Ichinose *et al.* [44, 45] cloned the human GCH1 gene, and discovered mutations in the GCH1 gene that caused a partial deficiency (2-20% of the normal value) in GCH1 activity and BH4 levels. Decreased GCH1 activity not only decreases BH4 levels but also decreased TH protein, as indicated by a study on BH4-deficient mice [46] as well as by one on the postmortem striatum of patients with autosomal-dominant GCH1 deficiency [47]. In this deficiency only 1 allele of the GCH1 gene is mutated and there is a lack of degeneration of nigrostriatal DA neurons. However, DA levels are decreased specifically in the nigrostriatal DA neurons; and the dystonia is controllable for many years by DA supplementation with L-DOPA without the development of dyskinesia (i.e., the "on-off" effect of L-DOPA, as frequently observed during L-DOPA therapy in PD patients) [42, 43]. In contrast, in autosomal-recessive GCH1 deficiency, which was first described by Niederwieser *et al.* in 1984 [48], both alleles of the GCH1 gene are mutated, resulting in GCH1 activity that is less than 2% of the normal value and in decreased levels of all of the neurotransmitters (DA, noradrenaline, adrenaline, serotonin, NO) produced by pteridine-dependent enzymes. The consequences are clinical phenotypes that are completely different and more severe than the phenotype of autosomal-dominant GCH1 deficiency: parkinsonism, hyperphenylalaninemia, muscle hypotonia, fever episodes, and epilepsy [44, 48]. Neither autosomal-dominant GCH1 deficiency nor autosomal-recessive sepiapterin reductase deficiency, a newly recognized BH4 deficiency disease recently reported by Blau *et al.* [49], show hyperphenylalaninemia, thus suggesting adequate PAH activity in the liver.

Autosomal-recessive TH deficiency was first reported in Germany in 1995 as autosomal-recessive DOPA-responsive dystonia or autosomal-recessive Segawa's syndrome [50, 51]. Several mutations were found in the human TH gene in this disorder; and the clinical symptoms depended upon the degree of decrease in TH activity ranging from mild DOPA-responsive dystonia to severe DOPA-nonresponsive parkinsonism [52]. Complete loss of TH would be lethal, for TH(-/-) mice were found to die around birth [53, 54] and Kobayashi *et al.* [53] found this neonatal death to be caused by cardiac dysfunction owing to noradrenaline deficiency in the sympathetic noradrenaline neurons innervating the heart. In the case of autosomal-dominant GCH1 deficiency, DA neuronal death does not occur; DA transporter levels are normal, as determined by single-photon emission computed tomography [57]. However, there is an indication of degneration in patients with autosomal-recessive DOPA-nonresponsive TH deficiency [52] and this deficiency was similar to that reported for autosomal-recessive juvenile familial PD (PARK2) caused by mutations of the *parkin* gene [5]. However, it should be underscored that differential diagnosis among autosomal-dominant GCH1 deficiency, autosomal-recessive TH deficiency, and autosomal-recessive juvenile PARK2 are sometimes difficult [55, 56].

GCH1, which is the first enzyme involved in the biosynthesis of BH4, is colocalized with TH in the nigrostriatal DA neurons [36]. Choi *et al.* [58] proposed a hypothesis that BH4 itself together with DA quinones may induce cell death. As described below, we propose that the selective neurotoxicity in the nigrostriatal DA neurons among the catecholamine neurons may be explained by the higher biosynthesis rate of both BH4 and DA in the residual nigrostriatal DA neurons in PD. The hypothesis proposed by Choi and co-workers is consistent with our proposition and helps explain why neuronal death is not produced in autosomal-dominant GCH1 deficiency, in which both BH4 and DA levels are decreased.

Although there is a lack of evidence concerning inflammatory responses in GCH1 deficient patients, microglia activation may occur in autosomal-recessive juvenile PD (PARK2) or sporadic PD. Furthermore, since BH4 is a cofactor of NOS, the induction of GCH1 with consequent increase in the BH4 levels may stimulate the formation of inducible NOS (iNOS) in microglia, thus causing the synthesis and release of more NO from the activated glial cells. It would be interesting to examine whether the neopterin/biopterin ratio in CSF increases in the severe forms of autosomal-recessive TH deficiency, in which microglial activation might be expected.

Since the elevated ratio of total neopterin/total biopterin in the CSF in PD patients indirectly suggests microglia activation in the brain, Mogi and co-workers in our group further examined, by using a highly sensitive enzyme-linked immunosorbent assay (ELISA), changes in the contents of pro-inflammatory cytokines in the CSF that may reflect glia activation and inflammation in the brain. Cytokines are unstable proteins, and so we examined the levels in both lumbar CSF and ventricular CSF. As shown in Table **1**, we found that the level of tumor necrosis factor-alpha (TNF-alpha) was significantly increased in the lumbar CSF and in the nigrostriatal region of the brain in PD [59]. As the concentrations of other cytokines were generally low in the lumbar CSF, we measured them in the ventricular one. We found elevated levels of the following cytokines: interleukin-1beta (IL-1beta), IL-2, IL-4, IL-6, transforming growth factor-alpha (TGF-alpha) [60], and TGF-beta1 [61]. In agreement with our data on increased cytokines in the CSF from patients with PD, Blum-Degan *et al.* [62] also reported increased levels of IL-1beta and IL-6 in the lumbar CSF from patients recently diagnosed with PD or with AD without drug treatment. Interestingly, a significant inverse correlation between severity of PD and IL-6 CSF levels was observed [63]. These results indicate that elevated IL-6 levels in the CSF of untreated PD patients may reflect the inflammatory reaction in the brain during the course of the disease. This inverse correlation may suggest a neuroprotective compensatory reaction of IL-6 in neuroinflammation. Increased levels of TGF-beta1 and TGF-beta2 in the ventricular CSF of PD patients were also reported [64]. These changes in the levels of cytokines as well as the relative increase in the neopterin in lumbar or ventricular CSF may be considered to reflect the changes in the nigrostriatal regions of the brain in PD, where glial activation and inflammatory responses might occur in a tissue-specific manner.

3. PRO-INFLAMMATORY CYTOKINES AND NEUROTROPHIC GROWTH FACTORS (NEUROTROPHINS) IN THE BRAIN IN PARKINSON' DISEASE

An increased neopterin/biopterin ratio and increased levels of pro-inflammatory cytokines, such as TNF-alpha,

IL-1beta, and IL-6 in lumbar and ventricular CSF from patients with sporadic PD may reflect increased production from activated glial cells, especially microglia caused by the immune reactions at nigrostriatal brain regions. Therefore, Mogi and colleagues in our group assessed pro-inflammatory cytokines and anti-apoptotic neurotrophin expression in the postmortem brain from patients with idiopathic (sporadic) PD. Many precautions regarding postmortem changes in cytokines or neurotrophins are necessary when interpreting the results, since these proteins are easily degraded by proteases in the brain. We examined postmortem samples from the brain bank at Würzburg University, Germany, in collaboration with Dr. Peter Riederer, and those from the Japanese brain banks of Dr. Yoshikuni Mizuno (Juntendo University, Tokyo, Japan) and Dr. Sadako Kuno (Utano Hospital, Kyoto, Japan). The results are summarized in Table 1.

In agreement with the CSF data described in the aforementioned section, we found increased levels of the following cytokines within nigrostriatal regions: TNF-alpha [59], IL-1beta [65], IL-2 [66], IL-6 [65], TGF-alpha [65], and TGF-beta1 [61]. We also found that levels of the TNF-alpha receptor R1 (TNFR1, p53) were elevated in the substantia nigra in PD in comparison with that of controls [67]. In agreement with our ELISA results, Boka *et al.* [68] found TNF-alpha immunoreactive glial cells in the substantia nigra of PD patients. They also reported immunoreactivity for TNF-alpha receptors in cell bodies and processes of most DA neurons, suggesting that TNF may participate in the degeneration process occurring in PD, at least after a primary insult capable of inducing reactive gliosis. Interestingly, expression of Fc epsilonRII/CD23 (low-affinity IgE/Fc epsilon receptor), whose engagement results in the production of nitric oxide and TNF-alpha, was found in glial cells of PD patients [69]. These immunohistochemical data suggest that proinflammatory cytokines are produced around DA neurons, probably in activated glial cells.

The presence of interferon-gamma (INF-gamma)-synthesizing cells in the brain has been a controversial issue. Hunot and Hirsch [70] found IFN-gamma-positive cells in the parkinsonian brain with morphology suggestive of a lymphocytic phenotype, indicating the possibility of infiltration of peripheral lymphocytes. Thus, these IFN-gamma

Table 1. Changes in Cytokines, Neurotrophins, and Apoptosis-related Proteins in the Brain (Nigrostriatal Regions), Ventricular Cerebrospinal Fluid (VCSF), and Lumbar CSF (LCSF) in Parkinson's Disease

	Brain	CSF	
	(striatum/substantia nigra)	VCSF	LCSF
Neopterin/Biopterin ratio			↑↑
TNF-alpha	↑↑		↑↑
IL-1beta	↑	↑	ND
IL-2	↑	↑	ND
IL-4		↑	ND
IL-6	↑	↑	↑
EGF	↑		
TGF-alpha	↑	↑	ND
TGF-beta1	↑	↑	ND
bFGF	→		
NGF	↓↓		
BDNF	↓↓		
GDNF	→		↑
sFAS	↑	ND	ND
TNF-alpha Receptor 1 (p55)	↑		
beta2-MG (MHC I)	↑	↓	↓
Bcl-2	↑	ND	ND
Caspase 1	↑		
Caspase 3	↑		

ND, not detectable; ↑↑ : markedly increased; ↑ : increased; → : no change; ↓ : decreased

positive cells were clearly distinguishable from TNF-alpha- and IL-1beta-positive cells, which appear to be of a neuronal or glial origin. Although T cell infiltration is typically not observed in the PD brain, there are a few cases where the presence of CD8-positive T cells were detected within the substantia nigra of patients with PD [71]. There is also a report indicating changes in blood vessels occur in the mesencephalon during PD [72]. Thus, the IFN-gamma-positive cells in the parkinsonian brain may be T cells that arrived there due to a malfunctioning blood-brain-barrier.

Neurotrophic growth factors, i.e., neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], neurotrophin-3, and neurotrophin-4/5) are proteins produced by glial cells and neurons that support the differentiation and survival of neurons, and also appear to act as anti-apoptotic factors. In contrast to the increased cytokine levels, nigrostriatal BDNF and NGF concentrations (on the order of ng/mg and pg/mg protein, respectively) were significantly lower in PD patients than controls [73]. The neurotrophins, BDNF and NGF, have neuroprotective consequences for DA neurons and their depletion triggers apoptotic processes. Thus, the lack of neurotrophins together with the increased levels of pro-inflammatory cytokines may be fundamental in the pathogenesis of PD by accelerating progressive apoptotic death of nigrostriatal DA neurons. Glial cell line-derived neurotrophic factor (GDNF) is also a neurotrophin that has attracted considerable interest in relation to PD, largely owing to its particularly potent effects on DA neurons [74-77]. We showed earlier that GDNF levels in the human brain were significantly higher in the nigrostriatal DA regions (substantia nigra, caudate nucleus, putamen) than in the cerebellum and frontal cortex from either control or PD brains; however, nigrostriatal GDNF did not significantly differ between PD and control patients [78]. This is in contrast to the markedly reduced levels of BDNF or NGF found specifically in the nigrostriatal region in PD. The unchanged levels of GDNF in PD could be due to compensatory production by glial cells, which does not occur for either BDNF or NGF [73]. Interestingly, the concentration of another DA neuron-protective growth factor, basic fibroblast growth factor (bFGF), which was also shown to be abundant in the nigrostriatal region (on the order of ng/mg protein), was unchanged between control and PD striatum [66], again suggesting the possibility of compensatory production by glial cells.

