Diverging mechanisms for TNF-α receptors in normal mouse brains and in functional recovery after injury. From gene to behavior.

Albert Quintana[1], Amalia Molinero[1], Sergi Florit[1], Yasmina Manso[1], Gemma Comes[1], Javier Carrasco[1], Mercedes Giralt[1], Rehannah Borup[2], Finn Cilius Nielsen[2], Iain L. Campbell[3], Milena Penkowa[4], and Juan Hidalgo[1*]

[1]Institute of Neurosciences and Department of Cellular Biology, Physiology and Immunology, Animal Physiology unit, Faculty of Sciences, Autonomous University of Barcelona, Bellaterra, Barcelona, Spain; [2]Department of Clinical Biochemistry, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark; [3]School of Molecular and Microbial Biosciences G08. Maze Crescent, University of Sydney, Australia; [4]Section of Neuroprotection, Centre of Inflammation and Metabolism, The Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

Running head: Role of TNFα receptors: analysis by microarrays

* Correspondence to: Dr. Juan Hidalgo, Departamento de Biología Celular, Fisiología e Inmunología, Unidad de Fisiología Animal, Facultad de Ciencias, Universidad Autónoma de Barcelona, Bellaterra, Barcelona, Spain 08193. Telephone: (34) 93 581 2037. Fax: (34) 93 581 2390. E-mail: Juan.Hidalgo@uab.es

ABSTRACT

Cytokines, such as tumour necrosis factor (TNF)-α□□ and lymphotoxin-α□□, have been widely described to play important roles in the brain both in physiological conditions and after traumatic injury. However, the exact mechanisms involved in their function have not been fully elucidated. Here we give some insight on their role by using animals lacking either type 1 receptor (TNFR1KO) or 2 (TNFR2KO) and their controls (C57Bl/6). Both TNFR1KO and to a greater extent TNFR2KO mice showed increased exploration/activity neurobehavioral traits in the hole board test, such as rearings, head dippings and ambulations, compared to wild type mice, suggesting an inhibitory role of TNFR1/TNFR2 signaling. In contrast, no significant differences were observed in the elevated plus maze test, ruling out a major role of these receptors in the control of anxiety. We next evaluated the response to a freeze injury to the somatosensorial cortex. The effect of the cryolesion on motor function was evaluated with the horizontal ladder beam test, and the results demonstrated that both TNFR1KO and TNFR2KO mice made fewer errors, suggesting a detrimental role for TNFR1/TNFR2 signaling for coping with brain damage. Expression of ~22600 genes was analysed using an Affymetrix chip (MOE430A) at 0 (unlesioned), 1 or 4 days post lesion in the three strains. The results show a unique and major role of both TNF receptors on the pattern of gene expression elicited by the injury but also in normal conditions, and suggest that blocking of TNFR1/TNFR2 receptors may be beneficial after a traumatic brain injury.

INTRODUCTION

Cytokines are low molecular weight proteins that are involved in many cell communication processes both in physiological and pathophysiological situations. Among them, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α have classically been considered as key players in diverse biological processes. These factors were first suggested as main participants in response to injury (for review; (Rothwell and Hopkins 1995), as they are involved in the establishment and maintenance of the inflammatory response occurring after injury, being produced by and/or act on various cell types such as lymphocytes, endothelial cells and macrophage/microglia, producing secondary damaging effects that in turn lead to lymphocyte recruitment and activation and may cause neuronal cell death (Bluethmann et al. 1994; Hansson and Ronnback 2003; Lee and Benveniste 1999; Lee and Chau 2002; Raivich et al. 2003). However, their role at a physiological level is also emerging as an important one, with effects being described in the periphery but also in the CNS (for review, (Gregersen et al. 2000; Muñoz-Fernández and Fresno 1998).

Although being considered as *classical* cytokines and being studied for many years, their exact actions within the brain are far from being completely elucidated. In this regard, TNF-α might be the most elusive one. TNFα is a pleiotropic cytokine that has been described to affect synaptic signaling (Stellwagen and Malenka 2006) and has been suggested to play a role on mouse exploratory activity (Fiore et al. 1998), suggesting physiological actions of this cytokine from development to learning processes. Following CNS injury TNF-α is dramatically upregulated, being involved in the activation, proliferation and hypertrophy of mononuclear, phagocytic cells and gliosis, and eventually in the initiation of apoptosis. TNFα exerts its biological functions via interaction with two receptors, 55 kDa type-1 receptor (TNFR1) and 75 kDa type 2 receptor (TNFR2) which seem to mediate significantly different functions in several diseases (MacEwan 2002a; MacEwan 2002b) as well as in animal models (Fiore et al. 1998; Rothwell and Hopkins 1995). TNFR1 presents a homologous cytoplasmic domain known as the death domain (DD), shared with other receptors from the same superfamily of death receptors, which also includes Fas/APO-1/CD95 (for review see (Debatin 2004). TNFR2, although not possessing a DD sequence, can also lead to apoptosis via adaptor proteins, and has been involved in the control of cell proliferation

