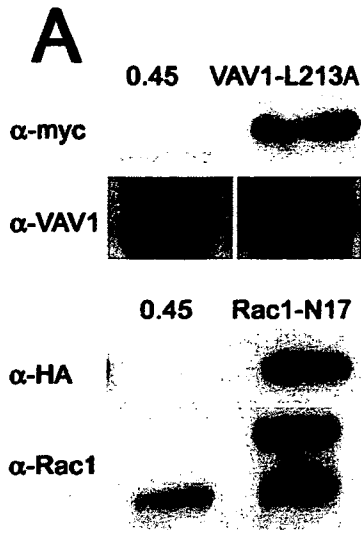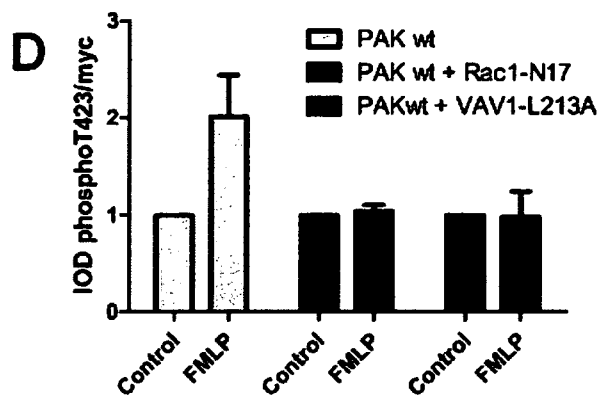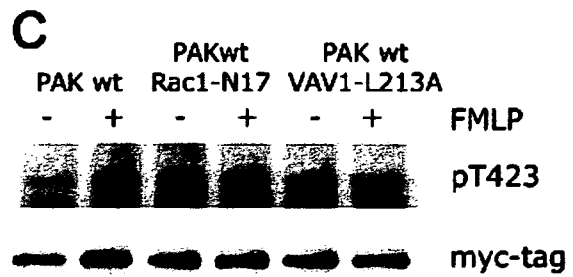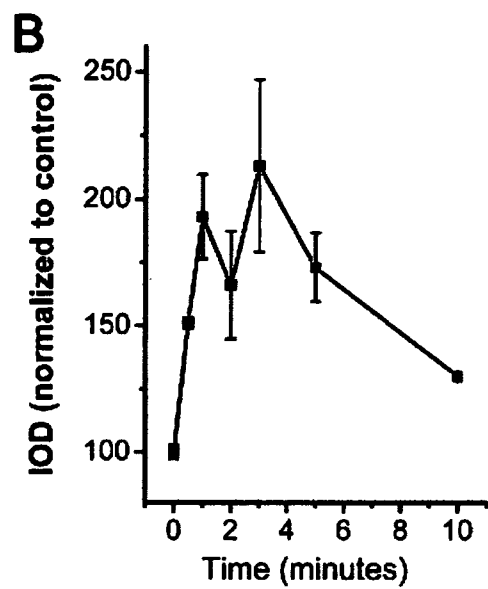