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Biochemical Pharmacology 64 (2002) 781–788

Biochemical Pharmacology

The c-Jun N-terminal kinases in cerebral microglia: immunological functions in the brain

Ute Hidding[a], Kirsten Mielke[a], Vicki Waetzig[a], Stephan Brecht[a], Uwe Hanisch[b], Alexander Behrens[c], Erwin Wagner[c], Thomas Herdegen[a,*]

[a]*Institute of Pharmacology, Hospitalstrasse 4, 24105 Kiel, Germany*
[b]*Max Delbrück Center for Molecular Medicine, Cellular Neurosciences, Robert-Rössle-Strasse 10, 13122 Berlin-Buch, Germany*
[c]*Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, 1030 Vienna, Austria*

Received 11 April 2002; accepted 6 May 2002

Abstract

The c-Jun N-terminal kinases (JNKs) exert a pleiotrophy of physiological and pathological actions. This is also true for the immune system. Disruption of the JNK locus results in substantial functional deficits of peripheral T-cells. In contrast to circulating immune cells and the role of p38, the presence and function of JNKs in the immune cells of the brain remain to be defined. Here, we report on the expression and activation of JNKs in cultivated microglia from neonatal rats and from mice with targeted disruption of the JNK locus and the N-terminal mutation of c-Jun (c-Jun*AA*), respectively. JNK1, 2 and 3 mRNA and proteins were all expressed in microglia. Following stimulation with LPS (100 ng/mL), a classical activator of microglia, JNKs were rapidly activated and this activation returns to basal levels within 4 hr. Following LPS and other stimuli such as thrombin (10–50 unit/mL), the activation of JNKs went along with the N-terminal phosphorylation of c-Jun which persisted for at least 8 hr. Indirect inhibition of JNK by CEP-11004 (0.5–2 μM), an inhibitor of mixed-lineage kinases (MLK), reduced the LPS-induced phosphorylation of both, JNK and c-Jun, by around 50%, and attentuated the LPS-induced the alterations in microglial morphology. Finally, JNKs are involved in the control of cytokine release since both, incubation with CEP-11004 and disruption of the JNK1 locus enhanced the release of TNFα, IL-6 and IL-12. Our findings provide insight in so far unknown functions of JNKs in cerebral immune cells. These observations are also important for the wide spread efforts to develop JNK-inhibitors as neuroprotective drugs which, however, might trigger pro-inflammatory processes.

Keywords: c-Jun; c-Jun N-terminal kinases; Immune cells; Microglia; Mixed-lineage kinases; Neurodegeneration

1. Multiple actions of JNKs

Besides ERK and p38, the c-Jun N-terminal kinases (JNKs) constitute the third major group of MAP-kinases (reviewed in [1]). Downstream of numerous signalling pathways JNKs (Fig. 1) which are triggered by extracellular stimuli, membrane receptors or peripheral cellular compartments such as presynaptic terminals and axons (reviewed in [2]). JNKs are involved in basic cellular functions underlying the integration of multiple responses and processes such as mitosis, cell cycle, activation of physiological transcriptional programs (predominantly AP-1 triggered expression) or the outgrowth of neurites as a marker for the differentiation of neural cells [2,3]. In contrast, JNKs are also involved in the realisation of degenerative and apoptotic programs after a variety of stressful stimuli, e.g. by antagonisation of anti-apoptosis (phosphorylation of Bcl-2) [4], reduction of radical scavengers (antagonisation of glutathion) [5] or by putative hyperphosphorylation of (cytoskeleton) proteins [6] with subsequent harmful aggregation of proteins [6].

The dichotomous actions of JNK are not restricted to the signalling within an individual cell, but are also relevant for the interaction of systemic processes. For example, apoptosis in postmitotic neurons represents a disastrous event which should be prevented, whereas apoptosis of activated immune cells or apoptosis during embryonic development is mandatory for the orchestrated control of the immune system and the coordinated organisation of the body, respectively. Thus, apoptosis by one given molecule such

* Corresponding author. Tel.: +49-431-597-3502; fax: +49-431-597-3522.
E-mail address: t.herdegen@pharmakologie.uni-kiel.de (T. Herdegen).
Abbreviations: AP-1, activatory proteins; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MLK, mixed-lineage kinase.

