

## Fractalkine-upregulated milk-fat globule EGF factor-8 protein in cultured rat microglia

Fernando Leonardi-Essmann<sup>a</sup>, Michael Emig<sup>b</sup>, Yoshihisa Kitamura<sup>c</sup>,  
Rainer Spanagel<sup>a</sup>, Peter J. Gebicke-Haerter<sup>a,\*</sup>

<sup>a</sup>Central Institute for Mental Health, Department of Psychopharmacology, J5, 68159 Mannheim, Germany

<sup>b</sup>Forschungslabor, III. Medizinische Klinik, Universitätsklinikum Mannheim, Germany

<sup>c</sup>Department of Neurobiology, Kyoto Pharmaceutical University, Japan

Received 23 August 2004; received in revised form 9 November 2004; accepted 10 November 2004

### Abstract

Fractalkine is the only known member of the CX<sub>3</sub>C-chemokine family, and so is its receptor CX<sub>3</sub>CR1. Fractalkine, typically is expressed by neurons where it is inserted in the plasma membrane (“chemokine on a stalk”). It can, however, be clipped off by a specific enzyme and diffuse into the extracellular space. CX<sub>3</sub>CR1 is primarily expressed by microglia, the phagocytes of the brain. This study was aimed at studying gene expression changes in cultured rat microglia upon fractalkine stimulation using gene chip technology. Six genes turned out to be upregulated, amongst which milk-fat globule EGF factor-8 protein (MFG-E8) was the most surprising, but also the most revealing one. We hypothesize that it serves as a bridging molecule between apoptotic cells (neurons) and microglia. Since the docking to microglia is, in part, mediated by members of the integrin family, six of these molecules have been—post hoc—included in real-time PCR confirmations of chip results. Two of them—integrin  $\alpha_2$  and integrin  $\beta_5$ —were upregulated as well. These data provide a much closer look into molecular mechanisms involved in apoptosis of neurons and their removal by microglia.

© 2004 Elsevier B.V. All rights reserved.

**Keywords:** Fractalkine; Milk-fat globule EGF factor-8 protein; Integrins; Microarrays; Microglia

Chemokines are widely distributed in mammalian organisms. As part of the immune system, they mediate recruitment of leukocytes to sites of inflammation. Until now, four families of chemokines (CXC, CC, C, CX<sub>3</sub>C) have been described (Power and Wells, 1996; Bazan et al., 1997; Pan et al., 1997; Fernandez and Lolis, 2002). Some receptors used by the first two groups ( $\alpha$ - and  $\beta$ -chemokines) have attracted considerable attention, when it turned out that at least two sites on blood leukocytes—CD4 and a chemokine receptor—were essential for binding of HIV-1 envelope protein gp120 (He et al., 1997; Huang et al., 1996; Paxton et al., 1996). Evidently, CXC-CKR4 (fusin) is an essential coreceptor for productive infection of CD4<sup>+</sup>-lymphocytes (Power and Wells, 1996), and CC-CKR5 is used for HIV-1 infection of macrophages (Alkhatib et al., 1996; Deng et al., 1996;

Samson et al., 1996). Furthermore, the fractalkine receptor CX<sub>3</sub>CR1 has been described as a coreceptor for HIV-1 (Combadiere et al., 1998). Fractalkine (neurotactin) is the only known member of the CX<sub>3</sub>C-chemokine family and so is its receptor. The chemokine is special in that it exists both in a membrane-anchored (“chemokine on a stalk”) and soluble form (Bazan et al., 1997). Like all chemokines, fractalkine and its receptor have been investigated predominantly in the periphery. Much less is known about their function in the central nervous system. Typically, CX<sub>3</sub>CR1 and other chemokine receptors are expressed by specialized glial cells, such as astrocytes or microglia, the macrophages of the brain, and are upregulated upon injuries or infections (Hughes et al., 2002). Microglia are, therefore, considered as an important gateway of HIV-1 infection of the brain (Price et al., 1988; Perry et al., 1994). In this context, the fractalkine receptor CX<sub>3</sub>CR1 appears to play an important role since it has been shown recently (Faure et al., 2000) that

\* Corresponding author.

E-mail address: gebicke@zi-mannheim.de (P.J. Gebicke-Haerter).

there is rapid progression to AIDS in individuals bearing a structural variant of this receptor. Increased fractalkine production possibly supported by HI-virions has been observed in patients with HIV-1 associated dementia (Erichsen et al., 2003). However, the chemokine may not necessarily be considered as a molecule triggering inflammation. There are also reports rather supporting an anti-inflammatory function of the molecule (Zujovic et al., 2000). Fractalkine has been found to be constitutively expressed in neurons (Harrison et al., 1998; Hatori et al., 2002). The relatively specific distribution of ligand and receptor raised the interesting hypothesis that upon injury or disease fractalkine may be released from its neuronal site (Pan et al., 1997; Chapman et al., 2000) and activate microglia. Normally, there is no direct interaction between neurons and microglia in the healthy brain. That separation may be relieved by disease processes and specific neuron-microglia crosstalk may ensue (Tarozzo et al., 2002). This investigation has been undertaken to gain more detailed insights into molecular responses of microglia to CX<sub>3</sub>CR1-activation by fractalkine. DNA-microarrays have been used to screen transcripts expressed by cultured rat microglia exposed to recombinant rat fractalkine. The results reveal tentative interactions of intracellular, plasma membrane-bound, and exosome-included proteins that are aimed at paving the way for activated microglia to migrate towards and dock to apoptotic cells (neurons).

## 1. Materials and Methods

### 1.1. Glial cell cultures

#### 1.1.1. Mixed astroglial cultures

'Mixed' astroglia cultures were prepared from cerebral hemispheres of newborn Wistar rats as described previously (Gebicke-Haerter et al., 1989). Meninges were carefully removed, forebrains were minced and gently dissociated by trituration in Dulbecco's phosphate-buffered saline. Tissue suspension was then filtered through 50- $\mu$ m-diameter nylon mesh (cell strainers, Falcon) into 50-ml Falcon tubes and cells were collected by centrifugation at 200 $\times$ g for 10 min. Cells were resuspended in Dulbecco's minimum essential medium (DMEM) (Gibco-BRL, Eggenstein, FRG) supplemented with 10% fetal calf serum (Biochrom-Seromed, Berlin, FRG), plated in 100-mm  $\emptyset$  culture dishes and incubated at 36.5 °C in a humidified atmosphere of 94.5% air and 5.5% CO<sub>2</sub>.

#### 1.1.2. Microglial cultures

Microglial cells can be harvested from "mixed" astrocyte cultures, provided extreme care is taken to avoid LPS contaminations. Glassware has to be heated to 210 °C for at least 2 h and fetal calf serum has to be tested for LPS contamination (Northoff et al., 1986a,b). At time of confluency (days 10–12 in vitro), media with floating

microglia is replaced by fresh media. New microglia can be collected in this way at weekly intervals thereafter from the same astrocyte (feeder) culture (Gebicke-Haerter et al., 1989). Floating microglial cells were plated in new culture dishes (100 mm  $\emptyset$ ), where they adhered within approximately 3 h. Isolated cells are pure as tested previously by several cell-type specific markers (Gebicke-Haerter et al., 1989). After attachment, cells were supplied with fresh media and fractalkine (100 ng/ml; R&D Systems, Wiesbaden, Germany) for 8 and 24 h or left untreated for the same periods of time (controls).