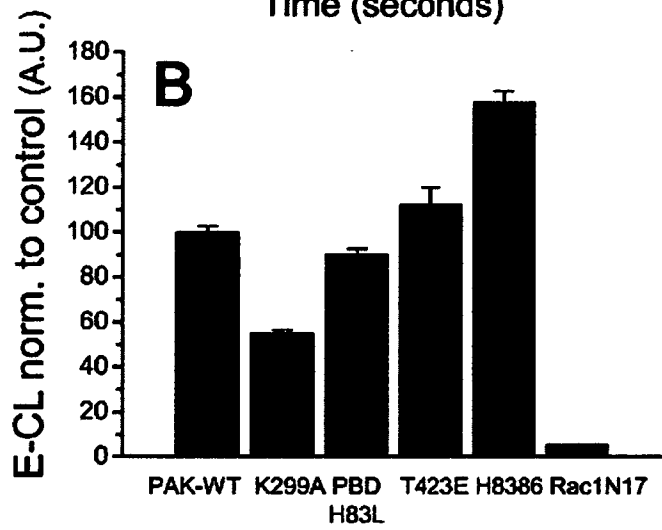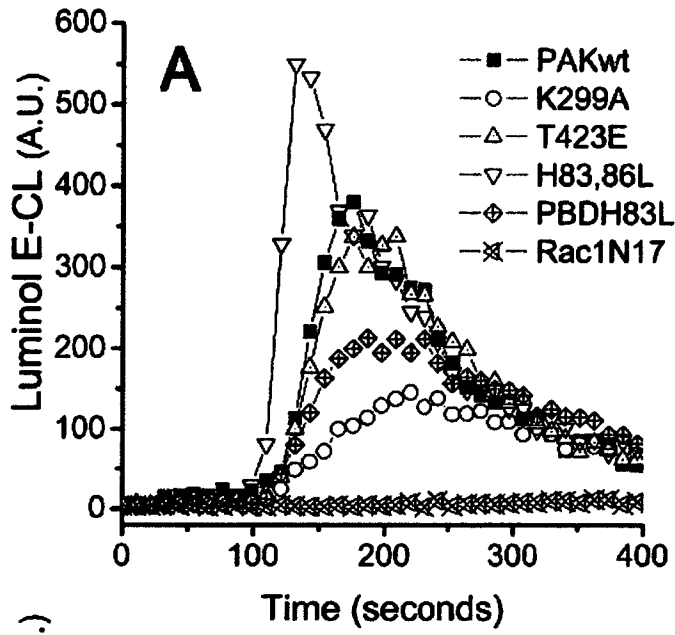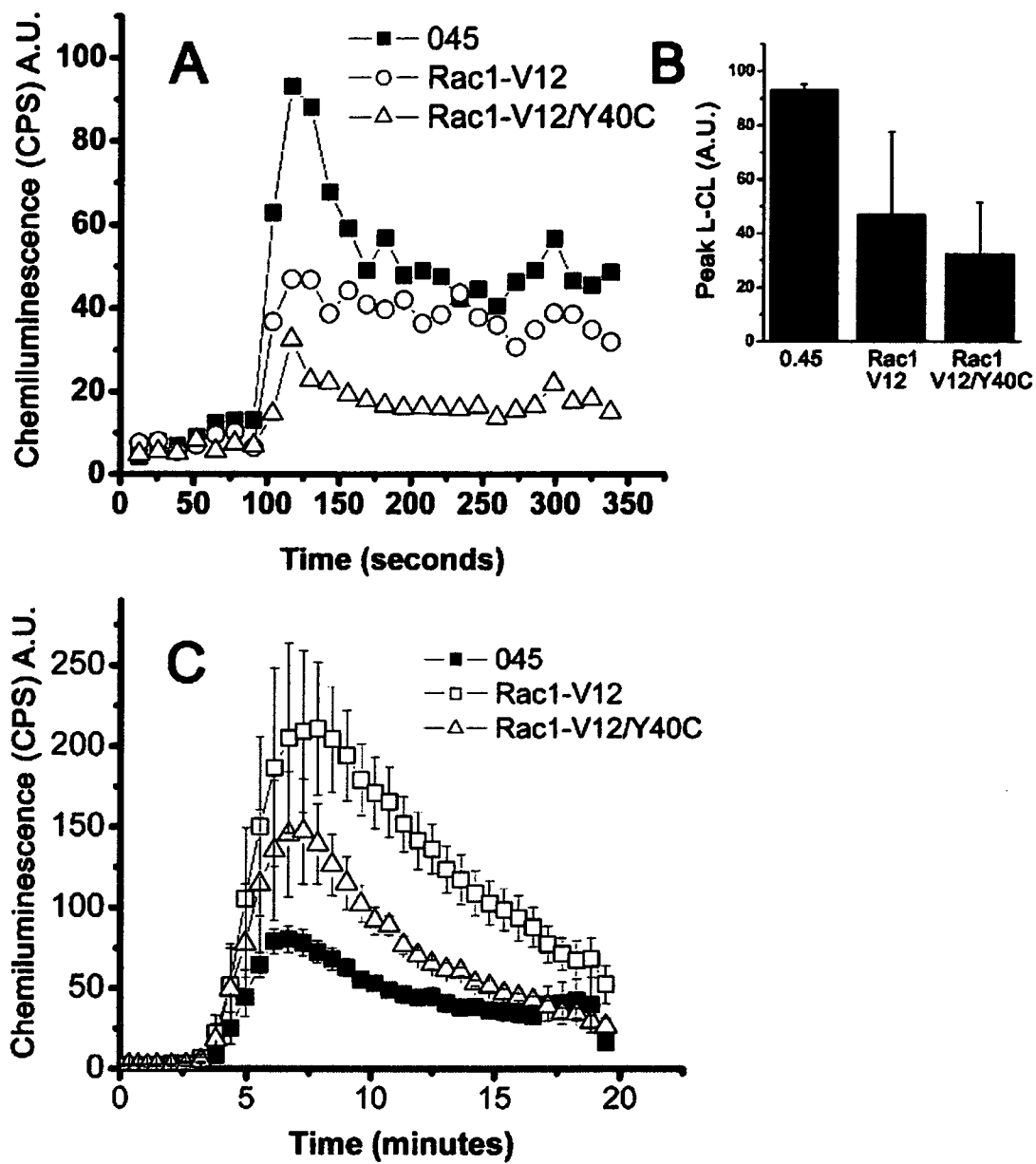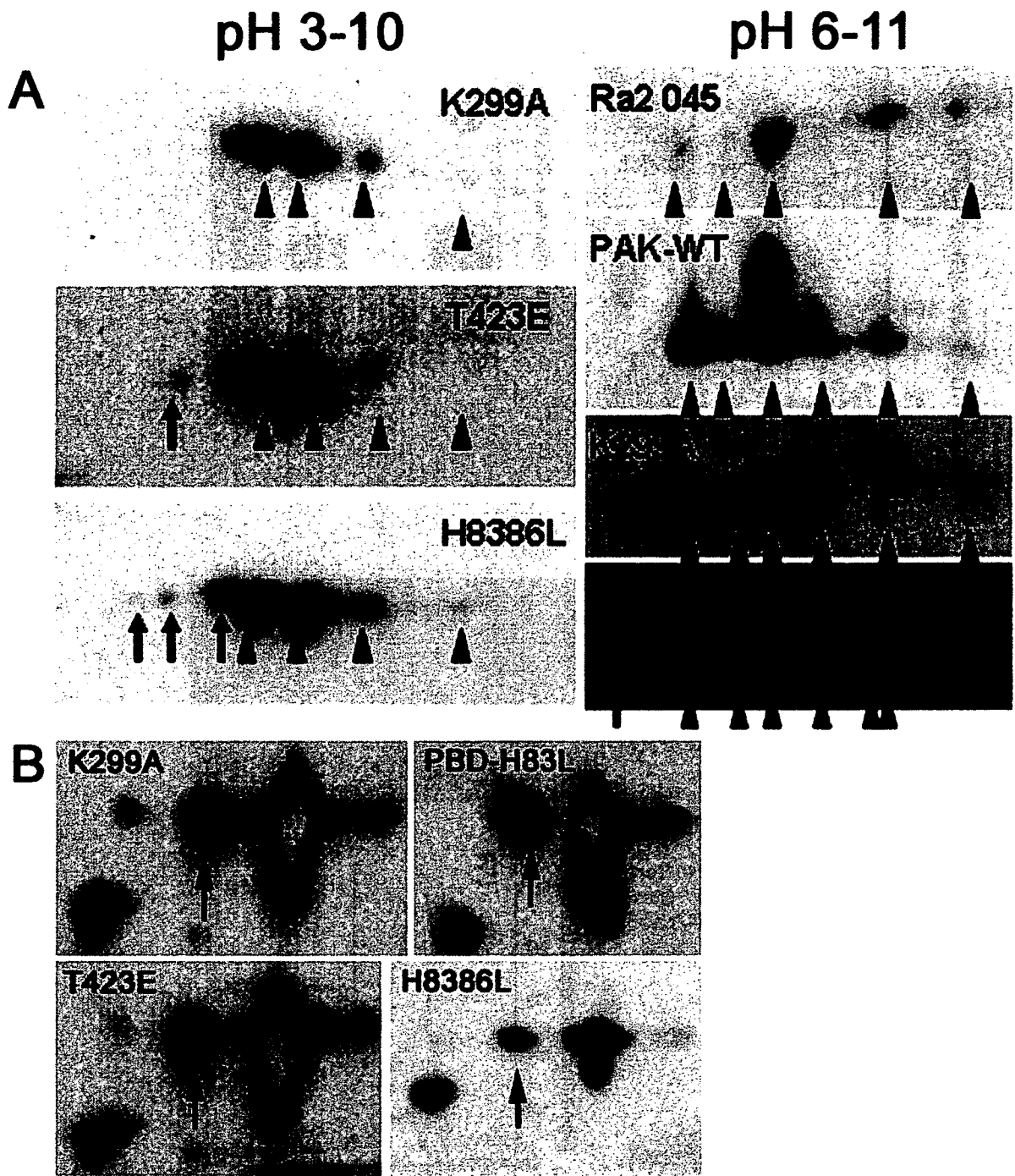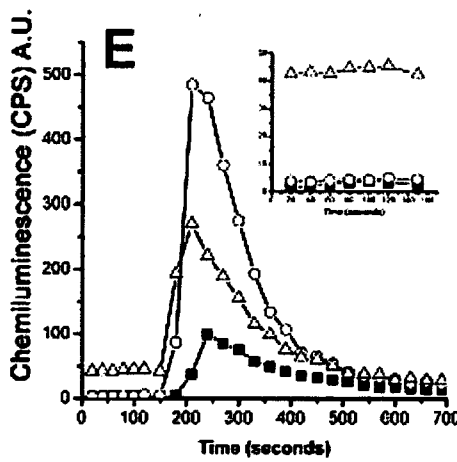