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PII: S0006-2952(02)01139-5

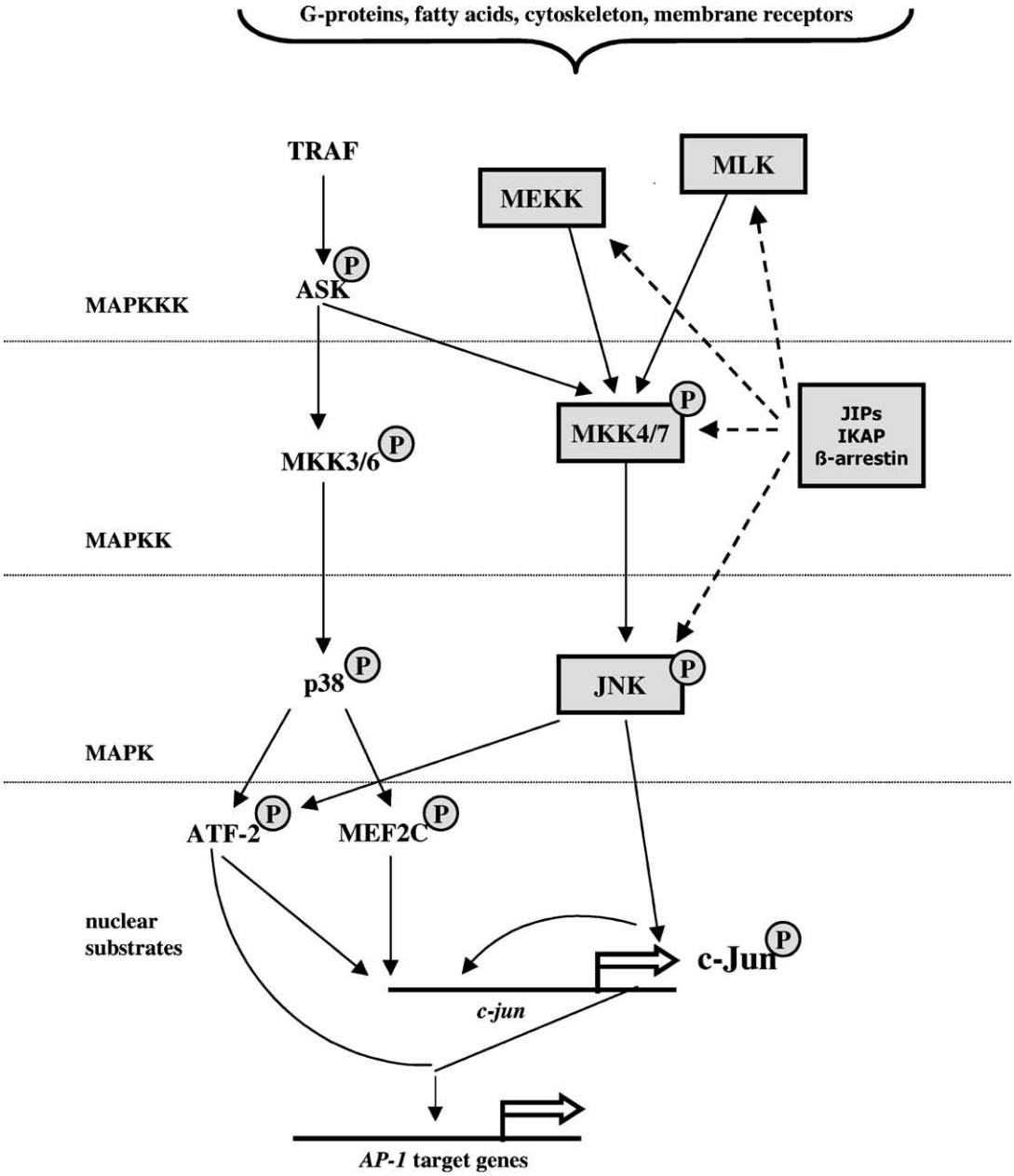

Fig. 1. Signal cascade of the JNK and p38 stress kinases. The dotted lines indicate the members of the JNK signalosom held by the scaffold proteins such as JIP, IKAP or β-arrestin.

as JNK cannot simply be considered as good or bad, but has to be assessed with regard to the physiological or pathological context of the cell.