4. APOPTOSIS-RELATED FACTORS DOWNSTREAM OF CYTOKINE OR NEUROTROPHIN SIGNALING PATHWAYS IN THE NIGROSTRIATAL REGION IN PARKINSON'S DISEASE

The presence of pro-inflammatory cytokines such as TNF-alpha, coupled with the deprivation of neurotrophins together may be a potent apoptotic signal [79]. Consequently, we examined changes in the levels of apoptosis-related factors in PD brains [Table 1]. Belonging to the TNF-alpha/NGF receptor family, the Fas antigen/APO-1/CD95 is a cell-surface receptor protein known to trigger apoptosis upon binding to the Fas ligand (FasL). Fas antigen and the two TNF receptors, p55 and p75, have been implicated in triggering cell death upon stimulation by their natural ligands, i.e., TNF-alpha and FasL [80, 81]. Activated Fas/CD95 was reported to induce pro-inflammatory cytokine responses by human monocytes and monocyte-related macrophages [82]. Thus, in addition to its classical intracellular apoptotic neuronal pathway, Fas may also induce pro-inflammatory responses by activated microglia.

Molecular cloning and nucleotide sequence analysis revealed a human Fas mRNA variant capable of encoding a soluble Fas (sFas) molecule lacking the transmembrane domain. Moreover, sFas is known to protect against Fas-mediated apoptosis. We found that the concentration of sFas in nigrostriatal DA regions was significantly higher in PD patients than controls [83], suggesting attenuation of the Fas-signaling pathway in PD. However, the content of Fas-FasL as well as that of Fas-associated death domain (FADD) was reported to be reduced in DA neurons of PD patients [84]. This finding suggests that a reduction in Fas signaling may be a defense mechanism of these neurons for PD-related pathology. We also found that the concentration of anti-apoptotic bcl-2 protein, which is localized in several cell components such as inner and outer mitochondrial membranes [85] in cells in the nigrostriatal DA region, was significantly higher in PD patients than in those of controls [86]. Marshall *et al.* [87] also reported upregulation of bcl-2 in the basal ganglia in PD. Importantly, the anti-apoptotic activity of bcl-2 was linked to reduced generation of ROS [88, 89]. One would expect the upregulation of sFas and bcl-2 and downregulation of Fas-FasL in the nigrostriatum to prevent the apoptosis that is presumably occurring in PD. These results are thus against the hypothesis that apoptosis is indeed responsible for the neurodegeneration. However, the possibility should be entertained that these changes in the apoptosis-related factors in PD may reflect responses of yet unknown compensatory mechanisms operative during ongoing Fas-mediated apoptosis.

Members of a novel family of aspartate-specific cysteine proteases, which include caspase-1 (IL-1beta-converting enzyme) and caspase-3, have been implicated as mediators of apoptotic cell death [90, 91]. Indeed, both caspase-1- and caspase-3-like proteases are involved in TNF- and Fas-receptor-mediated apoptosis. The activities of caspase-1 and -3 were significantly higher in the substantia nigra from PD patients relative to control patients [67]. Activated caspase-3 was also detected immunohistochemically and was proposed to be the final effector in the apoptotic cell death of DA neurons in PD [92]. Since both caspase-1 and -3 together with TNFR1 may be fundamental for apoptotic cell death through the TNF-alpha-induced signal pathway, the presence of a pro-apoptotic environment in the nigrostriatal region of the PD brain suggests vulnerability of neurons and glial cells towards a variety of noxious factors. In support for this contention, ribozyme-mediated inhibition of caspase-3 activity reduced apoptosis induced by 6-hydroxydopamine (6-OHDA) in PC12 cells [93]. In another human study, a significantly higher percentage of DA neurons than of other cells in the substantia nigra displayed caspase-8 activation, as indicated by their immunoreactivity [94]. As caspase-8 is known to cause the release of cytochrome c from mitochondria to trigger caspase-3 activation, it might be involved in the apoptotic cell death of DA neurons.

5. CYTOKINES AND NEUROTROPHINS IN THE BRAIN IN ANIMAL MODELS OF PARKINSON'S DISEASE

In several animal models of PD, we observed changes in cytokines and neurotrophins similar to those observed in postmortem PD brain. The dopaminergic neurotoxin, MPTP, which causes PD in humans [96], also produces apoptotic cell death of the nigrostriatal DA neurons in monkeys and rodents [95, 97]. MPTP is a synthetic N-methylated amine product that easily enters the brain from circulation and is oxidized to 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase type B (MAO-B) in glial cells. Subsequently, MPP^+ is transported into DA neurons *via* the DA transporter, where it causes neuronal death (possibly through apoptosis) by inhibiting complex I of mitochondria and producing ROS. MAO-B inhibitors such as deprenyl (selegiline) completely prevent PD induction by MPTP, and deprenyl is clinically effective for the treatment of PD.

It was also reported that MPTP had multiple immune/ inflammatory effects in mice, including upregulation of MHC expression in the nigrostriatum, microglial activation and infiltration of CD4- and CD8-T cells into the substantia nigra [98]. We observed that repeated intraperitoneal injection of MPTP increased IL-1beta concentration 23-fold and decreased NGF concentration by approximately 50% within the striatum [99]. These results agree with data from postmortem PD brains and hint at a possible induction of apoptosis within DA neurons. As well, in support for cytokine-mediated apoptotic and inflammatory pathway in PD, mice lacking in caspase 11 (an apical caspase mediating the activation of caspase 1) were somewhat resistant to MPTP toxicity [100]. Gene expression analysis in MPTP treated mice by cDNA microarray also revealed neuroinflammation to be an important event in the process of DA neuronal cell death [101]. Moreover, the anti-inflammatory drug, aspirin or salicylate, was found to protect against MPTP-induced dopamine depletion in mice [102], through inhibition of cyclooxygenase type 2 (COX2) [103], which is essential for synthesis of inflammatory prostaglandins. Correspondingly, increased expression of COX2 as well as the inducible form of NOS (iNOS) was evident in glial cells of the substantia nigra in patients with PD [104, 105]. Again, these data support a role for inflammatory pathways in PD, specifically involving COX2 and the oxidative stressor, iNOS, which may also be related to apoptotic factors (e.g. caspases).

Further support for a link between inflammation and apoptotic processes in PD comes from a recent study by Hayley *et al.* [106] that revelaed that MPTP increased nigral Fas expression and that genetically deficient Fas mice displayed attenuated DA neuron loss. As well, MPTP increased nigrostriatal microglia immunoreactivity within wild type but of the Fas null mice, however, it remains to be determined if these glial variations stemmed from direct actions of Fas or a secondary to variations of neuronal survival. Interestingly, nigrostriatal expression of a dominant-negative c-Jun adenovirus blocked the induction of Fas and also protected DA neurons from MPTP-induced damage. These data suggest the critical nature of the immediate early gene, c-Jun, and the Fas signaling pathway in MPTP induced degeneration. This group of investigators also reported that Fas-deficient mice displayed a pre-existing reduction in striatal DA levels and locomotor behavior, but were resistant to further MPTP induced decreases of DA levels. However, MPTP increased the metabolites, DOPAC and HVA in Fas mice, raising the possibility again that compensatory responses may have been engendered to maintain stable DA stores and behavioral responses.

Changes in cytokine levels were also observed in rats with 6-OHDA-induced PD. We found that in hemiparkinsonian rats, produced by injecting 6-OHDA unilaterally into the ventrotegmental bundle without or with L-DOPA treatment, levels of TNF-alpha were significantly increased only in the substantia nigra and striatum on the injected side. L-DOPA administration did not produce any significant change in TNF-alpha concentrations in the 6-OHDA-untreated control side of any of the brains [107]. These results agree with previous findings demonstrating increased TNF-alpha levels in the striatum and lumbar CSF of PD patients, and also suggest that the increased TNF-alpha levels in PD patients may not be due to the secondary effects of L-DOPA therapy. In a subsequent study, we showed that the increased TNF-alpha levels in the 6-OHDA-lesioned striatum was suppressed by the immunophilin ligand FK506 [108]. This finding is in keeping with the proposition that the immunosuppressant effects of FK506 may prevent 6-OHDA-mediated activation of microglial cells in the nigrostriatal DA region of rats. All these reports indicate that changes in cytokines and neurotrophins in the CSF and in the nigrostriatum of postmortem brains of idiopathic PD patients are consistent with data obtained from animal models using MPTP and 6-OHDA [109-111].

Although MPTP [96] is the only neurotoxin proved to produce PD in humans, the effects of neurotoxins similar to MPTP have also been examined in animals. Several MPTP-like neurotoxins were identified in the human brain [3, 109-117], but these compounds were found to produce PD only by direct injection into the striatum of animals. Among the various MPTP-like neurotoxins found endogenously in the PD brain, such as isoquinolines and beta-carbolines [3, 109-117], N-methyl(R)salsolinol (R-N-methyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisioquinoline) may be synthesized from DA within nigral neurons. Unilateral injection of N-methyl (R) salsolinol into the striatum of rats produced hemiparkinsonism, much like that associated with 6-OHDA [118, 119], and caused activation of caspase-3, suggesting possible apoptotic death of DA neurons [120]. This endogenous neurotoxin was reported to inhibit complex I in mitochondria, thereby producing ROS and ultimately provoked apoptotic cell death, as evidenced by DNA fragmentation [119].

Beta-calbolinium cations, which are produced from beta-carbolines by MAO-B, exist in the CSF from patients with PD at higher concentrations than those in controls [115]. These beta-calbolinium neurotoxins were also found to destroy the nigrostriatal DA neurons after their direct injection into the striatum of rats [113, 114]. Endogenous isoquinolines and beta-carbolines in the brain, like MPTP, are assumed either to be first N-methylated by N-methyltransferase and then oxidized by MAO-B to the corresponding isoquinolinium ions or beta-carbolinium ions, or to be

directly oxidized to more toxic metabolites by MAO-B [3, 109-117]. It is suggested that the mechanisms of the DA neuronal death induced by PD symptom-producing neurotoxins, i.e., MPTP/MPP^+, isoquinoline/isoquinolinium, or beta-carboline/beta-carbolinium, may be primarily apoptotic in nature. However, it remains to be determined whether isoquinoline/isoquinolinium or beta-carboline/beta-carbolinium produce changes in cytokine or neurotrophin levels along with inflammatory factors (e.g. COX2) in the nigrostriatum similar to those changes effected by MPTP/MPP^+.

Epidemiological studies have suggested that insecticide exposure is associated with an increased risk of developing PD. Rotenone is a naturally occurring, lipophilic compound from the roots of certain plants (*Derris* species), and is the main component of many insecticides. Rotenone is not structurally related to MPTP, but, just like MPP^+, its repeated peripheral injection selectively and partially inhibits mitochondrial complex I to produce a PD-like syndrome in rodents. Rotenone, as a highly lipophilic compound, may be able to affect both DA neurons and glial cells in the nigrostriatal region. Chronic systemic injection of rotenone into rats caused highly selective degeneration of nigrostriatal DA neurons and symptoms of muscle rigidity. An important morphological finding in rotenone-treated rats was that the nigrostriatal DA neurons accumulated fibrillar cytoplasmic inclusions, containing ubiquitin and alpha-synuclein, similar to the Lewy bodies seen in idiopathic PD [121]. This finding stands in contrast to the effects of MPTP in animals and humans, in which cytoplasmic inclusions or Lewy bodies are not observed. Lewy body formation is common in PD and is often taken to indicate failure of the ubiquitin-proteasome system; thus, rotenone may induce morphological changes closely aligned with idiopathic and familial PD involving PARK1 (alpha-synuclein mutation) [122].