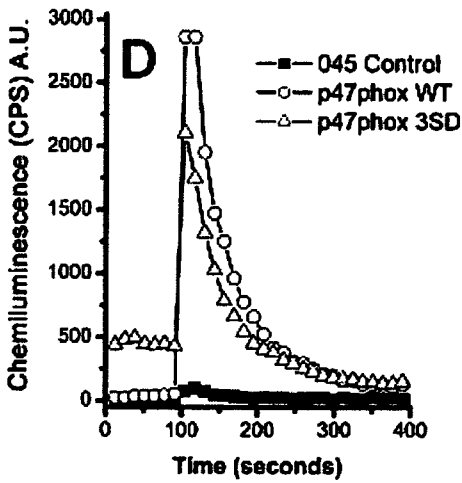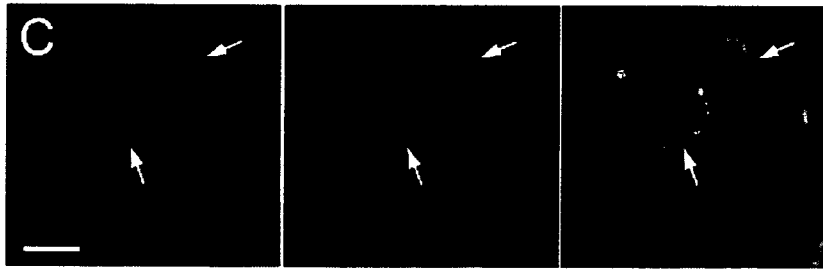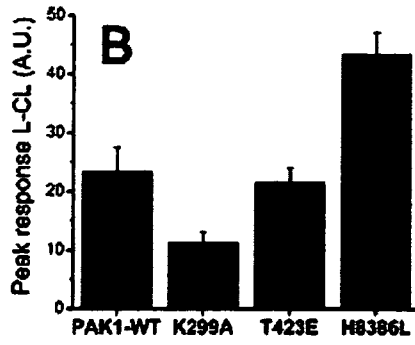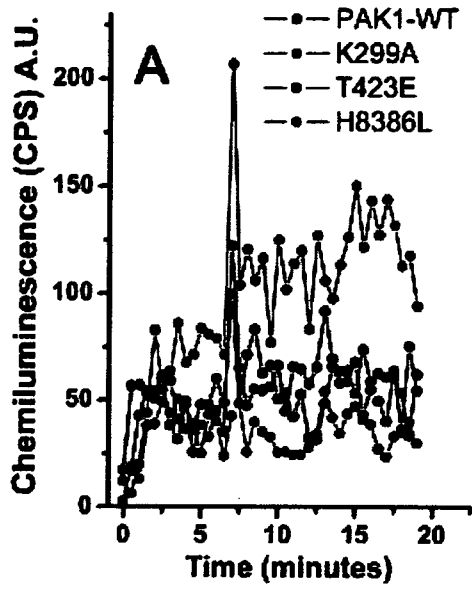
Figure 9