and in the elimination of autoreactive effector cells in a EAE paradigm (Suvannavejh et al. 2000). The related cytokine, lymphotoxin-α (formerly known as TNF-β), also signals through TNFR1 (Bossen et al. 2006).

Depending on the model used and on the timing studied, the effect of TNFα varies, showing beneficial as well as detrimental effects. This has given rise to the concept of dual role, suggesting that TNFα might be detrimental during the first stages after an injury but beneficial for the recovery (Shohami et al. 1999). It is interesting to realise that this concept of dual role is also being applied to inflammation itself, as it is still not clear the role that inflammation may have; it can be considered as a protective mechanism that isolates the injured area, destroys affected cells and repairs the extra-cellular matrix; however, chronic presence of inflammatory mediators may be followed by increased oxidative stress and cell death (for review see (Allan and Rothwell 2003; Hicks et al. 1999; Muñoz-Fernández and Fresno 1998; Sayer et al. 2002; Shohami et al. 1997). It is important to elucidate this concept as inflammation is common to many CNS pathologies, including stroke, seizures, trauma, ischemia (Carrasco et al. 2000; Gilks et al. 1998; Olanow 1993; Penkowa et al. 1999a), and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Campbell 1998; Yankner 1996).

Among all these CNS insults, Traumatic Brain Injury (TBI) is one of the leading causes of disability and death in the world, especially among young people (Prins and Hovda 2003), thus making it vital to understand the mechanisms involved. The study of the sequence of inflammatory cascade activation and the role of each mediator in the regulation of this process may provide some insight into when and why the damaging role of inflammation overrides the protective role. Cryolesion of the parietal cortex is an established experimental model (Cook et al. 1998) that features a standardized focal BBB disruption, massive brain edema, gliosis, recruitment of inflammatory cells, necrotic and apoptotic death, and upregulation of a number of genes attempting to increase neuronal survival and promoting the regeneration of brain parenchyma. We recently described that TNFR1 but not TNFR2 was involved in the induction of inflammation-related genes up to 24 hours post-lesion after cryolesion, showing a detrimental effect at 3 or 7 days post lesion(dpl) (Quintana et al. 2005). We herewith confirm and extend those results by taking advantage of the Affymetrix microarray

technology analyzing the expression of ~22600 genes in order to clarify the downstream signaling pathways of TNFR1 and TNFR2 receptors.

MATERIAL AND METHODS

Animals

Breeding pairs of homozygous TNFR1 and TNFR2 null mice (TNFR1 and TNFR2 KO, respectively) were kindly provided by Horst Bluethmann (Hoffmann-la Roche), and maintained on the inbred C57BL/6 genetic background. Control wild type (WT) mice (C57BL/6) were used in all experiments. All mice were kept under constant temperature and with free access to food and water. All experimental procedures were approved by the Ethical Committee of Comissió d'Experimentació animal del Departament d'Agricultura, Ramaderia I Pesca de la Generalitat de Catalunya.

Elevated Plus Maze test

The elevated plus-maze test (Pellow and File 1986) was adapted to fit mice size, and consisted of four white wooden arms (20 cm) at right angles to each other, connected to a central square. The apparatus is elevated 50 cm above the floor. Two of the opposite arms present high walls (enclosed arms, 15 cm high), whereas the other two are the open arms that have a 0.5 cm ridge to provide an additional grip. Each arm is divided into three equal sections. Mice were placed facing a closed arm and the subject was considered to be in a given arm when all four paws were inside. A black curtain surrounded the plus-maze to minimize external influences and a 40 W white bulb was placed 1.20 m above the centre of the apparatus. The procedure lasted 5 min. The time spent in open and closed arms, latency to the first open arm entry, number of entries (with all four paws) and ambulations in each type of arm (number of sections entered within a given arm), rearings, defecations and time spent in open/enclosed arms.