### 1.2. RNA preparation and labeling

#### 1.2.1. Extraction of total RNA and quality control

At the end of experiments, cells were lysed in guanidinium isothiocyanate/mercaptoethanol (1%) solution (GTC), and homogenized by passing the suspension 30 times through a 22 gauge needle. Total RNA was extracted according to Chomczynski and Sacchi (1987). However, to achieve better separation of organic and aqueous phases, Phase Lock Gel™ Heavy tubes (Eppendorf, Hamburg, Germany) were used. Upper phases were carefully removed by pipetting and RNA was precipitated in isopropanol. RNA pellets were resuspended in water and cleanup was carried out using RNeasy® MinElute™ Cleanup Kit (Qiagen, Hilden, Germany). RNA quality was evaluated by OD measurements (260 nm/280 nm) in a GeneQuant (Pharmacia, Freiburg, Germany) in 10 mM Tris-HCl, pH 7.6 and its integrity was determined by measuring ribosomal 28S/18S ratios using RNA 6000 Nano Assay RNA chips run in an AGILENT 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Ratios of 1.9–2.2 (OD 260/280) and >1.6 (28S/18S rRNA) as well as absence of a peak of DNA contamination in electropherograms at 29 s were chosen as inclusion criteria.

### 1.3. GeneChip® Rat Genome U34 A hybridization, scanning, and quality control

Since the amount of total RNA starting material recommended by Affymetrix is 5  $\mu$ g and the amounts of RNA obtained from isolated microglial cultures were between 1.5 and 2.1  $\mu$ g per 10 cm  $\emptyset$  dish, RNAs from four dishes had to be pooled for each condition. Double-stranded cDNA synthesis, in vitro transcription (IVT) into cRNA and GeneChip® Rat Genome U34 A (Affymetrix, Santa Clara, CA, USA) hybridizations were carried out according to the manufacturer's protocol. Briefly, 5  $\mu$ g pooled total RNA were reverse transcribed using T7-Oligo(dT)<sub>24</sub> primer (GeneSet Oligos, Evry, France) and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). Second strand cDNA was synthesized by adding DNA Polymerase I, RNase H, ligase and T4 DNA polymerase. After cDNA precipitation and resuspension, IVT was performed using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo, Farmingdale, NY,

USA). Biotin-labeled cRNA was purified with RNeasy® Mini Kit (Qiagen) and fragmented with RNA fragmentation buffer. IVT and cRNA fragmentation quality controls were carried out by running an mRNA Nano assay in the Agilent Bioanalyzer. Copy RNA electropherograms showed a single broad peak beginning at approximately 22 s and ending at 74 s. Fragmented cRNAs were resolved in a single peak starting at 19 s and declining at 26 s.

GeneChip®Rat Genome U34 A arrays were filled with hybridization cocktails containing 10 µg of fragmented cRNAs (after correction for total RNA carryover). After 16 h of hybridization in a GeneChip® Hybridization Oven (Affymetrix), chips were stained with streptavidin/R-phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA) in a GeneChip®Fluidics Station (Affymetrix) and immediately scanned in a GeneArray® Scanner (Agilent Technologies).

Chips were checked for performance by five evaluations: (1) comparable scaling factors between chips, (2) single chip average background <100 and similar between chips, (3) Sig (3' /5') of rat housekeeping genes GAPDH, hexokinase and β-actin <2, (4) increasing signals of hybridization controls within a chip and similar intensities for each hybridization control between chips and (5) visual assessment of scan image (DAT files) and grid alignment.

#### 1.4. Data mining

For broad initial screenings of differentially expressed genes in microglial cell cultures at two time points, four GeneChip®Rat Genome U34 A arrays were used corresponding to 8 h untreated (8Co), 8 h fractalkine-treated (8T), 24 h untreated (24Co), and 24 h fractalkine-treated (24T). After scanning, Affymetrix Micro Array Suite 5.0 (MAS 5.0) derived cell intensity files (CEL) were normalized by their scaling factors and analyzed by Affymetrix Data Mining Tool 3.0 (DMT 3.0).

We performed a first batch analysis between 8Co and 24Co and selected for genes showing “no change” between the two samples with the following filter: (1)  $-0.3 < \text{signal log ratio} < 0.3$ , (2)  $0.003 < \text{change } p\text{-value} < 0.997$ , (3) the same detection call in both samples, i.e., present–present (P-P) or absent–absent (A-A). The probe list obtained in this way represented genes that did not change due to time (Probe List I). A second batch analysis was performed between 8Co and 8T with the same filter as before. In this way, a second probe list was generated representing genes that did not change due to the treatment at 8 h (Probe List II). By cross-linking Probe Lists I and II and selecting common probes, a third probe list was obtained representing genes that did not change at 8 h with or without treatment (Probe List III). Then a third batch analysis was performed between 8T and 24T and selected for genes that showed changes, increases or decreases, between the two samples with the following filter: (1)  $1 < \text{signal log ratio} < -1$ , (2)  $0.997 < \text{change } p\text{-value} < 0.003$ , (3) control-treatment detec-

tion calls: increase–A-P or P-P, decrease–P-A or P-P. In this way, Probe List IV was obtained. By cross-linking Probe Lists III and IV and selecting common probes, a list of probes was generated representing genes that did not change at 8 h but at 24 h of fractalkine exposure (Probe List V). 8799 genes are represented in the GeneChip®Rat Genome U34 A array and 2017 of them had been reported as expressed sequence tags (ESTs) at the time of analyses. ESTs in Probe List V have not been included in further analyses.

#### 1.5. Relative quantitative real-time PCR

For confirmations of differentially expressed genes found with chip experiments, relative quantitative real-time PCR (qRT-PCR) determinations were carried out. Aliquots from samples hybridized to GeneChips were used as well as cDNAs from samples from another set of cultured cells prepared independently from additional animals at a different time.

Real-Time PCR was carried out in a total reaction volume of 20 µl using Absolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Hamburg, Germany) and run in an iCycler (BioRad, München, Germany). Ribosomal protein S12 cDNA was used as internal standard since it shows highly stable expression between samples. Reaction conditions were optimized for dilutions of sample cDNAs (1:100) and concentrations of primers (downstream 300 nM, upstream 150 nM). Cycling conditions were 95 °C for 15 min; 60 °C (56 °C for S12), 72 °C, 82–86 °C (depending on beginning of dsDNA melting temperature) and 95 °C for 30 s, each for 40 cycles. In prior runs of primer testing, melting curve analyses had been performed. During each cycle, fluorescence measurements were done at 82–86 °C (see above). Primers used for transcript confirmations are listed in Tables 1 and 2. Relative quantification was done according to the  $\Delta\Delta C_t$  method (Fink et al., 1998; Karsai et al., 2002). A  $C_t$  difference of at least 1.0 between control and fractalkine-treated samples (corresponding to a twofold difference of mRNA/cDNA) is widely accepted as threshold of significance.