Effects of Aging on Neuroprotective and Neurotoxic Properties of Microglia in Neurodegenerative Diseases

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Key Words

Microglia · Aging · Parkinson's disease

Abstract

The inflammatory process in the brain, which is accompanied by changes in the levels of proinflammatory cytokines and neurotrophins, along with the presence of activated microglia, has recently gained much attention in neurodegenerative diseases. Activated microglia produce either neuroprotective or neurotoxic factors. Many reports indicate that activated microglia promote degeneration of dopamine (DA) neurons in Parkinson's disease (PD). On the other hand, there are several lines of evidence that microglia also have a neuroprotective function. Microglia activated with lipopolysaccharide in the nigrostriatum of neonatal mice protect DA neurons against the PD-producing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), whereas activated microglia in aged mice promote DA cell death by MPTP. These results suggest that the function of activated microglia may change in vivo from neuroprotective to neurotoxic during aging as neurodegeneration progresses in the PD brain.   Copyright © 2008 S. Karger AG, Basel

Introduction

Parkinson's disease (PD) is characterized by degeneration of dopamine (DA) neurons in the substantia nigra. As a mechanism of neuronal death, the inflammatory process in the brain, which is accompanied by changes in the levels of cytokines and neurotrophins and the presence of activated microglia, has gained much attention in PD, AD, and other neurodegenerative diseases [1, 2]. Activated microglia may play neurotoxic roles by producing proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. On the other hand, activated microglia may also play neuroprotective roles by producing neurotrophic compounds such as brain-derived neurotrophic factor. Sawada et al. [3] proposed a hypothesis of two-step activation of microglia: cytokines from activated microglia in the substantia nigra and putamen may be initially neuroprotective, but may later become neurotoxic during progression of PD by toxic change of activated microglia. In the present study, the neuroprotective and neurotoxic roles of activated microglia have been examined in relation to aging.

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