2. JNKs and peripheral immune cells

Mitosis, differentiation and functions of peripheral immune cells such as T-lymphocytes or macrophages go along with the activation of MAP-kinases including JNKs and the downstream activation of AP-1 transcription including the expression and N-terminal phosphorylation of c-Jun. Observations from JNK knock-out mice demonstrated defined non-redundant functions of JNKs in the immune system. For example, JNKs trigger the depletion of positive T-cells in the thymus [7,8], and most likely, this process is mediated by JNK2 which is responsible for the differentiation of progenitor cells into Th1-helper cells [9]. AP-1 proteins such as JunB are involved in the differentiation of Th2-, but not Th1-lymphocytes [10]. A central function of JNKs is the activation of the IL-2 promoter through AP-1 transcription [11] and the stabilisation of the IL-2 mRNA [12]. JNK2-deficient mice show dysfunctions of peripheral T-cells, and T-lymphocytes without JNK1 or JNK2 reveal disturbances in the synthesis of cytokines [13]. Finally, JNKs promote the translocation of NFAT4/NFATc into the nucleus—the inhibition of this translocation is the central step of immunosuppression. It remains to

be clarified, to which extent JNKs modulate the functions of calcineurin. For example, we have shown that inhibition of calcineurin results in inhibition of JNKs [14], and on the other hand, JNKs are reported to be upstream of calcineurin as functional agonists [15].

The position of JNKs in the apoptosis of activated immune cells is controversially discussed. Thus, JNK was described as a mediator of TRAIL-, but not Fas-triggered apoptosis in T- and B-lymphocytes [16] and the MHC-1 triggered apoptosis [17,18]. On the other hand, JNK2-deficient T-lymphocytes are not altered in the apoptotic response [8]. Besides T-cells, JNK might be relevant for the differentiation of B-progenitor cells by IL-3 [19], and for the IL-6 or TNF release from macrophages [20,21] and mast cells [22]. In addition, functions of JNKs comprise the adhesion and infiltration of leucocytes mediated by expression of E-selectine in endothelial cells [23,24].

3. Regulation of microglial functions by MAP-kinases

Numerous cellular reactions of microglia are regulated by MAP-kinases. It is well established that ERK and p38 are involved in the secretion of cytokines [25], expression of adhesion molecules, and formation of Aβ proteins [26] or NO [27] (reviewed in [28]). Furthermore, the inhibition of ERK and p38 attenuate the activation of these kinases and subsequently prevent the activation of microglia with attenuation of their inflammatory and degenerative actions [25,29,30]. The release of pro-inflammatory and immune-modulatory molecules is only one aspect of the program underlying microglial differentiation and immuncompetence; further aspects include complex reactions such as MHC-I/II molecules and the morphological metamorphosis into large phagocyting and scar-forming activated microglia. Models with stimulation of microglia by LPS and TNFα have provided sound evidence that p38 and ERK are involved in these microglial programs (reviewed in [31]).

4. Expression and function of JNKs in microglia

Only few data exist on the expression, activation or putative function of JNKs in microglia *in vitro* and *in vivo* [29,32]. The expression of JNKs in the brain was considered to be restricted to neurons. It was not until recently that JNK immunoreactivities are localised in apparently vascular and ependymal cells as well as microglia and astroglia [29,32], but the correct identification of the JNK-immunoreactivities is still under discussion (reviewed in [2]). Some evidence for the functional presence of JNKs derives from the observation of N-terminal phosphorylation of c-Jun in non-neuronal cells [29,33]. Therefore, we investigated the expression and putative functions of JNKs in non-neuronal cells such as microglia.

4.1. JNKs are expressed and activated in microglia

At first, the expression of JNKs was analysed in microglial cell cultures. By RT-PCR, we isolated all the three JNK isoforms including JNK3 (Fig. 2), which is supposed to be exclusively expressed in neurons [34]. We wanted to know whether the microglial expression of JNK3 is a particular feature of the brain immune system. Comparison with the corresponding JNK expression pattern revealed the specific expression of JNK3 in cerebral microglia but it is not present in peripheral macrophages/monocytes (Fig. 2). It is an attractive speculation that the expression of JNK3 is a relevant step in the cerebral phenotyping of peripheral immune cells which immigrate and reside in the brain. Finally, Western-blot analysis confirmed the presence of all the three JNK isoforms in rat microglia (Fig. 3).

The next experiments addressed the activation of JNKs. For this purpose, we used lipopolysaccharide (LPS, 100 ng/mL), a widely used stimulus for microglia, which provoked a dramatic and rapid increase in JNK activity returning to basal levels within 4 hr (Fig. 4A). Interestingly, JNKs are not only activated by immunogenic stimuli such as LPS but also by blood derived factors. Thus, incubation of microglia with thrombin dose-dependently raised the N-terminal phosphorylation of c-Jun similar as LPS (Fig. 4B). The activation of microglia by thrombin was not reported until recently [35,36], and our data reveal that JNKs are downstream of multiple extracellular signals which are involved not only in immunogenic-inflammatory but also in hemostatic events. The activation by LPS and thrombin represent a novel hitherto unknown operational range of JNKs.