## 2. Results

Due to limited amounts of material, the relatively high amounts required for chip hybridizations and financial issues, RNA preparations had to be pooled before reverse transcription (Peng et al., 2003; Kendzierski et al., 2003). In this way, variations between cultures have been minimized. Using the settings outlined in Materials and methods, the genes listed in Table 1 turned out to be upregulated. Confirmation of upregulations of calnexin, TBF II and MFG-E8 has been carried out by semi-quantitative PCR (using S12 transcript as internal standard) and agarose gel electrophoresis (Fig. 1A). Here, it turned out that CD59,

Table 1  
Gene transcripts increased or unchanged by fractalkine between 8 and 24 h (10 gene transcripts)

Accession number	Name	Forw. primer	Inv. primer	$\Delta\Delta C_t$
L18889	calnexin	GCAGATCCAGATGCTGTCAA	CTGGCAACGCTACAGTCAGA	3.0
M38337	milk-fat globule EGF factor8 protein	CGGTGACTTCTGTGACTCCA	AACTCCTTGTCTCCGGTT	2.9
X74565	TBF ( $\alpha$ 1C1 collagen gene DNA binding protein)	TCAGCAGCCAACGGAAATGATAGC	TTGTAGATGTTCTGGCCATCCAGG	2.5
X04229	glutathione-S-transferase (GST) yb	CATGCAGCTCATCATGCTTT	CTTGGGCTCAAAAATGTGGT	2.1
X73653	tau protein kinase (GSK3 $\beta$ )	GGATCTGCCATCGAGACATT	GTGGCTCCAAAGATCAGCTC	1.5
J03627	S-100 related protein	TTGACAAAGGAGGACCTGAGA	CCCCGCCACTAGTGATAGAA	1.1
U39044	dynein intermediate chain 2A	CCCTCGAGAAATCGTCACAT	AGGGGGAGCTTTGCTATCAT	0.6
X05834	Fibronectin	GAGTGGAAAGTGTGAGCGACA	GCATCGTAGTTCTGGGTGGT	0
S63521	glucose-regulated protein GRP78	GAACGACCCCTGACAAAAGA	CCTGTCCCTTTGTCTTCAGC	0
U48255	CD59 antigen	CTCGGAGGGGATTTCATCTTA	CCCATTGTTTGGCTTGTCTT	0
X15962	s12 standard	ACGTCAACACTGCTCTACA	CTTGCCATAGTCCTTAAC	

which was also amongst the genes upregulated on chips, was false positive. Fig. 1B shows densitometric scanning results of agarose gels, as shown in Fig. 1A. They clearly reveal that MFG-E8 transcription was markedly elevated at 24 h of fractalkine treatment. Increased rates of transcription with calnexin and TBF II were observed at 8 and 24 h, whereas CD59 transcripts showed increased levels only at 8 h of treatment.

Subsequent qRT-PCRs confirmed only six of the genes listed in Table 1, including those already revealed by gel electrophoresis. Dynein showed a tendency but did not reach significance level. Gene products of the other genes are involved in correct folding of glycosylated proteins (calnexin), RNA-editing (polypyrimidine tract binding protein or TBF II), neutralization of reactive oxygen intermediates (glutathione-S-transferase), tau-protein phosphorylation and formation of A-beta peptides (tau-protein kinase or GSK3 $\beta$ ), and as markers of brain damage (S-100 related protein). One of the most upregulated transcripts, however, was milk fat-globule EGF factor-8 protein (MFG-E8) (Fig. 1A, upper left and Fig. 1B: MFG8). It has not been described in microglia until now and has only recently

found in murine peritoneal macrophages (Hanayama et al., 2002).

Due to the tentative mechanism of tagging of apoptotic cells by MFG-E8 and subsequent docking to microglial surface integrins, this family of transcripts has additionally been investigated in the same cellular samples by qRT-PCR. It turned out that only two integrin transcripts—integrin  $\alpha$ 2 and integrin  $\beta$ 5—were significantly upregulated upon fractalkine treatment. These integrins are not included on the Affymetrix chips used in this study. Integrins  $\alpha$ X,  $\alpha$ M, and  $\alpha$ E also appeared to be upregulated but did not reach significance levels (Table 2).

### 3. Discussion

Microarray technology has been used in this study to detect new transcripts upregulated upon stimulation of microglia by the unique chemokine fractalkine. The technology can be viewed as a screening method giving some idea of what molecular interactions may be going on. Evidently, there are some additional transcripts involved in

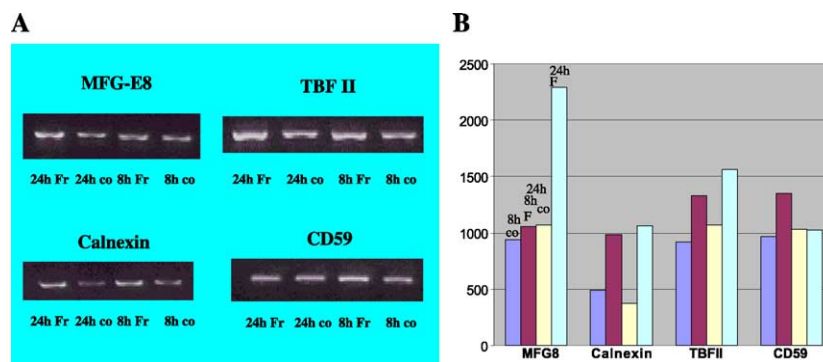


Fig. 1. Semi-quantitative RT-PCR of four upregulated transcripts as revealed by chip experiments. Gel electrophoretic separation (A) and subsequent scanning of cDNA bands (B) confirm three of them when ribosomal protein transcript s12 was used as standard (unchanged in each condition, not shown). Numbers at y-axis are arbitrary units.

Table 2

Additional gene transcripts increased or unchanged by fractalkine between 8 and 24 h (six gene transcripts)

Accession number	Name	Forw. primer	Inv. primer	$\Delta\Delta C_t$
XM_215492	Integrin $\alpha 2$	TTGCTGCATCAACTTTCCAG	GTGGGCACCTTCTGCTTTCTC	3.8
NM_147139	Integrin $\beta 5$	TCTGTACAGGGGTTTGGAGG	GTCAAAGACGACCAGGAAGC	1.1
NM_031691	Integrin $\alpha X$	ACCACGTGTTCAAGGTAGGC	TGACATCTCGTGCTGAAAGG	0.8
NM_012711	Integrin $\alpha M$	TCAGTTGTCGAGCCTTCTT	TGTCCACACAGTCCGGTAAA	0.5
NM_031768	Integrin $\alpha E$	CAGTGGAGGAGGAAGACGAG	TGAAATTTTGGCCTTCTGG	0.2
NM_017022	Integrin $\beta 1$	GCCAGTGTCACCTGGAAAAT	TGTGCCACTGCTGACTTAG	0

fractalkine-triggered microglial reactions that go unnoticed by the chip results. As revealed by the real-time data, at least integrins  $\alpha 2$  and  $\beta 5$  should have surfaced. Browsing the chip annotations, however, revealed that these two integrins are not represented on the chip.