Hole Board test

The hole-board (File and Wardill 1975), adapted to mice size, consisted of a white rectangular box (40 cm × 40 cm × 20cm) with a floor divided into 16 equal squares, containing four empty holes (diameter 3 cm). Each animal was placed initially in the periphery of the apparatus facing the wall. A black curtain surrounded the plus-maze to minimize external influences and a 40 W white bulb was placed 1.20 m above the centre of the apparatus. The procedure lasted for 5 min. The number of central and

peripheral ambulations (with all four paws into the above mentioned squares), defecations, rearings and latency and number of explored holes were measured.

Horizontal ladder beam test

The horizontal ladder beam (Columbus Instruments, USA) is designed for a specific test of gait errors in small subjects (rats or mice). It consists of an elevated (50cm), narrow ladder (125.5cm length), mounted in a horizontal position. At one end there is a dark hide box of 30.5cm length. Subjects are placed on the far end of the ladder, and normally make their way directly across the ladder to the security of the opaque hide. During the experiment room lights are switched off and a 40W light is placed in the far end of the ladder.

The subjects are preconditioned by using a shocker set at 3 mA. With the shock on continuously, any slip between the ladder rungs to touch the lower plate results in an aversive shock. There is a 2.5 cm separation between rungs. Preconditioning takes place during 4 consecutive days, with 3 trials of 2 minutes each. After preconditioning, the subjects are then tested by a sensor. In sensor mode, each slip or gait error that causes a touch on the lower plate is registered as a count on the front panel counter and rear panel output. Animals only perform one trial during this period. The test was performed prior to the lesion (day 5) and at 4 days post lesion (dpl) (see next section).

Cryolesion procedure

Adult mice (3-4 months age) were lesioned under 1.5% isofluorane anesthesia (1L/m oxygen flow). The skull over the right frontoparietal cortex was exposed and a focal cryoinjury was carried out as previously described (Penkowa et al. 1999a) using a 0.4cm diameter dry ice pellet. Animals were allowed to recover and returned to the animal room, and were subsequently killed at different time points after cryolesion: 1dpl or 4dpl for microarray studies, and 2 days post lesion (dpl), 3dpl or 6dpl for RNAse protection assay studies (RPA). An independent experiment was carried out to check whether the operation itself (SHAM animals) had any consequence in brain gene expression. Therefore animals were sacrificed at different time points after surgery (1, 2 or 3 days after surgery) and compared to naïve animals. Another set of animals were sacrificed at 8 hours post lesion (hpl) or 24hpl for in-situ hybridization (ISH) studies.

In all cases, the ipsilateral cortex were dissected, frozen in liquid nitrogen and stored at –80ºC for microarray and RPA analyses. We used the whole ipsilateral cortex

rather than attempting to use the damaged area only. This was done because cryolesion produces a large lesion but to determine the limits of the lesion by eye when carrying out the dissection is not straight forward, so in order to eliminate the risk of incurring significant errors from animal to animal the entire cortex was removed. We acknowledge, nevertheless, that this causes a diluting effect that decreases sensitivity in the assay and may cause the loss of significant changes in low-expression genes. For ISH, the whole brain was submerged in chilled isopentane prior to freezing in liquid nitrogen and stored at -80ºC.

Gene expression and DNA microarray analysis

Total RNA was isolated from each hemisphere cortex sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified with RNeasy1 kit (Qiagen, Albertslund, Denmark). Purified RNA (5 μg) was used to synthesize double-stranded cDNA using Superscript Choice System (Invitrogen) with an oligo-dT primer containing a T7 RNA polymerase promoter (GenSet, Evry, France). The cDNA was used as a template for an in vitro transcription reaction to synthesize biotin-labeled antisense cRNA (BioArray™ High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY, USA). After fragmentation at 94°C for 35 min in fragmentation buffer (40 mM Tris, 30 mM MgOAc, 10 mM KOAc), the labeled cRNA was hybridized for 16 h to Affymetrix MOE430A arrays (Affymetrix Inc., Santa Clara, CA, USA) which contain ~22.600 mouse probe sets. The arrays were washed and stained with phycoerytrin streptavidin using the Fluidics Station 400 (Affymetrix Inc.). The arrays were scanned in the GeneArray 2500 scanner (Affymetrix Inc.), as described in the GeneChip (Affymetrix Inc.) protocol, to generate fluorescent images.