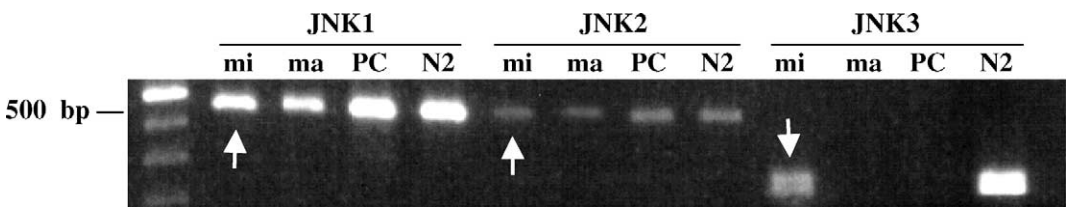

Fig. 2. RT-PCR of JNK1, JNK2 and JNK3 in microglia (mi), macrophages (ma), naïve PC12 (PC) and neuroblastoma 2A cells (N2). JNK3 is present in microglia and Neuro2A, but not in macrophages and PC12 cells. The arrows indicate the JNK1, 2 and 3 bands from microglia. Cultivation of cells and RT-PCR, and isolation of murine peritoneal macrophages were performed as described in detail elsewhere [42,43].

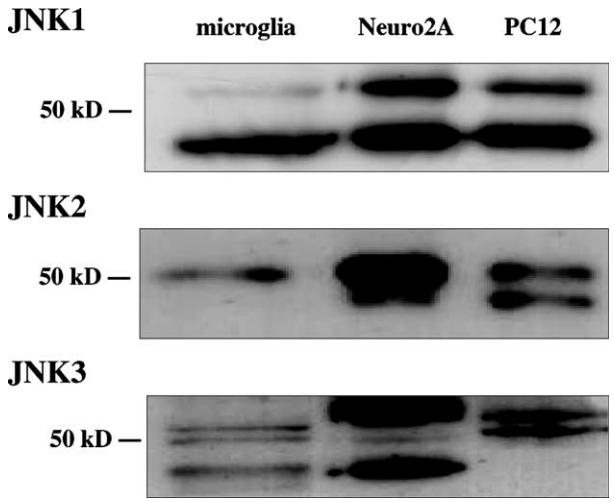

Fig. 3. Western blot of whole cell extracts from microglia, Neuro2A and naïve PC12 cells. The JNK3 immunoreactivity provides a band at 46 kDa which is absent in PC12 cells, and PC12 cells do not express JNK3 (see Fig. 2) [42]. For Western blotting, 10 μg protein of whole cell extracts were stained with either a monoclonal antibody against JNK1 (1:1500, Pharmingen) or polyclonal antibodies against JNK2 (1:500; Santa Cruz, N-18) or JNK3 (1:1000; UBI).

4.2. The functionality of activated JNKs

The subsequent experiments addressed the issue of the functionality of JNK activation. At first, we analysed the N-terminal phosphorylation of c-Jun in microglia. Under basal conditions, i.e. during the absence of any stimuli,

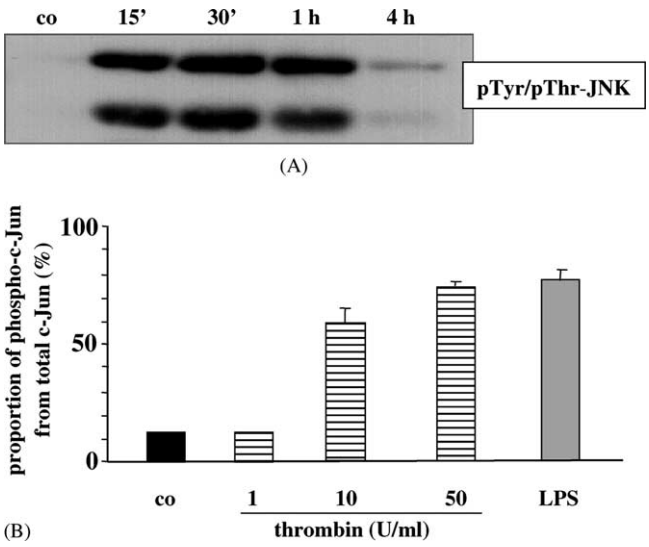

Fig. 4. (A) Western blot of phosphorylated JNK in untreated microglial controls (co) and 15 min to 4 hr following incubation with LPS (100 ng/mL). Fifteen micrograms protein from whole cell extract were stained with an polyclonal antibody against phosphorylated JNKs (1:2500; Promega). (B) Quantitative immunocytochemistry of phosphorylated c-Jun following various dosages of thrombin; stimulation with LPS (100 ng/mL) was used as positive control; co gives the untreated controls. The numbers give the percentage (%) of c-Jun positive microglia which are immunoreactive for N-terminal phosphorylated c-Jun. Immunocytochemistry for the N-terminal phosphorylation was performed with the polyclonal antibody against the serine-63 (New England Biolabs).