Nevertheless, the true positives, confirmed by qRT-PCR, already gave us some exciting insights into cellular and molecular interactions occurring upon fractalkine stimulation and set a solid framework to build hypotheses on for subsequent research. One of the outcomes, spontaneously pursued in this study, was the inclusion of the major integrins found in gene bank. Still, one point of caution has to be made: all results and the discussion reside on data obtained on the level of transcription. Whether or not transcriptional changes are reflected on the protein level remains to be determined.

### 3.1. Calnexin

Calnexin, which is localized in the endoplasmic reticulum, is a chaperone molecule that folds monoglucosylated glycoproteins (Ellgaard and Frickel, 2003; Fig. 2A). One of

those glycoproteins may be MFG-E8, which like calnexin is also upregulated (see below). Above that, calnexin appears to be involved in storage and release of intracellular calcium by interaction with calcium ATPase. The phosphorylation status of the cytosolic domain of calnexin determines this interaction inhibiting the calcium pump upon calnexin phosphorylation (Roderick et al., 2000). This important on/off switch of its activity immediately refers to the point made above about actions occurring on the protein level. Its function on the gene level has been more closely analyzed by using knockout mice (Denzel et al., 2002). The surviving animals showed motor disorders that were associated with a marked loss of large myelinated nerve fibers which points to a prominent function in the nervous system.

### 3.2. Glutathione-S-transferase Yb

Glutathione-S-transferases (GSTs) are dimeric, multi-functional cytosolic proteins involved in drug biotransformation and detoxification. Lai et al. (1986) cloned the cDNA of GST Yb. There are at least four isoforms of the enzyme, GST Yb1-4 (Lai et al., 1988). GST Yb3 has been identified

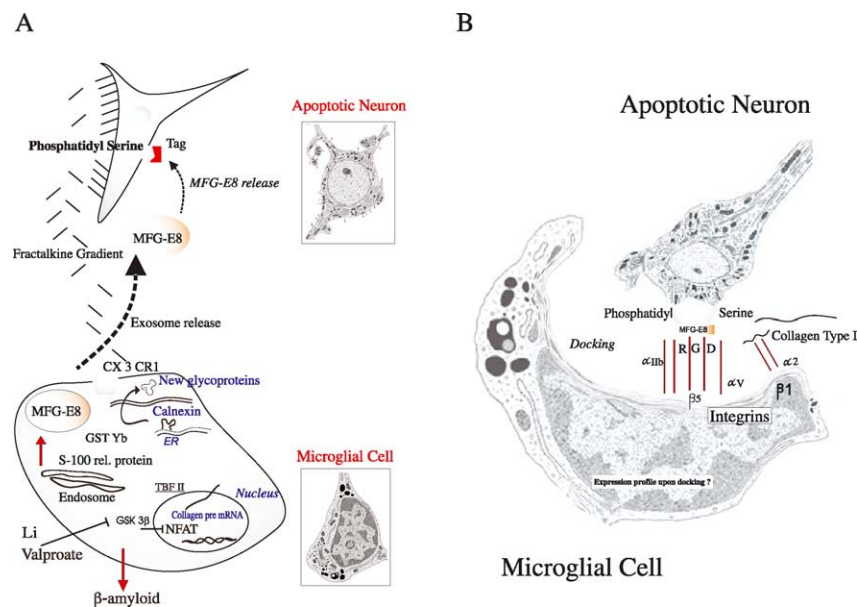


Fig. 2. Tentative molecular and cellular interactions associated with fractalkine release by neurons. A fractalkine gradient extends from the apoptotic neuron (where it is clipped off from its membrane-anchored position) to quiescent microglia in the brain parenchyma (where fractalkine receptor CX<sub>3</sub>CR1 is constitutively expressed in microglia). Fractalkine binding to this receptor activates microglia. Gene expression patterns changed by CX<sub>3</sub>CR1 stimulation have been investigated here and are outlined in (A). Integrin-mediated docking of the apoptotic cell through bridging of MFG-E8 and its RGD (Arg, Gly, Asp) domain by integrin  $\beta 5$  is shown in (B).

as the major isoform in rat brain (Abramovitz and Listowsky, 1987). Cammer et al. (1989, 1990), identified it localized in astrocytes, but not in neurons. Sherratt et al. (1990) reported on glucocorticoid-mediated increase of GSTs Ya, Yb, and Yc. Abramovitz et al. (1988) investigated its inhibition in brain by glucocorticoids, a number of neurotransmitters, by thyroxine, apomorphine, and benzodiazepines. GSTs are also involved in the synthesis of specific prostaglandins (Chang et al., 1987). Specifically, subunit Yb is rapidly translocated to the cell nucleus, suggesting a role in transcriptional regulation or transcript processing (Bennett and Yeoman, 1987). Glutathione-S-transferase has been shown to be downregulated in oxidative stress as revealed by microarray studies (Yajima et al., 2002).

### 3.3. Tau protein kinase I

Ishiguro et al. (1993) have shown that tau protein kinase is identical with glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). It can be phosphorylated at a number of sites, Ser9 being one of the most important residues. Lithium inhibits enzyme activity leading to reduced tau phosphorylation (Takahashi et al., 1999). Conversely,  $\beta$ -amyloid increased enzyme activity and subsequent tau phosphorylation (Takashima et al., 1998). Tau hyperphosphorylation has been hypothesized as a prerequisite of neuronal tangle formation in Alzheimer's disease. Immunostaining with anti-TPKI antibody indicated that this kinase is upregulated in AD brains (Imahori et al., 1998). Phiel et al. (2003) report on the pivotal involvement of GSK3 $\beta$  in production of A-beta peptides. Its inhibition by lithium reduced A-beta synthesis. In this way, GSK3 $\beta$  appears to serve a dual function in Alzheimer's disease contributing to tau-hyperphosphorylation and enhanced A-beta production (for review, see Bhat and Budd, 2002; Fig. 2A). Moreover, GSK3 $\beta$  has been shown to be expressed in tau-containing astrocytes and oligodendrocytes (Ferrer et al., 2003). Emamian et al. (2004) report on decreased AKT1 protein levels in both lymphocytes and brains of schizophrenic patients which results in reduced AKT1-GSK3 $\beta$  signaling. Calcineurin dephosphorylates NFATs in the cytoplasm and promotes their import into the nucleus, where they induce the transcription of many genes involved in lymphocyte activation and proliferation. Phosphorylation of NFATs by tau protein kinase I/GSK3 $\beta$  protein kinase decreases their DNA-binding activity and promotes their export from the nucleus, thereby inhibiting spontaneous activation (Beals et al., 1997). As well as lithium, valproate, another substance used in treatment of bipolar disorders, also inhibited activity of GSK3 $\beta$  (De Sarno et al., 2002). Furthermore, Nadri et al. (2003) and Kohen et al. (2003) have shown abnormal GSK3 $\beta$  expression in schizophrenia and learned helplessness, an animal model of depression, respectively. Ohkubo et al. (2003) showed inhibition of GSK3 $\beta$  by reelin/apolipoprotein E receptor mediated intracellular signaling events. Reelin, a protein absent in "reeler" mice, is required

for neuronal migration during brain development and is substantially reduced in brains of schizophrenic patients. Microglia have been reported to synthesize apoE and express apoE receptors (Ignatius et al., 1986; Christie et al., 1996; Marzolo et al., 2000).