The effects of other endogenous factors in the nigrostriatal DA neurons related to PD remain to be elucidated. For example, the formation of neurotoxic DA ortho-semiquinones [123] or DA quinones associated with high levels of neuromelanin and iron [124, 125]. As well, low levels of the normally protective factors calbindin (a calcium-binding protein), and glutathione (GSH) may render neurons vulnerable to excitotoxic and free radical damage.

An important question regarding the changes in the levels of cytokines, neurotrophins, and apoptosis-related factors in the nigrostriatum of the PD-animal models is whether these changes might be either the cause of neuronal cell death or secondary responses, and possibly neuroprotective reactions in glial cells, that occur in reaction to the degenerating DA neurons. Specifically, glial cells that are activated around degenerative DA neurons release cytokines that may either promote or inhibit neuronal survival. Thus, we asked the question of whether the microglia activation in the MPTP-mouse model is neuroprotective or neurotoxic by using histochemical methods. We compared the effect of single and repeated (7 doses) MPTP administration on microglia activation in the nigrostriatum. After a single dose of MPTP, no remarkable microglia activation was observed. However, after repeated administration, significant microglia activation was detected simultaneously with an approximately 50% decrease in the number of TH-positive cells. However, the numbers of TH-positive cells gradually recovered after the last dose of MPTP. We speculate from these histochemical findings that the activated microglia may not cause direct damage to TH-positive neurons but rather that a secondary neuroprotective reaction by them contributes to the recovery of TH-positive neurons in mice, since the glial cells were strongly associated with TH-positive cells [Sawada *et al.*, to be published].

There are also some reports indicating that the cytokines induced by the initial insults could be neuroprotective at least at the early stages of pathology in mice. It will be recalled; TNF-alpha is secreted from activated glial cells and acts *via* two different receptors, TNF-R1 and TNFR2. Rousselet *et al.* [126] reported that MPTP reduced motor activity in TNFR1 and TNFR2 double knock out mice but not in mice lacking only one of these receptors. The striatal DA level was slightly decreased in double TNF knockout mice and reduced even more in these mice after MPTP injection. From these results, the authors concluded that TNF-alpha may not directly participate in the death of DA neurons in PD, but may slightly alter DA metabolism, affecting the survival of DA terminals by a mechanism involving both TNFR1 and TNFR2 receptor subtypes. In another report by Suzuki *et al.* [127], microglia activated *via* the $P2X_7$ receptor through ATP, was found to release TNF to protect neurons against glutamate toxicity. These data suggest that TNF released from activated microglia may actually serve a neuroprotective role, at least in the initial stage of neuronal injury. However, several reports indicated that activated microglia might initiate or promote progression of DA neuronal death.

Intranigral or intrastriatal injection of the potent bacterial immunostimulant, lipopolysaccharide (LPS), produced PD-like pathology in rodents. Specifically, LPS induced microglia activation and damaged nigrostriatal neurons resulting in decreased levels of TH-positive neurons [128]. In another report [129], LPS-induced loss of DA neurons in the rat substantia nigra was accompanied by iNOS induction in fully activated microglia (with amoeboid morphology), suggesting that NO and/or its metabolites may play a crucial role in inflammation-mediated degeneration of DA neurons. The impact of glial activation provoked by IFN-gamma plus LPS was investigated in rat midbrain slices by Shibata *et al.* [130]. Application of IFN-gamma followed by LPS elicited an induction of iNOS and COX2 together with increased NO production, and resulted in DA neuronal death [130]. Aminoguanidine, an inhibitor of iNOS, or SB203580, an inhibitor of p38 mitogen-activated protein kinase (MAPK), prevented the IFN + LPS induced DA cell loss as well as NO production; whereas, selective COX-2 inhibitors such as NS-398 and nimesulide did not protect DA neurons. These results indicate that iNOS-derived NO in activated glial cells plays a crucial role in IFN-gamma /LPS-induced DA cell death.

MPTP-induced PD mice recover spontaneously after a few months. However, such recovery does not occur in humans and monkeys and this effect may be attributable to the progressive consequences of chronically enhanced glial activation. McGeer *et al.* [131] reported the presence of HLA-DR-positive reactive microglia, the accumulation of extracellular melanin, and the extensive loss of DA neurons

in the substantia nigra of monkeys administered MPTP 5 to 14 years before death. The monkeys had been drug free for at least 3 years before death, indicating that a brief exposure to MPTP had instituted an ongoing process. Highly reactive microglia appeared to surround DA neurons and a strong relationship was suggested between damage to DA neurons and the intensity of the neuroinflammatory response. Interestingly, such chronic neuroinflammation years after MPTP exposure is similar to that observed in humans with MPTP-induced PD [132]. Based on these changes at the chronic stage of MPTP-induced PD in monkeys, McGeer *et al.* [131] proposed that PD itself involves exposure to one of a variety of agents that disappear after instituting long-lasting inflammatory changes.

6. FAMILIAL PARKINSON'S DISEASE AND POSSIBLE IMMUNE REACTION

In idiopathic PD as well as in familial PD involving PARK1 (alpha-synuclein mutation), Lewy bodies consisting of mainly alpha-synuclein and spherical filamentous masses are common characteristic features. The question remains unanswered as to whether Lewy bodies are causative (neurotoxic) or compensatory (neurorpotective) in PD. Intraneuronal filamentous deposits similar to PD Lewy bodies are generally observed in Alzheimer's disease (AD), Huntington's disease, and other neurodegenerative conditions. Misfolded or unfolded proteins in cells are normally degraded by the ubiquitin-proteasome system thereby preventing their aggregation as intracellular inclusion bodies. Thus, dysfunction of the ubiquitin-proteasome system causes accumulation of these aggregates. The above neurodegenerative diseases in general are speculated to be "protein-conformational diseases or protein-misfolding diseases" due to a faulty ubiquitin-proteasome system [133]. However, in autosomal-recessive early-onset familial PD, PARK2, which is caused by the mutated *parkin* gene encoding inactive ubiquitin ligase E3 (loss of function), Lewy bodies are not observed. It is unclear why Lewy bodies are not formed in the presence of a dysfunctional ubiquitin-proteasome system in the case of PARK2 mutation. However, these results suggest that Lewy bodies may not be essential for the provocation of PD.

The Pael receptor (parkin-associated endothelin receptor-like receptor) is a substrate of parkin and is abundantly distributed in the nigrostriatal region. It would be expected that in PARK2 (autosomal-recessive juvenile PD), owing to the loss of functional parkin, Pael receptors may accumulate in the endoplasmic reticulum (ER) to cause ER stress [134], which in turn, may cause mitochondrial dysfunction and apoptotic death. The fact that TH-immunoreactive cells in the substantia nigra pars compacta are largely Pael receptor-positive may help explain the sensitivity of these DA neurons in PD [134]. A remaining question is the role of the Pael receptor in the sporadic PD. If sporadic PD with Lewy bodies might also be a "protein-conformational disease, " then oxidative stress of environmental factors coupled with the PD susceptibility genes might cause the unfolded protein response leading to ER stress [135]. ER stress arises from the failure of the ubiquitin proteasome system to remove misfolded or unfolded proteins. This build-up of abnormal proteins might finally induce the programmed cell death (apoptosis). However, the mechanism and the signal transduction pathway causing unfolded proteins to initiate apoptosis and the interaction between ER stress and oxidative stress, which is supposed to be the main trigger for sporadic PD, have not been made clear yet [136]. Since the parkin protein is a component of Lewy bodies, the formation of such bodies might contribute to neuronal death by the sequestration of functional parkin, ubiquitin ligase E3, which would prevent the removal of toxic proteins [137].

Alpha-synuclein (mutated protein in PARK1) is the main component of Lewy bodies and is speculated to act to presynaptically regulate DA release, synthesis or storage as well as regulation of synaptic plasticity. Curiously, in MPTP treated mice and in PD linked to PARK2 (parkin mutations) Lewy bodies are not produced. Thus, the contribution of alpha-synuclein to PD pathology is not clear. Thus, MPTP or rotenone was administered to alpha-synuclein(-/-) mice to assess the relation between ROS and alpha-synuclein. In these mice, DA neurons seem to be resistant to MPTP, but not to rotenone. This is curious given that both MPP^+ and rotenone inhibit complex I to produce PD in rodents. However, as mentioned before, the toxicity of MPP^+ formed from MPTP depends on the uptake of MPP^+ into DA neurons by the DA transporter, whereas rotenone is highly lipophilic and does not depend on this transporter. Therefore, one may speculate that alpha-synuclein is required for MPP^+ transport into DA neurons and that its deficiency would protect DA neurons. Accordingly, alpha-synuclein may be necessary for MPTP toxicity in mice.

Reactive oxygen species (ROS) may accelerate the process of alpha-synuclein aggregation and lead to the formation of more ROS through toxic alpha-synuclein fibrils, therein generating a repetitive cycle of death for the DA neurons. It is also important to note that DA itself, when oxidized, may stabilize the protofibril form of alpha-synuclein, thereby increasing the concentration of toxic protofibrils and of ROS in DA neurons [138]. Other reports indicated that the accumulation of normal alpha-synuclein in cultured human DA neurons resulted in ROS mediated apoptosis that also required endogenous DA production [139]. In contrast to alpha-synuclein toxicity in DA neurons, alpha-synuclein is not toxic in non-DA human cortical neurons, but rather exhibits neuroprotective activity, confirming the DA-dependent toxicity of alpha-synuclein. DA-dependent alpha-synuclein neurotoxicity is mediated by 5483-kDa soluble protein complexes (containing alpha-synuclein and 14-3-3 protein), which are elevated selectively in the substantia nigra in PD. Thus, the accumulation of soluble alpha-synuclein protein complexes can render endogenous DA toxic, suggesting a potential mechanism for the selectivity of neuronal loss in PD [139].

In accordance with *in vitro* data concerning DA specific toxicity of alpha-synuclein, nigrostriatal alpha-synucleopathy was induced *in vivo* by adeno-associated viral vectors overexpressing either wild-type or mutant human alpha-synuclein in the substantia nigra of adult marmosets. Indeed, the vectors promoted the loss of TH-positive neurons, formation of alpha-synuclein-positive inclusions and dystrophic neurites, as well as motor impairment, indicating a new primate model of PD [140].

A new gene implicated in familial PD, DJ-1 is beginning to receive attention. The crystal structure of DJ-1, which is the causative protein of another form of autosomal-recessive juvenile PD, PARK7 [9], was recently resolved [141, 142]; and studies revealed a highly conserved cysteine residue and an exsquisite sensitivity to oxidative stress. This cysteinyl structure suggests the possible involvement of DJ-1 protein in the cellular oxidative stress response and in the general etiology of sporadic PD [142].

All results concerning the causative mutated proteins of familial PD, i.e., alpha-synuclein in PARK1, parkin in PARK2, UCH-L1 in PARK5, and DJ-1 in PARK7, suggest the induction of apoptotic cell death of DA neurons due to the production of misfolded or unfolded proteins. Since the postmortem brain has not yet been adequately examined for the presence of cytokines or inflammatory response in familial PD, the relation between misfolded proteins and presumed neuroinflammation in the substantia nigra in familial PD is not clear yet. However, it is possibile that damaged DA neurons, either through misfolding or ER stress in familial PD, or by various insults in sporadic PD, may send unknown signal molecules to microglia to activate them [143]. Activated microglia would then produce pro-inflammatory cytokines, ROS, NO, etc. and accelerate the apoptotic pathway in the damaged DA neurons, which cells might finally be removed by phagocytosis by the activated microglia. Along these lines, caspase-12, a caspase that induces cytokine maturation, has been proposed as a mediator of apoptosis induced by ER stress and might contribute to the pathogenesis of AD [144].