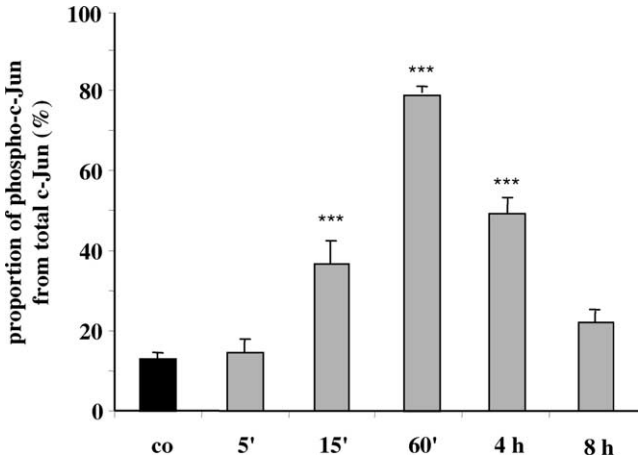

Fig. 5. Quantitative immunocytochemistry of phosphorylated c-Jun in untreated control microglia (co) and following LPS (100 ng/mL). The numbers give the percentage (%) of c-Jun positive microglia which are immunoreactive for phosphorylated c-Jun. Immunocytochemistry for the N-terminal phosphorylation was performed with the polyclonal antibody against the serine-63 (New England Biolabs). (***): $P < 0.001$.

almost all microglia expressed c-Jun. Only a minor proportion, however, is phosphorylated (Fig. 5), and this correlates with the low basal activity of JNKs (Fig. 4A). Already 15 min after stimulation, the pool of N-terminally phosphorylated c-Jun increased and within 1 hr, almost all microglial cells were immunopositive for N-terminal phosphorylated c-Jun (Fig. 5). The N-terminal phosphorylation persists for many hours and surpasses the period of total JNK activation. Several reason might account for this divergence: (i) the phosphorylation of c-Jun might reflect a particular stability of the phosphorylation, i.e. due to the absence of MAP kinase phosphatases; (ii) the total JNK activity might cover as 'background activity' the small pool of activated JNK in the nucleus which is responsible for c-Jun phosphorylation; this was already described in cerebellar neurons [37].

Functional analysis of JNKs was clarified by two approaches: (i) inhibition of the enzymatic activity of JNKs, and (ii) cultivation of microglia from JNK knock-out mice. For inhibition of JNKs, we used the MLK-inhibitor CEP-11004 [38], a shadow compound of the well described CEP-1347 [39,40]. MLKs are upstream of the JNK signalosom, and by direct antagonisation of MLKs, CEP-11004 as well as CEP-1347 achieve a substantial inhibition of JNK activation since. Numerous experiments have shown that the inhibition of JNK by CEP-1347 goes along with the protection of otherwise dying neurons in the brain [39,40] (reviewed in [2]).

Incubation of microglia with 0.5 and 1 μM CEP-11004 reduced the activity of JNK by around 50%, whereas the activity of p38 was not altered (Fig. 6A). This demonstrates that MLKs are upstream of JNKs in microglia; on the other hand, however, a substantial proportion of the JNK activation is regulated beyond the MLK cascade. Interestingly, CEP-11004 attenuated the N-terminal phosphorylation of