### 3.4. S-100 related protein

Masiakowski and Shooter (1988) first described expression of S-100 related protein in rat PC 12 cells. It belongs to the superfamily of calcium-binding proteins highly conserved in the central nervous system. Until now, it has not been described in microglia. One of the major S-100 proteins, S-100  $\beta$ , was substantially expressed in astrocytes and upregulated in periinfarcted areas (Matsui et al., 2002), or in striatum and substantia nigra after MPTP-treatment (Muramatsu et al., 2003). It was not found in microglia. In contrast, S-100 related proteins MRP-8 and -14 have been reported to occur in microglia (Postler et al., 1997).

### 3.5. Polypyrimidine tract binding protein or TBF II

TBF II has originally been described as a rat myoblast protein recognizing DNA sequences in the 3' -UTR of pro  $\alpha$ 1(CI) collagen gene. It is a member of the family of polypyrimidine tract binding proteins, is localized in the cellular nucleus and binds specifically to polypyrimidine sites of pre-mRNA introns (for review, see Valcarcel and Gebauer, 1997). It negatively regulates splicing and, hence, is involved in exon silencing. In this way, splicing of the c-src neuron-specific N1 exon is repressed (Chou et al., 2000). Conversely, its selective abrogation, e.g., by RNAi methodology, results in insertion of exons repressed by PTB-binding to silencer elements (Wagner and Garcia-Blanco, 2002). It has not been found in neurons in healthy brains, but was observed strongly expressed in microglia (McCutcheon et al., 2004). Intracellularly, it can shuttle between the nucleus and cytoplasm. Its exit from the nucleus appears to be only possible by specific phosphorylation of its conserved Ser-16 by cAMP-dependent protein kinase A (Xie et al., 2003). Its increased expression upon fractalkine treatment could lead to collagen repression or mRNA modification by its binding to collagen type I ( $\alpha$ 1) gene transcripts (Terraz et al., 2002; Fig. 2A). In this way, microglia may control formation of extracellular matrix during migratory activities.

### 3.6. Milk-fat globule EGF factor-8 protein

Milk-fat globule EGF factor-8 (MFG-E8) has originally been discovered in milk-fat globules of lactating mice. It is a glycoprotein with substantial homology to the blood-coagulation factors V and VIII (Stubbs et al., 1990). Specifically, it has been found later on dendritic cell-derived exosomes (They et al., 1999). Although the origin and function of exosomes is still a matter of debate, the data

reported until now speak for an endosomal origin rather than for a plasma membrane-derived origin (Thery et al., 2002). These multivesicular bodies carry a number of proteins required for intercellular communication. In this way, their composition appears to be very specific (Thery et al., 2001). Since they often transport proteins involved in immune reactions, such as MHC classes I and II, they are believed to play an important role in inflammatory processes (Denzer et al., 2000; Stoorvogel et al., 2002). They can bind to target cell membranes, decorate them with some of their content molecules, and confer new properties to these cells. Very likely, MFG-E8 is such a molecule. It has been found recently as a major protein in macrophage exosomes (Hanayama et al., 2002). But its presence in kidney, heart, lung, and brain has also been reported (Aoki et al., 1997; Oshima et al., 1999). Like annexin, another component of exosomes, MFG-E8 specifically binds to phosphatidyl serine exposed on plasma membranes of apoptotic cells (Verhoven et al., 1995; Azuma et al., 2002; Hanayama et al., 2002; Ezekowitz, 2002), tagging these cells for directed elimination. This report shows for the first time that microglia upregulate MFG-E8 upon fractalkine stimulation. It has been shown recently in another microarray investigation that GM-CSF can also upregulate MFG-E8 transcription in microglia (Re et al., 2002). We hypothesize that fractalkine is released from neurons only when they degenerate and under these conditions it can reach microglia. Upon that signal, microglia produce increased amounts of MFG-E8 that is assembled on the surface of exosomes. Released exosomes encountering the apoptotic neurons label them with MFG-E8 so microglia migrating along the fractalkine gradient can recognize their target cells (Fig. 2A). The apoptotic cell is then docked to the microglial cell via specific integrins (see below) expressed on the microglial cell and an RGD domain on MFG-E8. In this way, MFG-E8 forms a bridge between the two cell types and enables microglia to engulf and remove the dying cell (Fig. 2B). This view is also supported by a recent paper of Stolzing and Grune (2004) describing the phagocytosis of apoptotic neurons by microglia in culture. An even more recent paper (Hanayama et al., 2004) reports on MFG-E8-deficient mice that are still able to attach dying cells to their spleen and lymph macrophages, but that those macrophages are unable to phagocytose. MFG-E8, hence, not only appears to be important for docking apoptotic cells to macrophages, but also appears to be essential for their engulfment. Although this is still a theoretical view with respect to microglia which is based on the data by Hanayama et al. and the data of this report, it is a very reasonable notion to be tested in further in vivo experiments.

### 3.7. Integrins $\alpha_2$ and $\beta_5$

Integrins are critically involved in cell migration. Cell migration requires dual control mechanisms both on the level of the cytoskeleton and extracellularly on the level of

cell adhesion to extracellular matrices. Cytoskeletal changes include actin polymerization in apical parts of moving cells and docking of the actin cytoskeleton to extracellular matrix molecules, like laminin and collagen. Integrins function in this communication system as “inside-out” signaling molecules. Conversely, they pick up signals from the extracellular environment and transduce them to the cell interior, in this way mediating “outside-in” signaling events. This feature of dual signaling confers to them pivotal roles in leukocyte traffic and immune responses, development, and hemostasis, and in a variety of human diseases including cancer (Wehrle-Haller and Imhof, 2003).

The integrins form a large family of heterodimeric cell surface receptors consisting of an  $\alpha$ -subunit and a  $\beta$ -subunit. Following ligand binding, they generate cellular signals leading to the formation of cytoskeletal connections and specific cellular responses (Hynes, 2002). Many intracellular signaling pathways stimulated by integrins are similar to those triggered by growth factor receptors. Accordingly, they block apoptosis by activation of PI3-kinase and Akt and induce proliferation. Of the 24 different integrin receptors presently known, 5 function as collagen receptors. The collagen receptors are composed of the  $\beta_1$  subunit in complexes with either an  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_{10}$ , or an  $\alpha_{11}$ -subunit. Integrin  $\alpha_2\beta_1$  is an important collagen receptor on platelets and epithelial cells. Despite the structural similarity of the integrins  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$ , their binding specificity differs from each other. Integrin  $\alpha_1\beta_1$  prefers network-forming collagen type IV over fibrillar collagen type I, whereas  $\alpha_2\beta_1$  binds type I collagen more strongly than collagen type IV (Tulla et al., 2001; Fig. 2B). The collagen interactions of  $\alpha_2$ -containing integrins can be seen in line with modifications of collagen transcripts by upregulated polypyrimidine tract binding protein (TBF II, see above) in microglia upon fractalkine treatment. Moreover, integrins can form homodimers. For instance, Li et al. (2004) report on homodimerization of integrin  $\alpha_2$  rather than heterodimerization with  $\beta$ -integrins. Upregulation of  $\alpha_2$ -integrin in microglia upon fractalkine stimulation may, therefore, increase interactions of the cells with collagen of the extracellular matrix. Alpha2 integrins, however, may also be involved in internalization of cell debris via caveolae (Upla et al., 2004). In contrast, increased integrin  $\beta_5$  expression may be responsible for increasing binding sites on microglia surface for MFG-E8. Integrin  $\beta_5$  typically is associated with integrins  $\alpha_{IIb}$  or  $\alpha_V$  and in this configuration specifically binds molecules exhibiting RGD motifs, like MFG-E8 (Fig. 2B).