The image files were imported into the software package DNA-Chip Analyzer (dChip) developed by (Li and Wong 2001), which is freeware available at http://www.dchip.org. All the array files were normalized to the array with the median overall brightness using the multiarray invariant-set normalization method, which is based on probe values belonging to non-differentially expressed genes between the array being normalized and the baseline array (the invariant set). Expression values for each probe set were calculated according to the perfect match/mismatch difference model.

Gene filtering

A number of analysis methods for filtering the genes being up- or down-regulated in the different conditions were examined but finally we used a conservative strategy for the initial filtering followed by a standard statistical assay, two-way ANOVA with strain and cryolesion as main factors (see Results). Hierarchical clustering of the genes differentially expressed according to the ANOVA was carried out using the dChip software. Functional classification in gene-ontology categories of the genes belonging to each of the clusters identified was done with the DAVID program Version 2.1 (Dennis et al. 2003). All gene-ontology categories with three or more genes and a Fisher exact probability of less than 0.05 were selected.

RNase Protection Assay

Total RNA of injured ipsilateral cortex was isolated following standard methodology, using commercial TriPure Isolation Reagent Kit® (Roche). The construction and characterization of the multi-probe set for host-response (iNOS, Mac1, A20, ICAM 1, EB22/5, GFAP) was described elsewhere (Chiang et al. 1994).

For the synthesis of a radiolabeled antisense RNA probe set, the final reaction mixture contained 120 μCi, UTP (3000Ci/nmol, Amersham) UTP (73pmol), GTP, ATP, CTP (2.5 mmol each), DTT (100 nmol), transcription buffer (1X), Rnasin (20U), T7 polymerase (10U) (all from Promega) and an equimolar pool of *Eco*RI-linearized templates (150ng total). Dried probe was then dissolved (2.2 X 10^5 cpm/μl) in hybridization buffer and added to 10 μg of total RNA from brain, the samples were overlaid with mineral oil, heated to 96ºC, and then incubated at 56ºC for 12-16h. Single-stranded RNA was digested with RNAses A and T1. Hybridized RNA remained RNAse-protected. RNA duplexes were purified, precipitated and resolved on 5% acrylamide / 7M urea TBE sequencing gel. Dried gels were placed on Hyperfilm MP film (Amersham, UK) with Kodak MS intensifying screens (Kodak, Rochester, NY), and films were exposed at –70º during 16-168 hours depending on the probe set and the relative expression of the gene to quantify. The intensity of the bands was measured densitometrically (only for low-expression genes) and/or by phosphorimaging and normalized with L32 mRNA levels.

Metallothionein 1 mRNA In situ hybridization

Serial coronal cryostat sections (20 μm thick) were cut from the frozen brains with a cryostat (Reichert-Jung 2800 Frigocut E, Germany) and mounted on slides

coated with poly L-lysine, which were then maintained at −80 °C until the day of the analysis. *Mt1* was measured using the cDNA generously provided by Dr R. D. Palmiter (University of Washington, Seattle, WA). Preparation of sense and antisense probes and the *in situ* hybridization procedure were performed as previously described (Carrasco et al. 1998). Autoradiography was performed exposing the film (Hyperfilm-MP, Amersham, UK) to the slides for 3 days. All sections were prepared simultaneously and exposed to the same autoradiographic film. *Mt1* levels were semi quantitatively determined in three sections of each brain area per animal by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500MC system (Leica, Germany). The *Mt1* values shown are expressed in arbitrary units (number of pixels × optical density).

Microautoradiographies were obtained by exposing the sections to an autoradiographic emulsion (Amersham, UK) and developed according to manufacturer's instruction. Cells were counterstained with thionine.

Statistical analysis

Results were evaluated with one-way or two-way ANOVA depending on the experiment after checking the homogeneity of variances. When appropriated, post-hoc comparisons were carried out with SNK. All data in the text and figures are given as the arithmetic mean (± SEM) whenever possible.

RESULTS

Impairment of TNFR1 and TNFR2 signaling affects exploratory/activity neurobiological traits but not anxiety in mice

The hole board test mainly evaluates general locomotor activity and exploratory behavior of mice. The number of quadrants explored (ambulations) by mice is a mixed index of locomotor and exploratory activities, whereas the number of rearings and head dippings as well as the latency of each head dip serves as a more precise assessment for exploratory behavior. Although not being designed as an anxiety/fearfulness test, this can somewhat be assessed taking into account the number of inner quadrant ambulations (where the protection of walls is lost) and the number of defecations during the test.