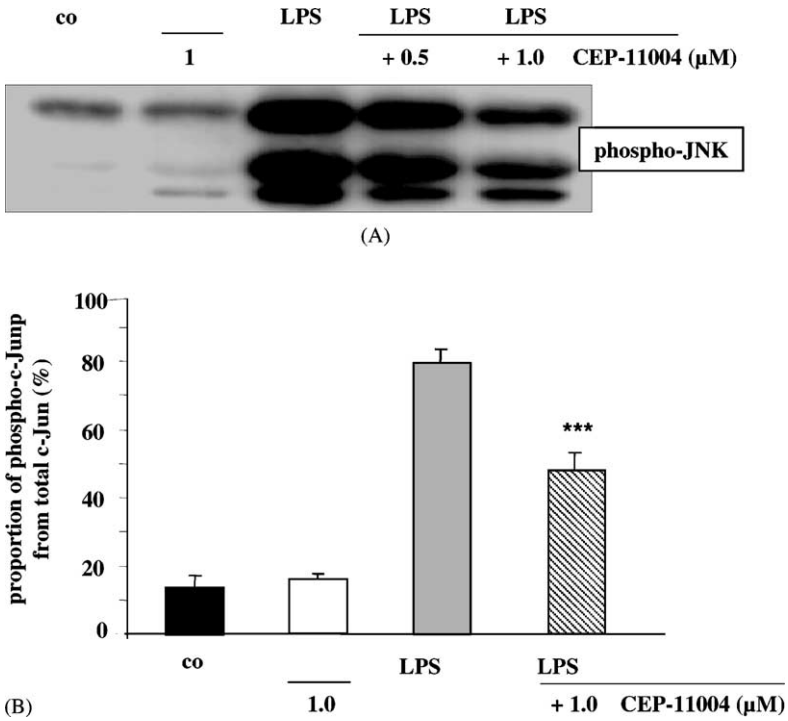

Fig. 6. (A) Pre-incubation of microglia with CEP-11004 reduces the LPS (100 ng/mL)-induced increase in phosphorylated JNK by around 50%. One microgram CEP-11004 alone had no effect on the basal phospho-JNK reactivity of untreated controls (co). Fifteen micrograms protein from whole cell extract were stained with an polyclonal antibody against phosphorylated JNKs (1:2500; Promega). (B) CEP-11004 significantly (***: $P < 0.001$) reduces the proportion of microglia with phosphorylated c-Jun from the total pool of c-Jun positive microglia when compared with LPS alone, but it has no effect on the basal phosphorylation of untreated controls (co). Immunocytochemistry for the N-terminal phosphorylation was performed with the polyclonal antibody against the serine-63 (New England Biolabs).

c-Jun to a similar degree as the JNK activation, i.e. by around 50% (Fig. 6B), and this supports the notion that JNKs contribute to the N-terminal phosphorylation of c-Jun in microglia.

4.3. JNKs control the release of cytokines

The control of the release of cytokines is a dominant effect of p38 and ERK in microglia [25,31]. Therefore, we analysed the putative modulation of cytokine release by JNKs. Microglia were incubated with different concentrations of CEP-11004 (50 nM–5 μM) 1 hr preceding the stimulation with LPS (100 ng/mL), and the concentration of cytokines in the supernatant were measured by ELISA 24 hr later. The inhibition of JNKs with CEP-11004 surprisingly *enhanced* the release of TNFα, IL-6 and IL-12 by 60–80% at concentrations which are known to inhibit JNKs (1–2 μM) (Fig. 7). Importantly, we were able to validate the inhibition of the cytokine release by JNKs in JNK knock-out mice; the generation of these mice is described in detail elsewhere [7,9,34]. Stimulation of microglia from JNK1 knock-out mice increased the release of TNFα by around 60% compared with the release in microglia from littermate controls (Fig. 8). These observations which suggest an antagonistic action of JNKs on the p38- and ERK-triggered release of cytokines [25], deserve major attention because of the actual therapeutic efforts to inhibit JNKs as novel strategy in the treatment of Alzheimer's or Parkinson's disease.

4.4. JNKs are modulators of the microglial morphology

Microglia undergo dramatic changes in their morphology as part of the differentiation process which extends from resting ramified phenotype to large confluent plaques. This morphological alteration has to be orchestrated with the mitosis, differentiation and maturation of the immunological competence. What might be the impact of JNKs on the cellular morphology since the components of the cytoskeleton are relevant (*in vitro*) substrates for JNKs (reviewed in [2]). Incubation of CEP-11004 reduced the size of growing microglia by around 40% (Fig. 9A) within 24 hr. We wanted to know whether the regulation of the individual microglial enlargement by JNKs was mainly triggered by post-translational modifications of already existing molecules or by *de novo* protein synthesis. For this purpose, we studied the enlargement in microglia from c-Jun*AA* mice in which the serines at positions 63 and 73 are exchanged for alanines [41]. In comparison with littermate controls, LPS provoked only a restricted enlargement of the individual microglial cells which could not be furthermore reduced by CEP-11004 (Fig. 9B). Again, the use of genetically altered mice confirmed the data from *in vitro* experiments. This observation