## 4. Conclusion

The transcripts identified in this report as upregulated in microglia upon fractalkine treatment are apt to provide a comprehensive close-up and new views into molecular mechanisms occurring upon cell (neuronal) death and

microglial activation in brain. Fractalkine appears to be a particularly good example to illustrate those mechanisms in that it is expressed by neurons and is not released immediately after its synthesis. We hypothesize that—on the contrary—it is enzymatically clipped off its membrane-standing position only in conditions when the parent neuron is no longer viable. Subsequently, the chemokine can reach its receptor on quiescent microglial cells and specifically activate them. Some gene transcripts involved in this activation process identified here are calnexin, GST, S100-related protein, and GSK3 $\beta$ . The most surprising gene transcript unveiled by this investigation, however, is MFG-E8. It provides an intriguing idea of how microglia may be conducted to the appropriate target cell undergoing apoptosis and becoming enabled to specifically attach to that cell via an MFG-E8 bridge. Finally, the additional results on integrin expression yield further insights into tentative molecular interactions between these microglial membrane receptors and the extracellular matrix as well as with MFG-E8 RGD domains. Although still hypothetical, the mechanisms depicted in Fig. 2 elucidate the seamless fitting of the corresponding gene products into a unifying concept.

## Acknowledgements

Supported by DFG-Schwerpunktprogramm “Mikroglia” Ge 486/11-4 and by EC-grant TARGALC QL63-CT-2002-01048.

## References

- Abramovitz, M., Listowsky, I., 1987. Selective expression of a unique glutathione-S-transferase Yb3 gene in rat brain. *J. Biol. Chem.* 262, 7770–7773.
- Abramovitz, M., Homma, H., Ishigaki, S., Tansey, F., Cammer, W., Listowsky, I., 1988. Characterization and localization of glutathione-S-transferases in rat brain and binding of hormones, neurotransmitters, and drugs. *J. Neurochem.* 50, 50–57.
- Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., Berger, E.A., 1996. CC CKR5: a RANTES, MIP-1  $\alpha$ , MIP-1 $\beta$  receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955–1958.
- Aoki, N., Ishii, T., Ohira, S., Yamaguchi, Y., Negi, M., Adachi, T., Nakamura, R., Matsuda, T., 1997. Stage specific expression of milk fat globule membrane glycoproteins in mouse mammary gland: comparison of MFG-E8, butyrophilin, and CD36 with a major milk protein, beta-casein. *Biochim. Biophys. Acta* 1334, 182–190.
- Azuma, Y., Inami, Y., Matsumoto, K., 2002. Alterations in cell surface phosphatidylserine and sugar chains during apoptosis and their time-dependent role in phagocytosis by macrophages. *Biol. Pharm. Bull.* 25, 1277–1281.
- Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., Schall, T.J., 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 385, 640–644.
- Beals, C.R., Sheridan, C.M., Turck, C.W., Gardner, P., Crabtree, G.R., 1997. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* 275, 1930–1934.
- Bennett, C.F., Yeoman, L.C., 1987. Microinjected glutathione-S-transferase Yb subunits translocate to the cell nucleus. *Biochem. J.* 247, 109–112.
- Bhat, R.V., Budd, S.L., 2002. GSK3beta signalling: casting a wide net in Alzheimer's disease. *Neurosignals* 11, 251–261.
- Cammer, W., Tansey, F., Abramovitz, M., Ishigaki, S., Listowsky, I., 1989. Differential localization of glutathione-S-transferase Yp and Yb subunits in oligodendrocytes and astrocytes of rat brain. *J. Neurochem.* 52, 876–883.
- Cammer, W., Tansey, F.A., Brosnan, C.F., 1990. Reactive gliosis in the brains of Lewis rats with experimental allergic encephalomyelitis. *J. Neuroimmunol.* 27, 111–120.
- Chang, M., Hong, Y., Burgess, J.R., Tu, C.P., Reddy, C.C., 1987. Isozyme specificity of rat liver glutathione-S-transferases in the formation of PGF2 alpha and PGE2 from PGH2. *Arch. Biochem. Biophys.* 259, 548–557.
- Chapman, G.A., Moores, K., Harrison, D., Campbell, C.A., Stewart, B.R., Strijbos, P.J., 2000. Fractalkine cleavage from neuronal membranes represents an acute event in the inflammatory response to excitotoxic brain damage. *J. Neurosci.* 20, RC87.
- Chomczynski, P., Sacchi, N., 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Chou, M.Y., Underwood, J.G., Nikolic, J., Luu, M.H., Black, D.L., 2000. Multisite RNA binding and release of polypyrimidine tract binding protein during the regulation of c-src neural-specific splicing. *Mol. Cell.* 5, 949–957.
- Christie, R.H., Chung, H., Rebeck, G.W., Strickland, D., Hyman, B.T., 1996. Expression of the very low-density lipoprotein receptor (VLDL-r), an apolipoprotein-E receptor, in the central nervous system and in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* 55, 491–498.
- Combadiere, C., Salzwedel, K., Smith, E.D., Tiffany, H.L., Berger, E.A., Murphy, P.M., 1998. Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *J. Biol. Chem.* 273, 23799–23804.
- Deng, H.K., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661–666.
- Denzel, A., Molinari, M., Trigueros, C., Martin, J.E., Velmurgan, S., Brown, S., Stamp, G., Owen, M.J., 2002. Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. *Mol. Cell. Biol.* 22, 7398–7404.
- Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., Geuze, H.J., 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* 113, 3365–3374.
- De Sarno, P., Li, X., Jope, R.S., 2002. Regulation of Akt and glycogen synthase kinase-3 beta phosphorylation by sodium valproate and lithium. *Neuropharmacology* 43, 1158–1164.
- Ellgaard, L., Frickel, E.M., 2003. Calnexin, calreticulin, and ERp57: teammates in glycoprotein folding. *Cell. Biochem. Biophys.* 39, 223–247.
- Emamian, E.S., Hall, D., Birnbaum, M.J., Karayiorgou, M., Gogos, J.A., 2004. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat. Genet.* 36, 131–137.
- Erichsen, D., Lopez, A.L., Peng, H., Niemann, D., Williams, C., Bauer, M., Morgello, S., Cotter, R.L., Ryan, L.A., Ghorpade, A., Gendelman, H.E., Zheng, J., 2003. Neuronal injury regulates fractalkine: relevance for HIV-1 associated dementia. *J. Neuroimmunol.* 138, 144–155.
- Ezekowitz, R.A., 2002. Local opsonization for apoptosis? *Nat. Immunol.* 3, 510–512.
- Faure, S., Meyer, L., Costagliola, D., Vaneensberghe, C., Genin, E., Autran, B., Delfraissy, J.F., McDermott, D.H., Murphy, P.M., Debre, P., Theodorou, I., Combadiere, C., 2000. Rapid progression to AIDS in HIV+ individuals with a structural variant of the chemokine receptor CX3CR1. *Science* 287, 2274–2277.