It is possible that misfolded proteins associated with familial PD, i.e., PARK1 (alpha-synuclein), PARK2 (parkin), PARK5 (UCH-L1), or PARK7 (DJ-1), may be produced not only in DA neurons but also in glial cells. Accordingly, immune responses may be initiated in microglia which then damage DA neurons. Biochemical and histological data from patients with familial PD may give clues about possible neuroinflammatory reactions in familial PD. In a study using cultured neural cells, inhibition of the ubiquitin-proteasome system was shown to induce a pro-inflammatory response manifested by upregulation of COX2, its accumulation as ubiquitin conjugates and the production of prostaglandin E(2) [145]. Thus, ubiquitin-proteasome system disturbances might be a common mechanism in both familial PD and sporadic PD. Similar neuropathlogical findings that have been reported between familial and sporadic PD, except for the difference in the presence or absence of Lewy bodies, strongly suggest involvement of common molecular mechanisms for neuroinflammation leading to the death of nigrostriatal DA cells.

7. NEUROTOXIC AND NEUROPROTECTIVE EFFECTS OF CYTOKINES IN NEUROINFLAMMATION IN PARKINSON'S DISEASE

7.1. Roles of Microglia in Neuroinflammation

As already indicated, microglia together with astroglia play critical roles in the pathogenesis of various neurological disorders by acting as cytokine-producing and MHC class II antigen-positive immunoregulatory cells. Microglia produce IL-1alpha, IL-1beta, IL-5, IL-6, IL-10, IL-12, TNF-alpha, and TGF-beta, whereas astrocytes produce IL-1alpha, IL-1beta, IL-5, IL-6, IL-8, IL-10, INF-alpha, and -beta, RANTES (regulated on activated normal T cell expressed and secreted), macrophage inflammatory protein-1, TNF-alpha, TGF-beta, G-CSF, GM-CSF, and M-CSF (granulocyte-, granulocyte-macrophage-, and macrophage-colony-stimulating factors). Microglia also express mRNAs of cytokine receptors for IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, and M-CSF. These cytokines affect functions of both neuronal and glial cells *via* specific receptors on the cells, and form a unique cytokine network in the brain [146-151].

Changes in levels of pro-inflammatory cytokines and the upregulation of inflammation-associated factors such as iNOS or COX2 in activated microglia in the brain from patients with PD and in animal PD models strongly suggest that the loss of the nigrostriatal DA neurons may be triggered by glial derived pro-inflammatory cytokines. However, owing to their pleiotropic effects, cytokines may have either neurodeleterious or neuroprotective effects depending upon the circumstances at a particular time in the progression of PD. In terms of neurodeleterious effects, pro-inflammatory cytokines might start the cascade of the events leading to apoptotic cell death of the nigrostriatal DA neurons [18, 149-151]. Microglial activation may also occur as a secondary reaction to the various primary insults to the DA neurons. The process of oxidative stress in DA neurons involves decreased complex I activity in mitochondria, a decreased level of GSH, an elevated level of iron, and increased lipid peroxidation, all of which initiate the apoptosis cascade from mitochondria finally leading to DNA damage in the nucleus and subsequent apoptotic cell death. Damage to DA neurons in the nigrostriatum may initiate neuroinflammation and aggravate the disease process by causing glial cell activation, especially, microglia activation, to produce pro-inflammatory cytokines, ROS, and NO [152, 153]. However, a neurotoxin such as rotenone, which is highly lipophilic and penetrates any cells without depending upon DA transporter action, may inhibit complex I of mitochondria in both DA neurons and glial cells. As a result rotenone might also produce direct glial activation to produce cytokines, ROS, and NO.

Viruses have been suspected for many years as a cause of certains instances of PD, ever since the pandemic von Economo's disease in the early 20th century [154]. In fact, a rat model of PD was induced by Japanese encephalitis virus [155]. Interestingly, neither viral antigen nor viral genome could be detected in the substantia nigra. This fact suggests that a virus infection may trigger the chronic process of PD long after disappearing from the brain. As mentioned before, reactive microglias exist in the substantia nigra of monkeys [131] and humans [132] with MPTP-induced PD years after MPTP exposure.

The signal transduction pathway from the initial trigger of neurodegeneration of DA neurons to the final cell death has been extensively examined in various animal models of PD, particularly aspects involving oxidative regulatory factors. For instance, Hunot *et al.* [156] showed that nuclear translocation of transcription factor nuclear factor kappa B (NF-kappa B), which is activated by oxidative stress, is increased in DA neurons of patients with PD. As described

in the article by Barger in this issue, NF-kappa B is also known to be upregulated by TNF-alpha, IL-1beta or IL-6. As well, NF-kappa B induces iNOS expression to increase NO production, which may finally cause apoptotic death of DA neurons. Additionaly, oxidative stressors may be provoked by cytokine induced activation of the low-affinity IgE receptor Fc epsilonRII/CD23 on glial cells. Indeed, cytokines were reported to stimulate Fc epsilonRII/CD23 expression, which resulted in the induction of iNOS (with the subsequent release of NO) and TNF-alpha in substantia nigra glial cells of PD patients [69]. The activation of iNOS, which mediates the synthesis of high levels of NO, is supposed to be the key intermediate to produce apoptosis of DA neurons [105]. Activated microglia produce ROS and NO *via* induction of NADPH oxidase and iNOS and release ROS, NO, and H_2O_2, which may mediate DA cell injury. Accordingly, MPTP treated mice displayed elevated NADPH oxidase activity that was shown to regulate oxidative stress induced injury [157]. Thus, the immune/inflammatory pathology and oxidative stress caused by induction of iNOS and NADPH oxidase may be tightly linked in PD, and activated microglia may play an important deleterious role in propagating and amplifying oxidative neuronal injury and possibly even in initiating such injury [153].

In mesecephalic-microglia co-culture models, both LPS as well as IgGs from patients with PD activated microglia and provoked DA neuronal death [158]. However, PD IgGs only provoked neuronal demise in the presence of DA quinone- or H_2O_2-modified DA cell membranes, suggesting possibly important interactions between the IgGs and altered DA epitopes. Importantly, activated microglia released several proinflammatory cytokines, ROS, H_2O_2 and NO, which appeared to mediate the DA cell injury [158]. It is likely that PD IgGs activate Fc receptors on microglia, thereby promoting glial responding against DA neurons. Initially, the reactive IgGs may arise in response to altered DA epitopes associated with ongoing degeneration in PD.

As already alluded to, PD has been associated with an induction of MHC-I and MHC-II and increased expression of complement components, HLA-DR antigens, and Fc epsilonRII/CD23 in glial cells [12, 69]. We have also demonstrated increased beta2-microglobulin expression, i.e., the light chain of MHC-I, specifically in the striatum in PD patients [14]. Since TNF-alpha levels (which may induce beta2-microglobulin) were also increased in the striatum, it may be the case that a local immune reaction may occur within the nigrostriatal region in PD. Indeed, MHC expression of microglia enable these cells to present antigen to any infiltrating lymphocytes as well as elicit respiratory oxidative activity. Although immune responses are kept to a minimum in the healthy brain by active neuronal inhibition of such processes [159], microglia with MHC-regulated antigen-presenting capacity induced in response to degeneration may further precipitate DA loss. Further, increased proinflammatory cytokine expression would further serve to stimulate microglia MHC expression and phagocytosis, thereby perpetuating a cycle of inflammation and degeneration.

Neurotoxins such as MPTP/MPP^+ can produce apoptotic cell death in the PD animal models and this effect may be mediated by inflammatory glia. Indeed, human microglia express CD95 (Fas) ligand (CD95L) and can induce apoptosis in CD95-expressing target cells *in vitro* [160]. However, the issue of apoptotic cell death of the DA neurons in the PD brain is still controversial. Apoptotic cells in postmortem brain in PD have been demonstrated using histochemistry, however, the numbers of such cells are admittedly few [20, 21, 161]; and in one report, they were undetectable [162]. Furthermore, the intracellular signaling pathway causing the deleterious effects of activated microglia leading to the apoptotic cell death of DA neurons in the substantia nigra in PD is not yet clearly understood. The tumor suppressor protein p53 plays a pivotal function in neuronal apoptosis triggered by oxidative stress. However, oxidative stress induces p53-mediated apoptosis also in glial cells, although the signaling pathway may differ between neural and glial cells. Pifithrin-alpha (PF-alpha), which has been reported to protect neurons from ischemic insult by specifically inhibiting p53 DNA-binding activity, was unable to prevent oxidative stress-induced apoptosis of astrocytes. In astrocytes p53, acting *via* caspase-dependent and transcription-independent pathways, mediated an apoptotic response by directly promoting mitochondrial release of cytochrome c release and nucleosomal fragmentation [163].

Cytokines, such as TNF-α, released from microglia can activate the apoptotic pathway *via* intracytoplasmic death domains on DA neurons. In fact, caspase-3 and caspase-8, which are the effectors of apoptosis, are activated in PD. However, caspase inhibitors or blockade of the TNF-alpha or IL-1beta receptors has not yielded substantial protection of DA neurons against degeneration in experimental models of PD. Perhaps, manipulation of a single signaling pathway may not be sufficient to completely protect DA neurons. Neuroprotection strategies for PD may require a poly-therapy acting on different cell-death pathways to block the degeneration process of the DA neurons, as in the case of poly-therapies used in the clinical treatment of AIDS [163].

It should be realized that the glial response in PD might not only be toxic to the DA neurons, but also neuroprotective in certain cases owing to the secretion of neurotrophic factors that can buffer against ROS and gluatamate toxicity. In fact, delivery of GDNF by neural stem cells induced neuroprotection in a mouse model of PD [74] and GDNF expressed by viral vectors prevented DA neuron cell death in primate models of PD *in vivo* [76] or in mecencephalic cells *in vitro* [77]. Interestingly, Sawada *et al.* [164] discovered that intravenous injection of immobilized microglial cells promoted brain-specific gene expression, which may make these cells useful for the delivery of GDNF into the nigrostriatal region.

In order to address the question as to whether microglial activation is neurotoxic or neurotrophic [165] *in vivo* in PD, we examined activated microglia in the autopsy brain from PD patients by immunohistochemistry using HLA-DR antibody. We found 2 types of activated microglia, one associated with and one without neuronal degeneration; the former was found in the nigrostriatum; and the latter, in the hippocampus and cerebral cortex. We observed the expression of genes related to pro-inflammatory cytokines in the nigrostriatum, but not in the cerebral cortex or hippocampus.

In contrast, increased gene expression of neurotrophic growth factors such as BDNF, GDNF, or TGF-beta was observed in the hippocampus. We also found that in postmortem brains from early stage-PD patients double-stained for microglia and TH, such that the activated microglia were in close contact with TH-positive nerve fibers [166]. We speculate that since TH staining was intense in these nerve fibers, some neuroprotective role may be played by microglia, at least in early stages of disease and in regions outside the nigrostriatal pathway. Along these lines of Sawada and coworkers [166], Hirsch *et al.* [167] also proposed separate popula-tions of microglia. One subpopulation of glial cells may play a neuroprotective role by metabolizing DA and scavenging oxygen free radicals and another that may be deleterious to DA neurons by producing NO and pro-inflammtory cytokines.