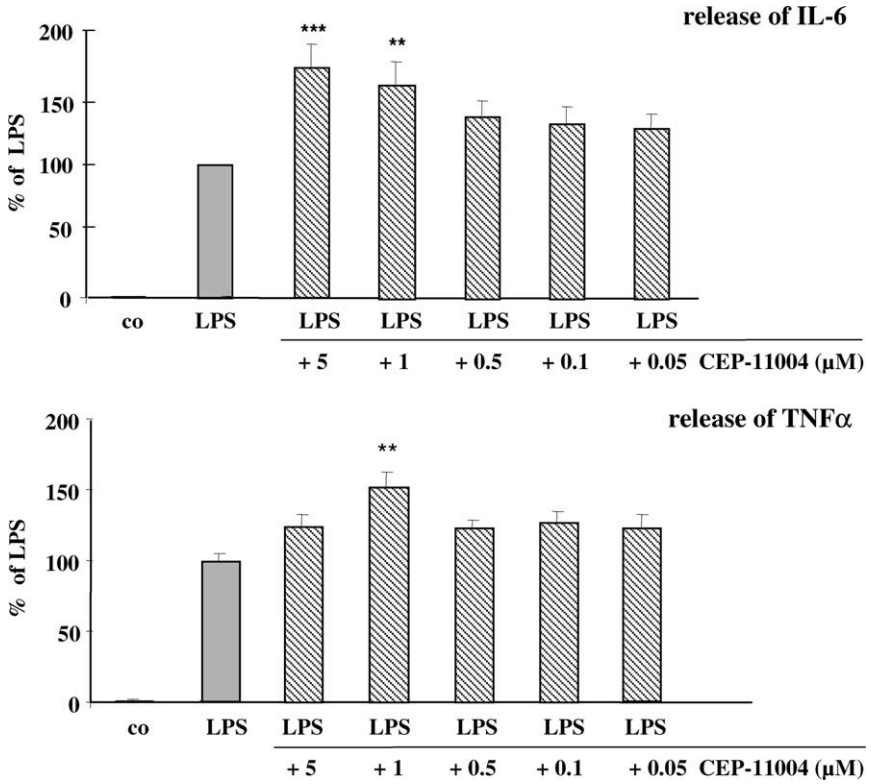

Fig. 7. Incubation of microglia with 0.05–5 μM CEP-11004 dose-dependently increased the release of TNFα and IL-6 when compared with LPS alone (**, ***: $P < 0.05$ and $P < 0.001$). The concentration of the cytokines in the supernatant was collected for 24 hr and determined by ELISA as published recently [25]; co gives the untreated controls.

contributes to the pool of data which strongly suggest that the effect of CEP-11004 is mainly mediated by the suppression of c-Jun/AP-1 triggered transcription downstream of JNKs.

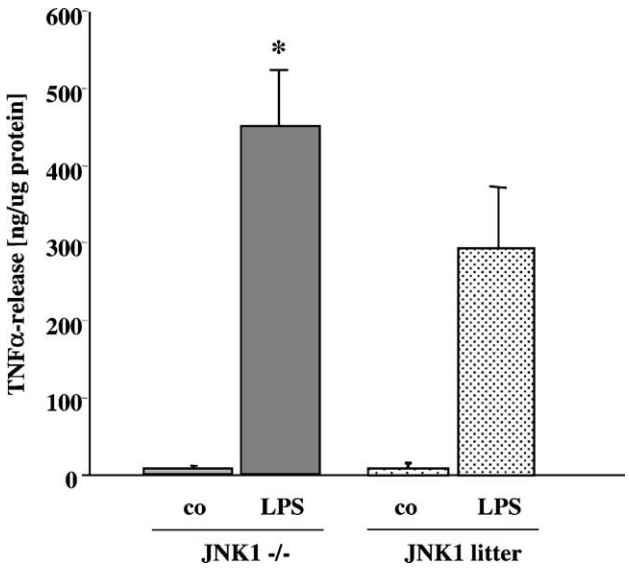

Fig. 8. Microglia from JNK1 knock-out mice (dark bar) revealed an significant increase in TNFα-release ($P < 0.05$) within 24 hr following LPS (100 ng/mL) compared with the corresponding littermates (bright bars). TNFα release was determined by ELISA as published recently [25]; co gives the untreated controls.

5. Discussion: inhibition of JNKs in neurons and microglia—antagonisation or reinforcement of therapeutic effects?

Our data provide substantial evidence that JNKs interfere with central features of microglial functions following immunogenic-inflammatory and hemostatic events, i.e. the release of cytokines, the mitosis and the morphological alterations. These modulations of microglial activation by JNKs gain particular interest in the context of JNK inhibition as novel therapeutic strategy for the treatment of Alzheimer's disease and Parkinson's disease; JNK-inhibitory drugs have successfully passed phase I and are ready to enter phase II for the treatment neurodegenerative disorders such as Parkinson's disease.