- Fernandez, E.J., Lolis, E., 2002. Structure, function, and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.* 42, 469–499.
- Ferrer, I., Barrachina, M., Tolnay, M., Rey, M.J., Vidal, N., Carmona, M., Blanco, R., Puig, B., 2003. Phosphorylated protein kinases associated with neuronal and glial tau deposits in argyrophilic grain disease. *Brain Pathol.* 13, 62–78.
- Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W., Bohle, R.M., 1998. Real-time quantitative RT-PCR after laser-assisted cell picking. *Nat. Med.* 4, 1329–1333.
- Gebicke-Haerter, P.J., Bauer, J., Schobert, A., Northoff, H., 1989. Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. *J. Neurosci.* 9, 183–194.
- Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., Nagata, S., 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature* 417, 182–187.
- Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., Nagata, S., 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304, 1147–1150.
- Harrison, J.K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R.K., Streit, W.J., Salafra, M.N., Adhikari, S., Thompson, D.A., Botti, P., Bacon, K.B., Feng, L., 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc. Natl. Acad. Sci. U. S. A.* 95, 10896–10901.
- Hatori, K., Nagai, A., Heisel, R., Ryu, J.K., Kim, S.U., 2002. Fractalkine and fractalkine receptors in human neurons and glial cells. *J. Neurosci. Res.* 69, 418–426.
- He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C.R., Sodroski, J., Gabuzda, D., 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* 385, 645–649.
- Huang, Y., Paxton, W.A., Wolinsky, S.M., Neumann, A.U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K., Erickson, D., Dragon, E., Landau, N.R., Phair, J., Ho, D.D., Koup, R.A., 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat. Med.* 2, 1240–1243.
- Hughes, P.M., Botham, M.S., Frentzel, S., Mir, A., Perry, V.H., 2002. Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia* 37, 314–327.
- Hynes, R.O., 2002. *Cell* 110, 673–687.
- Ignatius, M.J., Gebicke-Haerter, P.J., Skene, J.H.P., Schilling, J.W., Weisgraber, K.H., Mahley, R.W., Shooter, E.M., 1986. Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc. Natl. Acad. Sci. U. S. A.* 83, 1125–1129.
- Imahori, K., Hoshi, M., Ishiguro, K., Sato, K., Takahashi, M., Shiurba, R., Yamaguchi, H., Takashima, A., Uchida, T., 1998. Possible role of tau protein kinases in pathogenesis of Alzheimer's disease. *Neurobiol. Aging* 19, S93–S98.
- Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T., Imahori, K., 1993. Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* 325, 167–172.
- Karsai, A., Muller, S., Platz, S., Hauser, M.T., 2002. Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. *Biotechniques* 32, 790–792, 794–796.
- Kendzioriski, C.M., Zhang, Y., Lan, H., Attie, A.D., 2003. The efficiency of pooling mRNA in microarray experiments. *Biostatistics* 4, 465–477.
- Kohen, R., Neumaier, J.F., Hamblin, M.W., Edwards, E., 2003. Congenitally learned helpless rats show abnormalities in intracellular signaling. *Biol. Psychiatry* 53, 520–529.
- Lai, H.C., Grove, G., Tu, C.P., 1986. Cloning and sequence analysis of a cDNA for a rat liver glutathione-S-transferase Yb subunit. *Nucleic Acids Res.* 14, 6101–6114.
- Lai, H.C., Qian, B., Grove, G., Tu, C.P., 1988. Gene expression of rat glutathione-S-transferases. Evidence for gene conversion in the evolution of the Yb multigene family. *J. Biol. Chem.* 263, 11389–11395.
- Li, R., Gorelik, R., Nanda, V., Law, P.B., Lear, J.D., DeGrado, W.F., Bennett, J.S., 2004. Dimerization of the transmembrane domain of integrin alpha IIb subunit in cell membranes. *J. Biol. Chem.* 1 (April 2, Epub ahead of print).
- Marzolo, M.P., von Bernhard, R., Bu, G., Inestrosa, N.C., 2000. Expression of alpha(2)-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. *J. Neurosci. Res.* 60, 401–411.
- Masiakowski, P., Shooter, E.M., 1988. Nerve growth factor induces the genes for two proteins related to a family of calcium-binding proteins in PC12 cells. *Proc. Natl. Acad. Sci. U. S. A.* 85, 1277–1281.
- Matsui, T., Mori, T., Tateishi, N., Kagamiishi, Y., Satoh, S., Katsube, N., Morikawa, E., Morimoto, T., Ikuta, F., Asano, T., 2002. Astrocytic activation and delayed infarct expansion after permanent focal ischemia in rats: Part I. enhanced astrocytic synthesis of s-100beta in the periinfarct area precedes delayed infarct expansion. *J. Cereb. Blood Flow Metab.* 22, 711–722.
- McCutcheon, I.E., Hentschel, S.J., Fuller, G.N., Jin, W., Cote, G.J., 2004. Expression of the splicing regulator polypyrimidine tract-binding protein in normal and neoplastic brain. *Neuro-oncology* 6, 9–14.
- Muramatsu, Y., Kurosaki, R., Watanabe, H., Michimata, M., Matsubara, M., Imai, Y., Araki, T., 2003. Expression of S-100 protein is related to neuronal damage in MPTP-treated mice. *Glia* 42, 307–313.
- Nadri, C., Lipska, B.K., Kozlovsky, N., Weinberger, D.R., Belmaker, R.H., Agam, G., 2003. Glycogen synthase kinase (GSK)-3beta levels and activity in a neurodevelopmental rat model of schizophrenia. *Brain Res. Dev. Brain Res.* 141, 33–37.
- Northoff, H., Kabelitz, D., Galanos, C., 1986a. Interleukin 1 production for detection of bacterial polysaccharide in fetal calf sera and other solutions. *Immunol. Today* 7, 126–127.
- Northoff, H., Glück, D., Wölpl, A., Kubanek, B., Galanos, C., 1986b. Lipopolysaccharide-induced elaboration of interleukin-1(IL-1) by human monocytes: use for the detection of LPS in serum and influence of serum-LPS interactions. *Rev. Infect. Dis.* 9 (Suppl. 5), 599–602.
- Ohkubo, N., Lee, Y.D., Morishima, A., Terashima, T., Kikkawa, S., Tohyama, M., Sakanaka, M., Tanaka, J., Maeda, N., Vitek, M.P., Mitsuda, N., 2003. Apolipoprotein E and Reelin ligands modulate tau phosphorylation through an apolipoprotein E receptor/disabled-1/glycogen synthase kinase-3beta cascade. *FASEB J.* 17, 295–297.
- Oshima, K., Aoki, N., Negi, M., Kishi, M., Kitajima, K., Matsuda, T., 1999. Lactation-dependent expression of an mRNA splice variant with an exon for a multiply O-glycosylated domain of mouse milk fat globule glycoprotein MFG-E8. *Biochem. Biophys. Res. Commun.* 254, 522–528.
- Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J.-A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J.C., Gearing, D., 1997. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* 387, 611–617.
- Paxton, W.A., Martin, S.R., Tse, D., O'Brien, T.R., Skurnick, J., vanDevanter, N.L., Padian, N., Braun, J.F., Kotler, D.P., Wolinsky, S.M., Koup, R.A., 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. *Nat. Med.* 2, 412–417.
- Peng, X., Wood, C.L., Blalock, E.M., Chen, K.C., Landfield, P.W., Stromberg, A.J., 2003. Statistical implications of pooling RNA samples for microarray experiments. *BMC Bioinformatics* 4, 26.
- Perry, V.H., Lawson, L.J., Reid, D.M., 1994. Biology of the mononuclear phagocyte system of the central nervous system and HIV infection. *J. Leukocyte Biol.* 56, 399–406.
- Phiel, C.J., Wilson, C.A., Lee, V.M., Klein, P.S., 2003. GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. *Nature* 423, 435–439.
- Postler, E., Lehr, A., Schluesener, H., Meyermann, R., 1997. Expression of the S-100 proteins MRP-8 and -14 in ischemic brain lesions. *Glia* 19, 27–34.