Thus, immune responses by activated microglia in the nigrostriatal region in PD patients appear to be either neuro-protective or neurotoxic depending upon the circumstances present at a particular time during the progression of the disease. Activated microglia may be neuroprotective at least at an early stage of PD, but may assume a deleterious role after chronic activation over the course of disease. The fragments of DA neurons produced by apoptosis of DA neurons may be removed by phagocytosis by activated microglia. Phagocytosis of damaged DA neurons by microglia would be an important process in PD pathology.

7.2. Roles of Astrocytes in Neuroinflammation

In addition to microglia, astrocytes may contribute, although to a lesser extent, to the neurodegenerative process in PD [168]. Along these lines, astrocytes can express MHC class II antigens and can produce a wide range of cytokines. The presence of reactive astrocytes often leads to substantial neuronal degeneration in the injured adult brain [169]. Although astrocyes typically release neurotrophins or small antioxidants with free radical-scavenging properties (GSH, ascorbic acid, GDNF, BDNF, NGF, bFGF), in certain disease conditions they may produce toxic products such as NO, pro-inflammatory cytokines [170]. Thus, altered astro-cyte function might also contribute to nigral degeneration in PD [171]. Indeed, neuroprotection of mesencephalic DA neurons elicited by cyclic AMP may require the repression of astrocytes through inhibition of cyclin-dependent kinase 1 (CDK1) [172].

In terms of a protective role for astrocytes, de Bernardo *et al.* [173] reported that mesencephalic astroglia-condi-tioned medium greatly increased the expression of the DA phenotype and protected cells from spontaneous or neuro-toxin-induced death through a complex signaling network involving protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulation kinase (ERK)/ mitogen-activated protein kinase (MAPK), and phos-phatidylinositol-3 kinase (PI-3K). Thus, neuroprotection and differentiation of DA neurons mediated by astroglia may require the activation of PKA, PKC, ERK/MAPK, and PI-3K pathways. Astrocytes also influence the formation and maintenance of the blood-brain-barrier. *In vivo*, astrocyte foot processes are in close apposition to the abluminal surface of the microvascular endothelium of the blood-brain-barrier [174].

8. INTERACTIONS BETWEEN GLIA AND DOP-AMINE NEURONS *VIA* CYTOKINES IN INFLA-MMATORY PROCESS OF PARKINSON'S DISEASE

Communication between neurons and glia plays a key role in shaping the quiescent and reactive states of microglia. The demonstration of numerous receptors for brain signaling molecules (neurotransmitters, neuropeptides, ATP) and neurotophins on microglial cells has suggested that these cells not only monitor but are also under the strict control of the neurochemical environment. Given the heterogeneity of neuronal populations within distinct neuroanatomical regions, the effects of the neurochemical environment on microglia are site-specific, and this could account for differences in the degree of microglia activation and inflammatory reactions in different brain regions [175, 176]. Therefore, the interaction between the nigrostriatal DA neurons and activated microglia in PD may have complex sequences, resulting in neurotoxicity or neuroprotection. It is important that we obtain a better understanding of the neuron-glia communication that takes place during the development of PD.

Use of anti-inflammatory drugs may prove to be a viable adjunct treatment strategy to augmnent the efficacy of current pharmacological regimens for PD. Indeed, ongoing clinical trials for AD have suggested a tremendous potential for such compounds. Furthermore, animal models have shown the anti-inflammatory drugs, pioglitazone, a PPAR (peroxisome proliferators-activated receptor)-gamma agonist, and minocycline, a tetracycline derivative, provide clinical benefit for neurotoxin treated mice [151]. In summary, microglial cells may thus regulate tissue changes that confer either harm or benefit, depending on the specific circumstances. A hypothetical scheme of neuroinflammation in PD is shown in Fig. (**3**).

CONCLUSIONS

Recent discoveries of the causative genes and the products of familial PD, such as alpha-synuclein, parkin, UCH-L1, and DJ-1, suggest that the neuronal death in PD as well as in other neurodegenerative diseases such as AD may be "protein conformational diseases" caused by accumula-tion of disease-characteristic misfolded proteins that have adopted some non-native conformation often rich in beta-sheets. However, most (about 95%) cases of PD or AD are sporadic, without any finding of mutated causative genes. Recent findings of increased levels of pro-inflammatory cytokines and reduced neurotrophin levels accompanied by inflammatory responses in the nigrostriatal regions in sporadic PD rasise the possibility that immunogenic pro-cesses are fundamental to PD.

Although sporadic and familial PD are thought to be different with respect to the initial trigger, they might have in common apoptotic neuronal death as the final event, reached by multiple signaling pathways. Inflammatory response accompanied by glial activation and cytokine release may be neuroprotective in the early stages of PD but exacerbate pathology later in the disease. In this respect, Swada and colleagues [165] recently proposed different microglial populations that may act in either a protective or destructive capacity, depending upon the cytokines and other factors

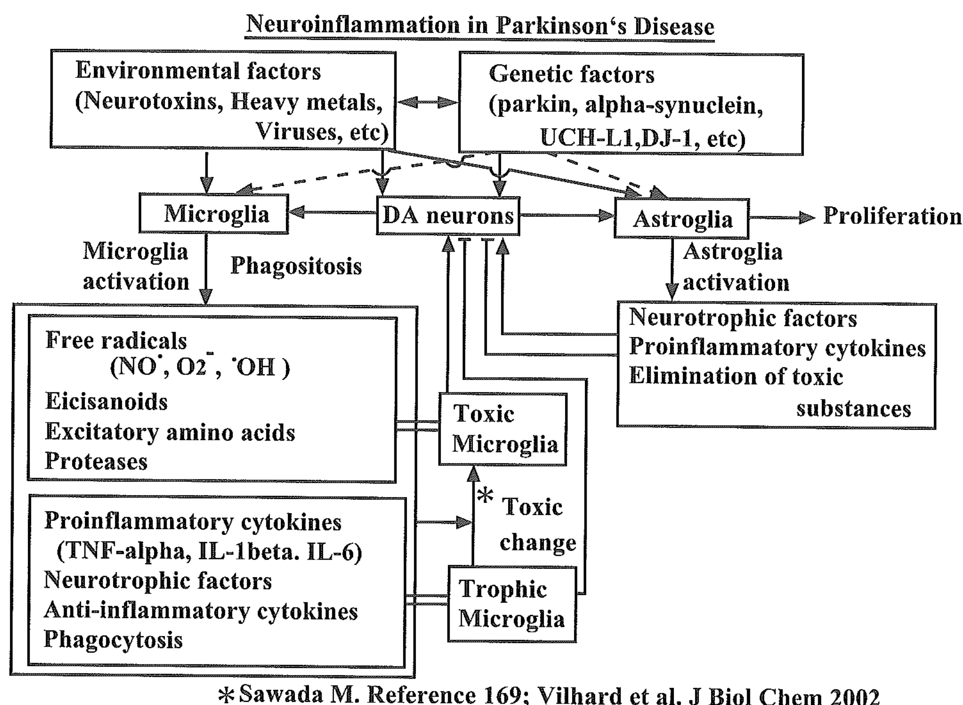

Fig. (3). Hypothetical scheme depicting neuroinflammation and interactions among the triggers, dopamine (DA) neurons, and glial cells in Parkinson's disease (PD).

Lines: possible relations; dotted lines: unsettled relations.

released within the microenvironment. A more comprehensive understanding the neuroinflammatory response and the roles of cytokines in PD should prove to be useful for the designing of new drugs to prevent or protect against the neurodegenerative process that occurs in the disease.

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Rabies Virus-Induced Activation of Mitogen-Activated Protein Kinase and NF-κB Signaling Pathways Regulates Expression of CXC and CC Chemokine Ligands in Microglia

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Following virus infection of the central nervous system, microglia, the ontogenetic and functional equivalents of macrophages in somatic tissues, act as sources of chemokines, thereby recruiting peripheral leukocytes into the brain parenchyma. In the present study, we have systemically examined the growth characteristics of rabies virus (RV) in microglia and the activation of cellular signaling pathways leading to chemokine expression upon RV infection. In RV-inoculated microglia, the synthesis of the viral genome and the production of virus progenies were significantly impaired, while the expression of viral proteins was observed. Transcriptional analyses of the expression profiles of chemokine genes revealed that RV infection, but not exposure to inactivated virions, strongly induces the expression of CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5) in microglia. RV infection triggered the activation of signaling pathways mediated by mitogen-activated protein kinases, including p38, extracellular signal-regulated kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase, and nuclear factor κB (NF-κB). RV-induced expression of CXCL10 and CCL5 was achieved by the activation of p38 and NF-κB pathways. In contrast, the activation of ERK1/2 was found to down-regulate CCL5 expression in RV-infected microglia, despite the fact that it was involved in partial induction of CXCL10 expression. Furthermore, NF-κB signaling upon RV infection was augmented via a p38-mediated mechanism. Taken together, these results indicate that the strong induction of CXCL10 and CCL5 expression in microglia is precisely regulated by the activation of multiple signaling pathways through the recognition of RV infection.

Microglia, the ontogenetic and functional equivalents of macrophages in somatic tissues (10), exert a central role in immune surveillance and host defense against infectious agents in the central nervous system (CNS) (50). Microglia act as scavengers (phagocytes) and antigen-presenting cells in the CNS, control the proliferation of astrocytes, and produce soluble factors associated with an immunologic response (16, 59). Under normal conditions, microglia exist in a quiescent state lacking many of the effector functions and receptor expression patterns observed in macrophages within other tissues. However, in response to brain infection, microglia readily transform into an activated state, acquiring numerous if not all of the macrophage properties required to launch effective immune responses (2). Upon activation, microglia respond to viral infections through a highly regulated network of cytokines and chemokines, which subsequently facilitate the recruitment of peripheral leukocytes into the CNS and orchestrate a multicellular immune response against the infectious agent (2).

Leukocyte recruitment into the CNS is a multistep process that can be mediated by chemokines. Chemokines are low-molecular-weight and structurally related molecules that are divided into four subfamilies, designated C, CC, CXC, and CX_3C chemokine ligands based on the positions of their cysteine residues (65). These molecules control trafficking and recirculation of the leukocyte population among the blood vessels, lymph, lymphoid organs, and tissues, a process important in host immune surveillance and in acute and chronic inflammatory responses (51). A growing body of evidence suggests that CNS-resident cells secrete various kinds of chemokines upon injury or infection and that peripheral leukocytes, such as lymphocytes, monocytes, and natural killer cells, transmigrate toward the chemokine gradient, cross the blood-brain barrier, and gain access to the brain parenchyma (11).

Expression of most chemokines is regulated primarily at the level of transcription through activation of a specific set of transcription factors, such as nuclear factor κB (NF-κB) and interferon (IFN) regulatory factors (32). It has also been shown that signal transduction pathways mediated by the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38, contribute to the activation of transcription factors (22, 33). ERK1/2 is activated primarily by stimulation with growth factors, cytokines, and phagocytosis, while p38 and JNK are involved in the MAPK activation induced by environmental stresses, such as bacterial endotoxins, proinflammatory cytokines, osmotic shock, UV irradiation, and virus infections (15, 28).

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Rabies virus (RV) is a negative-strand RNA virus belonging to the *Rhabdoviridae* family, genus *Lyssavirus*. Most RV strains are highly neurotropic, which usually results in a fatal infection in warm-blooded animals, and viral replication occurs primarily in neurons as a cellular target (27). In vitro, it has also been reported that some RV strains can infect nonneuronal CNS-resident cells, including microglial cells (47, 49, 60). Considering the cell tropism of RV, which is restricted to neurons, it is unlikely that microglia support the productive replication of RV in the infected CNS. However, based on the previous data showing that virions and viral antigens are detected in microglia in the RV-infected brain (60), it is possible that microglial cells can engulf RV virions released from the infected neurons. Recently, we have shown that endocytosis of inactivated RV virions, as well as infectious viruses, triggers the activation of the ERK1/2-mediated signaling cascade, leading to chemokine expression in cells of the macrophage lineage, despite the fact that these cells have extremely low susceptibility to RV infection (35). From these lines of evidence, we postulated that RV infection of microglia might activate an undefined signaling pathway that leads to chemokine production. Still, the molecular mechanism underlying cellular responses of microglia to RV infection remains totally unknown.