The present data demonstrate the necessity to sharpen the integrative view for JNK functions and for the risky side effects by JNK inhibition. Thus, inhibition of apoptotis in neurons is a desirable target for neuroprotection, but inhibition of apoptosis in immune cells might prolong their pro-inflammatory status with pro-degenerative effects. Here, we have shown that JNKs exert restrictive control of cytokine release, and inhibition of JNK result in enhanced release of pro-inflammatory cytokines such as TNFα. On the other hand, inhibition of JNKs and neutralisation of the transcriptional actions of c-Jun in c-Jun*AA* mice [41] substantially restrict the growth of microglia following LPS stimulation. In consequence, inhibition of JNK could attenuate the

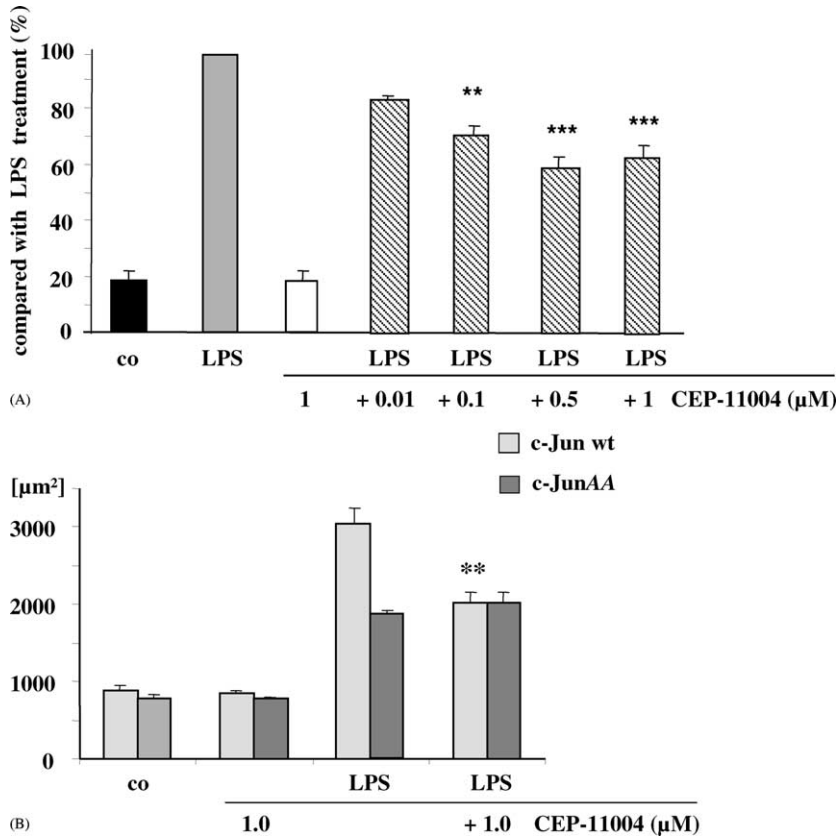

Fig. 9. (A) LPS increases the size of microglia within 24 hr (given as 100%) by around 5-fold compared with untreated controls (co). Pre-incubation with CEP-11004 significantly (**, ***: $P < 0.05$ and 0.001) reduces this morphological enlargement, whereas the size of untreated microglia controls (open column) is not affected by 1 μM CEP-11004. (B) Compared with the littermates, the exchange of the serine-63/73 into alanines in c-Jun*AA* mice resulted in a significant reduction ($P < 0.05$) of the size of individual microglial cells following LPS (100 ng/mL). This limited growth was not furthermore lowered by 1 μM CEP-11004; co gives the untreated controls.

differentiation into the phagocytotic-immuncompetent phenotype with subsequent beneficial restriction of immune reactions. At present, it is purely speculative whether (pharmaceutical) inhibition of JNKs provokes antagonisation or reinforcement of the therapeutic effects. Moreover, the contribution of the individual JNK isoforms to each of these processes remain to be elucidated. Finally, inhibition of MLKs by CEP-11004 as well as knock-out of individual JNK isoforms block only a major proportion, but not all JNK activity. Thus, the effects of total JNK inhibition might reveal more prominent pro- or anti-inflammatory effects.

Acknowledgments

We thank A. Dorst for excellence technical assistance. This work was supported from DGF grants (He 1561) and Boehringer-Ingelheim Incorp. (Germany).

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