- Power, C.A., Wells, T.N.C., 1996. Cloning and characterization of human chemokine receptors. *Trends Pharmacol. Sci. (TIPS)* 17, 209–213.
- Price, R.W., Brew, B., Sidtis, J., Rosenblum, M., Scheck, A.C., Cleary, P., 1988. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. *Science* 239, 586–592.
- Re, F., Belyanskaya, S.L., Riese, R.J., Cipriani, B., Fischer, F.R., Granucci, F., Ricciardi-Castagnoli, P., Brosnan, C., Stern, L.J., Strominger, J.L., Santambrogio, L., 2002. Granulocyte-macrophage colony-stimulating factor induces an expression program in neonatal microglia that primes them for antigen presentation. *J. Immunol.* 169, 2264–2273.
- Roderick, H.L., Lechleiter, J.D., Camacho, P., 2000. Cytosolic phosphorylation of calnexin controls intracellular Ca(2+) oscillations via an interaction with SERCA2b. *J. Cell Biol.* 149, 1235–1248.
- Samson, M., Libert, F., Doranz, B.J., Rucker, J., Liesnard, C., Farber, C.-M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R.J., Collman, R.G., Doms, R.W., Vassart, G., Parmentier, M., 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382, 722–725.
- Sherratt, A.J., Banet, D.E., Prough, R.A., 1990. Glucocorticoid regulation of polycyclic aromatic hydrocarbon induction of cytochrome P450IA1, glutathione-S-transferases, and NAD(P)H:quinone oxidoreductase in cultured fetal rat hepatocytes. *Mol. Pharmacol.* 37, 198–205.
- Stolzing, A., Grune, T., 2004. Neuronal apoptotic bodies: phagocytosis and degradation by primary microglial cells. *FASEB J.* 18, 743–745 (Epub February 6, 2004).
- Stoorvogel, W., Kleijmeer, M.J., Geuze, H.J., Raposo, G., 2002. The biogenesis and functions of exosomes. *Traffic* 3, 321–330.
- Stubbs, J.D., Lekutis, C., Singer, K.L., Bui, A., Yuzuki, D., Srinivasan, U., Parry, G., 1990. cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. *Proc. Natl. Acad. Sci. U. S. A.* 87, 8417–8421.
- Takahashi, M., Yasutake, K., Tomizawa, K., 1999. Lithium inhibits neurite growth and tau protein kinase I/glycogen synthase kinase-3beta-dependent phosphorylation of juvenile tau in cultured hippocampal neurons. *J. Neurochem.* 73, 2073–2083.
- Takashima, A., Honda, T., Yasutake, K., Michel, G., Murayama, O., Murayama, M., Ishiguro, K., Yamaguchi, H., 1998. Activation of tau protein kinase I/glycogen synthase kinase-3beta by amyloid beta peptide (25–35) enhances phosphorylation of tau in hippocampal neurons. *Neurosci. Res.* 31, 317–323.
- Tarozzo, G., Campanella, M., Ghiani, M., Bulfone, A., Beltramo, M., 2002. Expression of fractalkine and its receptor, CX3CR1, in response to ischaemia-reperfusion brain injury in the rat. *Eur. J. Neurosci.* 15, 1663–1668.
- Terraz, C., Brideau, G., Ronco, P., Rossert, J., 2002. A combination of cis-acting elements is required to activate the pro-alpha 1(I) collagen promoter in tendon fibroblasts of transgenic mice. *J. Biol. Chem.* 277, 19019–19026.
- Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., Amigorena, S., 1999. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* 147, 599–610.
- Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., Amigorena, S., 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* 166, 7309–7318.
- Thery, C., Zitvogel, L., Amigorena, S., 2002. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2, 569–579.
- Tulla, M., Pentikainen, O.T., Viitasalo, T., Kapyla, J., Impola, U., Nykvist, P., Nissinen, L., Johnson, M.S., Heino, J., 2001. Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. *J. Biol. Chem.* 276, 48206–48212.
- Upla, P., Marjomaki, V., Kankaanpaa, P., Ivaska, J., Hyypia, T., Van Der Goot, F.G., Heino, J., 2004. Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. *Mol. Biol. Cell.* 15, 625–636.
- Valcarcel, J., Gebauer, F., 1997. Post-transcriptional regulation: the dawn of PTB. *Curr. Biol.* 7, R705–R708.
- Verhoven, B., Schlegel, R.A., Williamson, P., 1995. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med.* 182, 1597–1601.
- Wagner, E.J., Garcia-Blanco, M.A., 2002. RNAi-mediated PTB depletion leads to enhanced exon definition. *Mol. Cell* 10, 943–949.
- Wehrle-Haller, B., Imhof, B.A., 2003. Integrin-dependent pathologies. *J. Pathol.* 200, 481–487.
- Xie, J., Lee, J.A., Kress, T.L., Mowry, K.L., Black, D.L., 2003. Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8776–8781.
- Yajima, N., Masuda, M., Miyazaki, M., Nakajima, N., Chien, S., Shyy, J.Y., 2002. Oxidative stress is involved in the development of experimental abdominal aortic aneurysm: a study of the transcription profile with complementary DNA microarray. *J. Vasc. Surg.* 36, 379–385.
- Zujovic, V., Benavides, J., Vige, X., Carter, C., Taupin, V., 2000. Fractalkine modulates TNF-alpha secretion and neurotoxicity induced by microglial activation. *Glia* 29, 305–315.