In the present study, we have systemically examined the growth characteristics of RV in microglia and the activation of cellular signaling pathways leading to chemokine expression. We demonstrate that the viral genome synthesis and the production of progeny virus are significantly impaired in microglia, while RV can enter these cell types and express viral proteins. We also show that RV infection of microglia strongly induces the gene expression and protein production of two chemokines, CXC chemokine ligand 10 (CXCL10) and CC chemokine ligand 5 (CCL5). Furthermore, our data indicate that the RV-induced production of CXCL10 and CCL5 is positively and negatively regulated by the activation of cellular signaling pathways mediated by p38, ERK1/2, and NF-κB.

MATERIALS AND METHODS

Reagents and antibodies. Highly purified bovine serum albumin (fatty acid free), 4′,6-diamidino-2-phenylindole (DAPI), 1,4-diazabicyclo-2,2,2-octane (DABCO), and bafilomycin A1 (BA1) were purchased from Sigma (St. Louis, MO). Chemical inhibitors and inactive analogues, U0124, U0126, SB202190, SB202474, BAY 11-7082, and caffeic acid phenethyl ester (CAPE), were obtained from EMD Biosciences, Inc. (San Diego, CA). SP600125 was purchased from Biomol (Plymouth Meeting, PA). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) specific for RV nucleoprotein (N) were obtained from Centocor, Inc. (Malvern, PA). Rabbit antibodies against inhibitory NF-κB α (IκBα), the p65 subunit of NF-κB, phosphorylated ERK1/2 (p-ERK1/2), and phosphorylated JNK (p-JNK) were purchased from Santa Cruz Biotechnology (Hercules, CA). Antibodies specific for ERK1/2, p38, JNK, and α-tubulin, as well as horseradish peroxidase- or FITC-linked secondary antibodies, were purchased from Sigma. Antibodies against phosphorylated forms of p38 (p-p38) and IκBα (p-IκBα) were obtained from New England Biolabs (Beverly, MA). Granulocyte macrophage colony-stimulating factor was obtained from Genzyme (Cambridge, MA).

Cells. A murine microglial cell line, Ra2, was established by spontaneous immortalization of primary microglia from normal brain tissue (53, 61). Ra2 cells closely resemble primary microglia with respect to morphology, phagocytic functions, expression of microglia-specific molecules, and high migrating activity to the brain (18, 21, 53, 61). Ra2 cells were cultivated in Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), insulin (5 μg/ml), 0.2% glucose, granulocyte macrophage colony-stimulating factor (2 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Murine neuroblastoma (NA; C1300 clone) cells were cultured in RPMI1640 medium containing 10% FCS and the above-named antibiotics. All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2 in air.

Viruses. The highly neurovirulent RV strain, challenge virus standard-11 (CVS-11), and the attenuated strain high egg passage (HEP)-Flury were propagated in NA cells as previously described (57). Preparation of RV virions was performed as described before (35). Briefly, virions in the culture supernatant of RV-infected NA cells were purified by polyethylene glycol (no. 6000) precipitation, followed by sucrose density ultracentrifugation. Finally, RV virions were further purified and resuspended in EMEM by using ultrafiltration with an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA) according to the manufacturer's instructions. Virus titers were determined by a focal infectivity assay using the FITC-coupled anti-N protein MAbs (35). Alternatively, purified virions were inactivated by UV irradiation for 15 min just prior to the experiments.

Immunofluorescence. Ra2 cells, which had been plated in 24-well culture plates (3×10^5 cells/well), were incubated with or without 2 focus-forming units (FFU) per cell of CVS-11 virus suspended in EMEM containing 0.5% FCS, 0.2% glucose, and the above-named antibiotics (hereafter called test medium) for 2 h at 37°C, washed, and overlaid with culture medium. Fluorescent staining of cultured cells was performed as described in previous papers (34, 36, 57). Briefly, the cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. The cells were stained with FITC-conjugated MAbs specific for viral N protein and with DNA dye (DAPI; 0.1 μg/ml). Samples were overlaid with a solution containing 90% glycerol, 2.3% DABCO, and 20 mM Tris-HCl (pH 8.0) and were examined under a fluorescence microscope (Eclipse TE200; Nikon, Tokyo, Japan). The percentages of N-positive cells were calculated with reference to the total cell number (more than 100 cells per sample). For indirect immunofluorescence of NF-κB p65, the fixed cells were labeled with anti-p65 antibodies and stained with FITC-coupled secondary antibodies and with DAPI. The samples were observed under a confocal laser scanning microscope (LSM510; Zeiss, Heidelberg, Germany).

Measurement of virus production. Cells were incubated with or without virus, washed, and overlaid with culture medium as described above. At different times after inoculation, culture supernatants were separated by centrifugation at 5,000 × *g* for 5 min and subjected to virus titration on NA cell monolayers.

Real-time PCR analysis of RV genome replication. In order to assess the replication of the viral RNA genome, cells were mock infected or infected with the CVS-11 strain of RV at a multiplicity of infection (MOI) of 2 (2 FFU/cell) as described above. After incubation for the indicated times, total RNA was isolated from the 1.5×10^6 cells by using an RNA extraction reagent (Isogen; Nippon Gene, Tokyo, Japan), and the first-strand cDNAs were generated by reverse transcription (RT) reaction as described previously (35). In order to obtain cDNAs from RV genome and cellular mRNAs, the RT reaction was performed by using an oligonucleotide primer termed N5-a (57) and oligo(dT) primers, respectively. PCR primers for the amplification of GAPDH (glyceraldehydes-3-phosphate dehydrogenase) cDNA as an internal control of RNA preparation have been published elsewhere (38). A pair of primers termed SYBR RV1 (5′ TCAGGGCTGGTATCGTTTACAGGG 3′) and SYBR RV2 (5′ GGATGAAATAAGAGTGAGGAACAGC 3′) was designed to amplify the viral cDNA sequence, and their specificity was confirmed by using a plasmid which contains viral genomic cDNA from RV-infected cells as a PCR template (data not shown). Real-time PCR was performed on each cDNA preparation using the above-mentioned primers, a LightCycler DX400 (Roche, Penzberg, Germany), and LightCycler DNA Master SYBR Green I (Roche) according to the manufacturer's protocol. The relative amounts of viral cDNAs were normalized with reference to those of GAPDH cDNAs.

Measurement of cell viability. Cell viability of the RV-infected cells was determined as described in the previous report (34). Briefly, Ra2 cells were mock infected or infected with CVS-11 virus at an MOI of 2 (2 FFU/cell) and stained with trypan blue solution at a final concentration of 0.2% (wt/vol). The percentages of cells excluding trypan blue dye were calculated with reference to the total cell number (more than 50 cells per sample).

RT-PCR analysis of chemokine gene expression. Semiquantitative RT-PCR analysis to determine the transcription profiles of chemokine genes has been described in the previous report (35). Ra2 cells were plated at a density of 3×10^6 in the 60-mm-diameter dishes and incubated in test media with or without CVS-11 virus (2 FFU/cell), which had been untreated or inactivated with UV irradiation, for 2 h at 37°C. The cells were then washed, overlaid with test media, and further incubated at 37°C. After a 20-h incubation period, total RNA was extracted, and then the first-strand cDNAs were obtained by using the oligo(dT) primer as described above. Oligonucleotides were synthesized on the basis of the

published primers for cDNA amplification of chemokines (25, 29, 38), and their specificities and optimum PCR conditions have been described previously. To control for possible contamination of genomic DNAs in total RNA extracts, primer sequences for GAPDH were separated by introns so that the amplified products from genomic DNAs would be longer than the amplified cDNAs (38). The absence of contaminating DNA was also verified by PCR using RNA preparations not treated with reverse transcriptase. PCRs were performed with serially fivefold-diluted cDNA preparations, and the amplified products were separated by using 1% agarose gel electrophoresis and visualized by ethidium bromide staining (35).

Measurement of chemokine production. The protein levels of CXCL10 and CCL5 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (35). Ra2 cells were plated at a density of 3×10^6 in the 60-mm-diameter dishes and incubated in the absence or presence of RV, which had been untreated or inactivated with UV irradiation for 1 h at 37°C. The cells were washed and overlaid with test medium, and then the culture fluids were separated after incubation of the cells for the appropriate times. The protein levels of CXCL10 and CCL5 were determined by using a Quantikine mouse IP-10 immunoassay kit (R&D Systems Inc., Minneapolis, MN) and a mouse RANTES ELISA kit (BioSource International, Inc., Camarillo, CA), respectively. As to the inhibition of vacuolar pH acidification, cells were preincubated in the presence of BA1 for 1 h at 37°C at a concentration that did not cause any cytotoxicity and were infected with CVS-11 virus. The cells were incubated in the test media with or without BA1, and the protein levels of CXCL10 and CCL5 were determined as described above.

Western blot analysis. The activation of MAPK and NF-κB pathways were measured by immunoblotting using antibodies against phosphorylated forms of ERK1/2, p38, JNK, and IκBα as described elsewhere (35). Ra2 cells were incubated in test media with or without RV virions (2 FFU/cell), which had been untreated or inactivated with UV irradiation, washed, and overlaid with test media as described above. At the appropriate time points, the cells were washed with PBS and lysed directly with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Complete Mini; Roche), and phosphatase inhibitor cocktails (Sigma). Extracts were clarified by centrifugation at 12,000 × g for 20 min at 4°C. Each sample, containing 10 μg of proteins, was separated under reducing conditions in 0.4% sodium dodecyl sulfate-12% polyacrylamide gels and was transferred to polyvinylidene difluoride membranes (Millipore). The blots were blocked for 1 h with 2% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.1% Tween 20 (TBST) and incubated with the primary antibodies in TBST. The proteins were reacted with the horseradish peroxidase-linked secondary antibodies and visualized with enhanced-chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) and photographed using an enhanced-chemiluminescence mini camera (Amersham Biosciences). For quantification of IκBα phosphorylation, the digital images of each blot were prepared, and the band densities were measured by using Scion Image (Scion Corp., Frederick, MD) according to the manufacturer's recommendations.

Inhibition of cellular signaling pathway. Inhibition of MAPK and IκB–NF-κB signaling in microglia was carried out as described elsewhere (19, 62, 64). Briefly, cells were incubated for 1 h at 37°C in test media containing U0126, SB202190, SP600125, BAY 11-7082, or CAPE just prior to the experiment and were subjected to the above-mentioned analyses in the presence of these inhibitors. Under the assay conditions, these inhibitors did not induce any cytotoxic effects as judged by a dye exclusion test using trypan blue (34).

Statistics. The significance of differences between groups was statistically determined by Student's *t* test.

RESULTS

Infection of microglia by RV. Previous studies demonstrated that some RV strains are able to infect cultured microglia and that the efficiency of viral multiplication in infected cells depends on virus strains (49). Although an attenuated Evelyn-Rotnycki-Abelseth strain, which had been adapted to tissue culture, grew well in primary microglia, the productive replication of pathogenic RV, such as a street isolate of bat origin, was strictly restricted in these cell types (49). We initially examined the overall growth performance of CVS-11, a highly neurovirulent laboratory strain of RV, in microglial Ra2 cells (Fig. 1). Figure 1A shows the expression pattern of the viral N protein, which accumulates in the cytoplasm of RV-infected cells, in microglia. Cells were inoculated with CVS-11 virus at an MOI of 2 (2 FFU/cell), and the viral N protein and cell nuclei were stained with anti-N MAbs and with the DNA dye DAPI, respectively, at 24 h postinfection. As shown in Fig. 1A (right), the majority of the RV-inoculated Ra2 cells exhibited a strong signal of the N protein, while anti-N MAbs did not react with the mock-inoculated cells (Fig. 1A, left). Figure 1B shows the percentages of N-positive cells during the course of viral infection of microglial Ra2 and neuroblastoma NA cells. When Ra2 cells were inoculated with CVS-11, the fluorescent signal of N proteins was observed in about 80 and 90% of the total cell population at 24 and 48 h postinfection, respectively (Fig. 1B). The plating efficiency of CVS-11 in Ra2 cells was at a level similar to that seen in neuronal NA cells, and its infectivity was readily diminished by UV irradiation (Fig. 1B). These data suggest that CVS-11 virus is capable of entering microglial cells and that virus-encoded proteins were expressed in these cell types.

Defective growth of RV in microglia. We next assessed the growth characteristics of CVS-11 virus in Ra2 cells. The cells were inoculated with virus, and the culture supernatants were subjected to virus titration (Fig. 1C). In either cell type, low levels of infectious virions, which might be detached from the cells, were detected in culture supernatants at 0 h postinfection. In neuronal NA cells, CVS-11 exhibited a marked increase in virus titers, reaching a near-plateau level at 24 h postinfection (Fig. 1C). In contrast, virus titers in culture supernatant fluids from RV-inoculated cells at 24 and 48 h postinfection were slightly higher than those at 0 h postinfection, and these differences were not statistically significant, suggesting that the production of virus progenies of CVS-11 in microglia is severely impaired. To further assess a defective growth of RV in microglia, Ra2 cells were infected with CVS-11, and the relative amounts of negative-strand RV genome in the infected cells were determined by real-time PCR analyses. As shown in Fig. 1D, the amounts of viral genome in RV-infected microglia at 20 and 40 h postinfection were about 1.3- and 1.2-fold higher than that at 0 h postinfection, respectively, but these differences were not statistically significant, indicating that the viral genome synthesis of CVS-11 is impaired in microglia. In the experiments shown in Fig. 1E, the viability of microglia infected with CVS-11 was examined by using a dye exclusion assay as described above. The dye exclusion capability of RV-infected cells was at the level similar to that of mock-infected cells at all the time points tested. These observations indicate that CVS-11 infection does not induce any cytotoxicity in microglia.

RV infection stimulates chemokine gene expression in microglia. Recently, we have shown that endocytic processing of inactivated RV virion, as well as infectious virus, triggers the activation of a cellular signaling pathway, leading to the selective expression of the chemokine CXCL10 in macrophages (35). To examine whether RV selectively induces CXCL10 expression in microglia, Ra2 cells were inoculated with live CVS-11 virus or exposed to UV-inactivated virion, and the expression profiles of multiple chemokine genes were examined by RT-PCR 20 h after incubation. Figure 2 shows the transcription patterns of two major chemokine subfamilies,

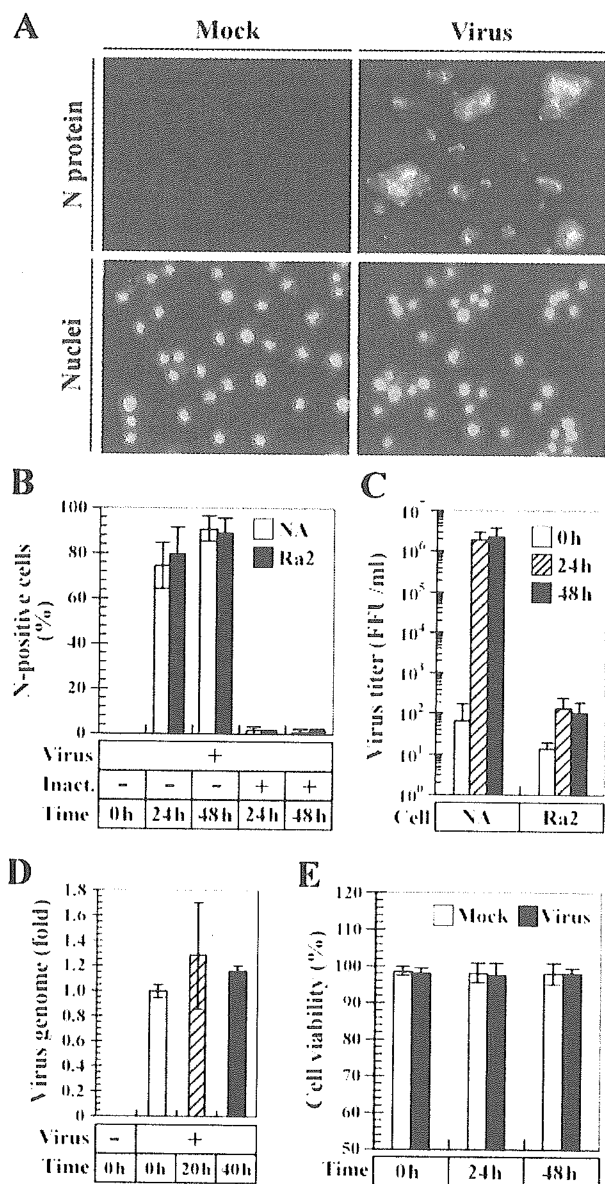

FIG. 1. Growth characteristics of RV in microglia. (A) Indirect immunofluorescence assay of viral N protein in RV-inoculated cells. Microglia were incubated with CVS-11 virus and then further incubated. After 24 h of incubation, the cells were fixed and stained for N protein (top) and cell nuclei (bottom). The experiments were repeated three times, and representative areas of each culture are shown. Magnification, ×200. (B) Infectivity of CVS-11 virus in microglia. Neuroblastoma (NA) and microglial (Ra2) cells were incubated with or without CVS-11 virus, which had been untreated or inactivated with UV irradiation (Inact.). After incubation for the indicated times, the percentages of N-positive cells were determined with reference to the total cell number as described in the text. The values are averages from three independent experiments, and the error bars indicate standard deviations. (C) Production of progeny virus in RV-inoculated microglia. Cells were infected with CVS-11 virus, and immediately thereafter (0 h) and at the indicated time points, virus progenies in culture supernatant fluids were titrated. The data are averages from three independent experiments, and the error bars indicate standard deviations. (D) Replication of the viral genome in RV-infected microglia. Ra2 cells were mock infected or infected with CVS-11 virus, and the relative amounts of viral cDNA were measured by using real-time PCR analyses immediately thereafter (0 h) or 20 and 40 h after infection. The results are shown as n-fold increases in the amount of viral cDNA with reference to the cDNA levels in RV-inoculated cells at 0 h postinfection. Mean values and standard errors from the results of three independent experiments are shown. (E) Viability of microglia following RV infection. Cells were mock infected or infected with CVS-11 virus, and the percentages of cells excluding trypan blue dye were calculated with reference to the total cell number at the time points indicated. Values are averages from three separate experiments, and the error bars represent standard deviations.

CXC and CC of chemokine ligands in microglia. When Ra2 cells were inoculated with infectious CVS-11 virus, the strong signals of amplified products of CXCL10, CCL4, and CCL5 were observed at highly diluted concentrations of PCR templates compared to those of mock-inoculated cells, while CVS-11 infection had little or no effect on the expression of CCL2 and CCL3. In particular, the expression of CXCL10 and CCL5 genes in RV-infected microglia was approximately 3,000-fold higher than that in mock-infected cells. We also examined the transcription patterns of the other chemokines, such as CXCL2, CXCL9, CXCL11, and CX_3CL1, in RV-infected microglia, but the expression levels of these chemokine genes were not affected or were less affected by RV infection (data not shown). The chemokine expression patterns in Ra2 cells, which had been exposed to UV-inactivated RV virions, were similar to those seen in mock-treated cells. Thus, these results indicate that RV infection, but not exposure to inactivated virions, greatly induces the expression of CXCL10 and CCL5 in microglia. To assess whether the enhanced expression of CXCL10 and CCL5 mRNAs in RV-infected cells correlates with protein production, we measured the levels of CXCL10 (Fig. 2B) and CCL5 (Fig. 2C) production in microglia upon RV infection. When Ra2 cells were infected with CVS-11 virus, the onsets of chemokine production were detected 10 h after infection, and the amounts of chemokines increased steeply between 10 and 15 h after infection, reaching near-plateau levels at 20 h postinfection. In the experiments shown in Fig. 2D and E, we examined whether virion uncoating is required for the RV-induced expression of CXCL10 and CCL5 in microglia. BA1 is a selective and potent inhibitor of vacuolar H^+-ATPase, prevents endosomal acidification (13), and is applicable to the suppression of an acid-induced fusion of lyssaviral envelope with cell membrane (24). As shown in Fig. 2D, when Ra2 cells were treated with BA1 ranging from 60 to 240 nM and then infected with CVS-11, the virus infectivity was significantly impaired in a dose-dependent manner. Figure 2E shows the RV-induced expression of CXCL10 and CCL5 in BA1-treated Ra2 cells. Pretreatment with BA1 at concentrations of 120 and 240 nM significantly diminished the expression of either chemokine in RV-infected Ra2 cells, suggesting that the virion uncoating is necessary for RV-induced chemokine expression in microglia. In order to examine whether viral gene expression is required for the RV-induced chemokine expression in microglia, we compared the chemokine production in Ra2 cells, which had been exposed to UV-inactivated virions, to that in RV-infected cells. As shown in Fig. 2F and G, the CVS-11-induced expression of CXCL10 and CCL5 was nearly completely abrogated by UV inactivation of virions. To examine whether the RV-induced chemokine expression depends on virus strains, we measured the expression of CXCL10 and

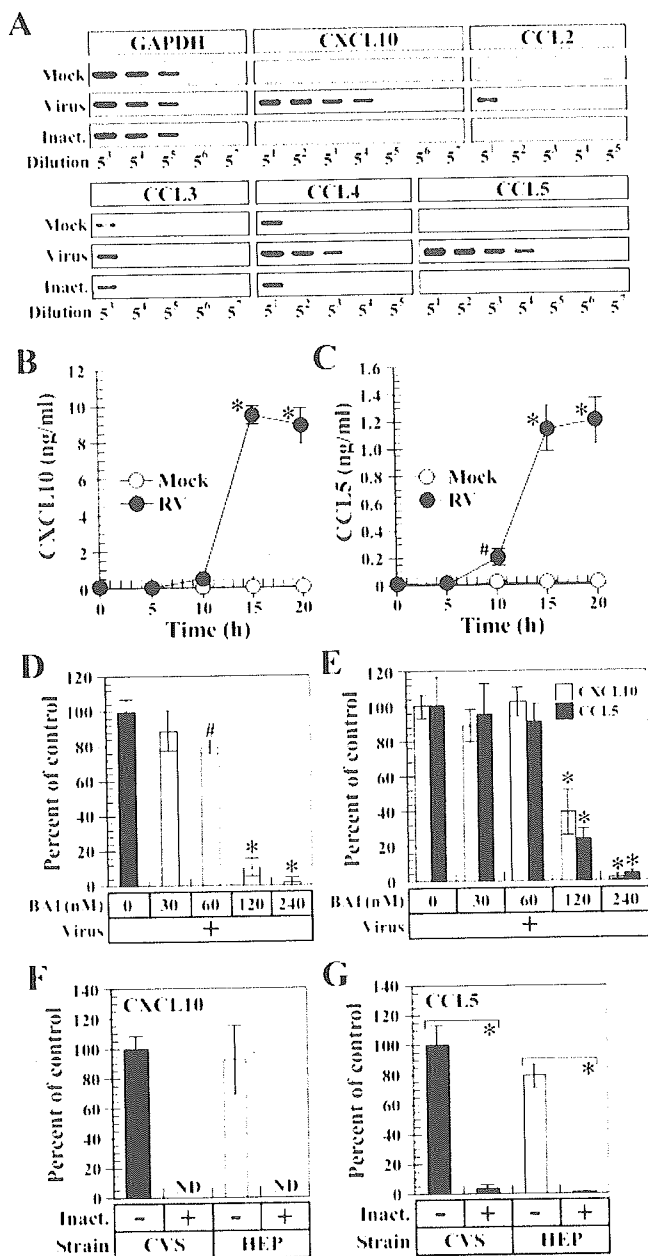

FIG. 2. Chemokine responses in microglia against RV infection. (A) Transcription patterns of chemokine genes in microglia following RV infection. Cells were incubated in the absence (Mock) or presence of infectious (Virus) or UV-inactivated (Inact.) CVS-11 virions and were subjected to RT-PCR analyses for the chemokine gene expression by using serially fivefold-diluted cDNA preparations as described in the text. The data are from one of three separate experiments with similar results. (B and C) Time course of RV-induced chemokine production by microglia. Cells were mock infected or infected with CVS-11 virus. After additional incubation for the indicated times, the protein contents of CXCL10 (B) and CCL5 (C) in culture supernatants were determined by using ELISA. (D and E) Effects of lysosomotropic agents on virus infectivity and chemokine expression in microglia. Ra2 cells, which had been treated with the indicated doses of BA1, were infected with CVS-11 virus and were further incubated in the presence or absence of BA1. After a 24-h incubation period, virus infectivity (D) and chemokine expression (E) were determined as described in the text. The results are shown as percentages of virus infectivity and chemokine expression with reference to the values for the drug-untreated controls. (F and G) RV-induced expression of chemokines is dependent on viral gene expression. Ra2 cells were incubated with neurovirulent CVS-11 (CVS) and attenuated HEP-Flury (HEP), which had been untreated (−) or inactivated with UV irradiation (+). At 20 h postinfection, protein contents of CXCL10 (F) and CCL5 (G) were determined. The percentages of chemokine expression were calculated with reference to the protein levels in culture fluids of the CVS-11-infected cells. For each panel, mean values and standard deviations from three separate experiments are shown, and statistically significant differences are indicated by asterisks ($P < 0.01$) and pound signs ($P < 0.05$).

CCL5 in microglia infected with HEP-Flury, a tissue culture-adapted and highly attenuated RV strain. In Ra2 cells, the induction of CXCL10 (Fig. 2F) and CCL5 (Fig. 2G) production was also observed following incubation with infectious HEP-Flury virus but not with inactivated virions. Taken together, these data demonstrate that the RV-induced expression of CXCL10 and CCL5 is triggered at the stage after viral gene expression and is not dependent on the virus strains.

RV infection activates MAPK signaling in microglia. The enhanced production of CXCL10 and CCL5 in RV-infected microglia implies the possibility that RV infection may stimulate the cellular signaling pathway underlying chemokine expression. Considering the important roles of MAPK-mediated signaling pathways in host defense, we examined the activation of three major MAPK subfamilies, p38, JNK, and ERK1/2, in microglia upon RV infection (Fig. 3). In order to assess the activation of MAPK signaling pathways in microglia during the course of RV infection, cells were mock infected or infected with CVS-11, and the degrees of MAPK phosphorylation were examined by Western blotting. To avoid an additional effect of growth factors on MAPK activation, the cells were incubated in test medium containing extremely low concentration of FCS that did not induce MAPK phosphorylation. As shown in Fig. 3A, when microglia was infected with CVS-11 virus, the strong signals of p-p38 were detected between 10 and 15 h after infection (Fig. 3A, lanes 7 and 8). In RV-infected cells, the amounts of p-JNK1/2 and p-ERK1/2 were also increased between 10 and 20 h (Fig. 3A, lanes 7 to 9), and the maximal induction of either MAPK was seen at 15 h postinfection (Fig. 3A, lane 8). The increased levels of MAPK phosphorylation were not due to the enhanced production of these molecules or the difference in protein extracts loaded, as the protein levels of total amounts of MAPKs, as well as α-tubulin, in each sample were comparable. As shown in Fig. 3B, the RV-induced phosphorylation of each MAPK was also observed for microglia infected with attenuated HEP-Flury virus (Fig. 3B, lane 4). When virions were inactivated, RV-induced MAPK activation was nearly completely diminished (Fig. 3B, lanes 3 and 5). Thus, these data indicate that RV infection, but not exposure to inactivated virions, induces the activation of cellular signaling pathways mediated by multiple MAPK subfamilies, p38, ERK1/2, and JNK.

RV-induced MAPK activation regulates chemokine expression in microglia. Based on the above-mentioned results, we hypothesized that the activation of MAPK signaling pathways underlies the up-regulation of CXCL10 and CCL5 expression in RV-infected microglia. To examine this possibility, we assessed the effects of MAPK inhibitors on the induction of chemokine expression in RV-infected microglia. Ra2 cells

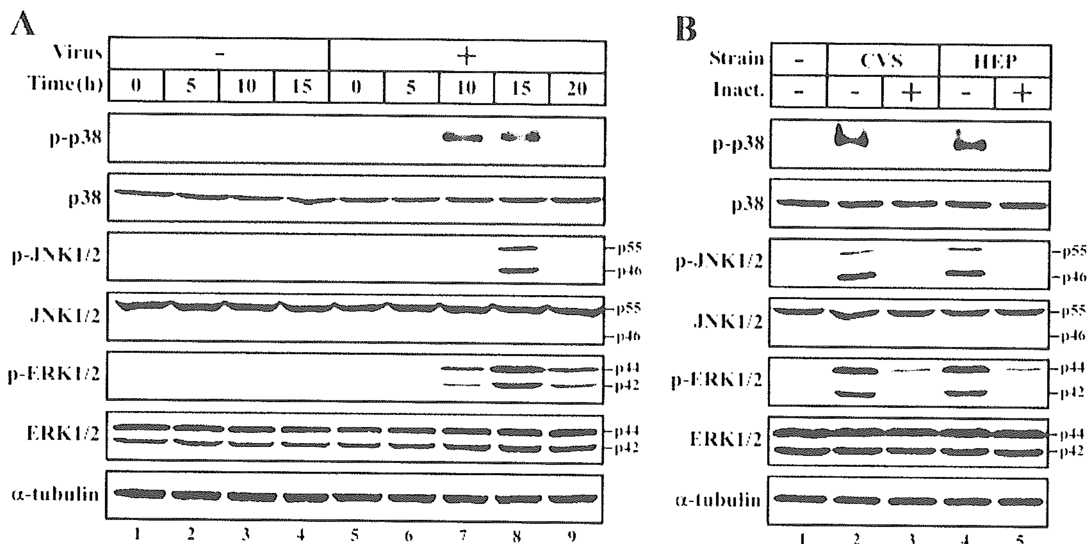

FIG. 3. RV infection activates MAPK signaling pathways in microglia. (A) Time course of MAPK activation in microglia following RV infection. Cells were mock infected (−) or infected with CVS-11 (+). At the indicated time points, cells were harvested, and equal amounts of protein extracts from each sample (10 μg/lane) were loaded on the gel. The protein levels of phosphorylated and total MAPKs were analyzed by Western blotting as described in the text. The amounts of α-tubulin were also assessed to monitor the equal loadings of protein extracts. (B) Viral gene expression is required for RV-induced MAPK activation. Cells were incubated with CVS-11 and HEP-Flury virions, which had been untreated (−) or inactivated with UV irradiation (+) (Inact.), washed, and further incubated for 15 h. The phosphorylation levels of MAPKs were examined as described for panel A. The data are from one of three separate experiments with similar results.

were pretreated with increasing doses of MAPK inhibitors and then infected with CVS-11 virus. Of the inhibitors tested, p38 inhibitor SB202190 significantly reduced the RV-induced expression of CXCL10 in a dose-dependent manner, and the maximal concentration of this compound (10 μM) diminished the expression level of CXCL10 by about 80% (Fig. 4A). Treatment with the ERK1/2 inhibitor U0126 resulted in a partial but statistically significant diminution of CXCL10 expression upon RV infection, while the JNK inhibitor SP600125 had little or no effect on the CXCL10 expression levels (Fig. 4A). These observations suggest that RV-induced CXCL10 expression is partly achieved through the activation of p38 and ERK1/2 pathways. Figure 4B shows CCL5 production in RV-infected microglia in the presence of MAPK inhibitors. The inhibition of p38 led to a considerable diminution of RV-induced expression of CCL5, and the amounts of CCL5 were not affected, or were less affected, by the treatment with JNK inhibitor (Fig. 4B). Interestingly, treatment with ERK1/2 inhibitor at concentrations ranging from 1 to 10 μM led to an up-regulation of CCL5 expression in RV-infected cells (Fig. 4B). These data suggest that the p38-mediated signaling pathway partly contributes to the RV-induced expression of CCL5 but that ERK1/2 activation participates in the down-regulation of CCL5 production in microglia. To examine the regulation of the RV-induced expression of CXCL10 and CCL5 through the p38 and ERK1/2 pathways in more detail, we measured chemokine production in RV-infected microglia under conditions in which both the p38 and ERK1/2 pathways were disrupted. In these experiments, SB202474 and U0124 (inactive analogues of SB202190 and U0126, respectively) were used to control the nonspecific effect of simultaneous treatments with SB202190 and U0126. As shown in Fig. 4C, simultaneous treatments with SB202190 and U0126 additively reduced the RV-induced CXCL10 expression compared to that with SB202190 and U0124 ($P < 0.05$) or with SB202474 and U0126 ($P < 0.01$) (Fig. 4C), suggesting that the p38 and ERK1/2 pathways individually facilitate CXCL10 expression in microglia in response to RV infection. Figure 4D shows RV-induced CCL5 expression in microglia, which had been treated with p38 and ERK1/2 inhibitors in combination. Under conditions in which both the p38 and ERK1/2 pathways were inhibited, CCL5 expression was at a level similar to that seen in microglia treated with p38 inhibitor alone, suggesting that ERK1/2 activation participates in the down-regulation of an excessive production of CCL5 in response to RV infection. In addition, CVS-11 virus was able to infect microglia normally even when the cells were treated with maximal doses of MAPK inhibitors tested (10 μM). Taken together, our findings indicate that the RV-induced expression of CXCL10 and CCL5 in microglia is positively and negatively regulated by the activation of the p38 and ERK1/2 pathways.

RV infection activates the IκB–NF-κB pathway in microglia. Analyses of the RV-induced expression of CXCL10 and CCL5 in the presence of MAPK inhibitors have revealed that the p38 and ERK1/2 pathways regulate the production of these chemokines. In particular, we found that p38 activation is responsible for the RV-induced expression of these chemokines. However, RV-induced chemokine expression was not completely blocked by MAPK inhibitors even at maximal concentrations that did not cause cytotoxicity. These lines of evidence imply that another signaling pathway may also contribute to the chemokine responses of microglia to RV infection. NF-κB plays critical roles in transcriptional regulation of numerous genes involved in host defense mechanisms (3). This transcription factor is normally found in the cytoplasm in a latent form associated with IκB, of which various isoforms exist (23). Fol-