Animals lacking either TNFR1 or TNFR2 (to a greater extent) presented an overall increase in the number of ambulations, suggesting an increase in locomotor/exploratory activity (Figure 1, top). This increase was exclusively due to a higher activity in outer quadrants, whereas the number of inner ambulations was not significantly different, suggesting that anxiety may not be affected by signaling through these receptors. In agreement with this, the number of defecations did not differ between strains. A more significant role of TNFR2 in exploratory behavior was also demonstrated by the increased number of head dippings (and the same tendency for their length) and rearings observed in the TNFR2KO mice compared to TNFR1KO and/or WT mice, indicating different roles for each receptor in these behavioral traits.

As stated before, the hole board test was not designed to test anxiety, although it likely had an influence on the results, so this trait was further evaluated by means of the elevated plus maze test (Figure 1, middle). The time an animal spends in the open arms or the number of ambulations in the open arms is used as an inverse index of anxiety in mice. Although there was some tendency for decreased time spent in the open arms for both KO strains, this did not reach statistical significance. Further supporting the hypothesis that anxiety/fearfulness is not affected by TNFR deficiency, defecations did not differ from WT mice.

Locomotor impairment caused by a focal brain injury is reduced in animals lacking TNF receptors

Putative clinical symptoms elicited by the cryolesion of the cortex were evaluated with the horizontal ladder beam. Interestingly, a difference between WT and TNFR1 or TNFR2 KO mice showed up during the training phase: WT mice scored worse that both KO strains from the very beginning (not shown), possibly, at least in part, because of the differences in locomotor activity described above. Therefore, before lesioning, emphasis was put into achieving a comparable baseline for all strains, which was obvious after 4 days of training (not shown) and the fifth day, when the mice were tested in the ladder beam before being cryolesioned (Figure 1, bottom). As expected, cryoinjury of the cortex significantly increased the number of errors recorded in all strains at 4dpl, but this number was higher in the WT mice than in the two KO strains (Figure 1, bottom). These results strongly suggest a detrimental role for TNFR1/TNFR2 in the recovery of the lesion.

Analysis of global gene expression by microarrays

Taking into consideration the results shown above, we decided to look for gene expression changes occurring in the brain due to the cryolesion that may be responsible for the beneficial effects observed in animals lacking TNF receptors. To this end, we took advantage of a valuable tool such as microarray chips to assess the expression of as many as ~22600 genes at the same time. Unlesioned animals were also included.

As described by our group in a previous paper (Poulsen et al. 2005), using dChip software (http://www.dchip.org for more details) we generated a combined gene list by comparing the genes up- and down-regulated at each time point for each strain with the unlesioned WT mice. The used criteria to identify the genes were: 1.5-fold change (that is, a 50% increase or decrease) and an absolute change in expression values of 100 expression units. With this procedure a list of 555 IDs (corresponding to 477 genes) was produced. Since several animals per group were analyzed we identified which among these genes were statistically significantly affected. To this end, the expression values were exported to a standard statistical software, SPSSx (SPSS, Chicago, IL, USA), where a conventional two-way ANOVA was performed (timings of lesion and strain as main factors, $p < 0.05$). However, we would like to point out that, although comparing to similar published array analysis the number of animals has been elevated (n=3 for each time point, except for C57 4dpl n=2, making a total of 26 mice), it is still not high enough to ensure that ANOVA is able to pick up all affected genes, since in many occasions a given gene only changed its expression in a single timing, thus making it

difficult to obtain statistical significance between strains when analyzing all groups together as needed for this experimental design. On the other hand, ANOVA is able to identify genes changing only moderately but in several timings.

This approach reduced the number of genes to 419. As we have recently described (Poulsen et al. 2005), cryolesion had a huge impact on gene expression, both at 1 and 4dpl. Out of the 419 genes, 258 were affected by cryolesion only, 80 genes were found to be affected by strain only, and 81 were affected by both factors (Supplementary table I). The pattern of expression of these 419 genes was analyzed using the high-level analysis "Hierarchical clustering" utility of dChip, which clusters the genes according to their pattern of expression (not shown because of its large size which makes impossible to read the gene names). For the sake of clarity this large hierarchical clustering has been split into six smaller ones: genes mainly up-regulated by the cryolesion at 1 dpl (Figure 2), at 1-4dpl (Figure 3), or mainly at 4dpl (Figure 4); genes that in contrast were down-regulated either at 1dpl (Figure 5) or at 4dpl (Figure 6); and those only affected by either TNFR1 or TNFR2 deficiency (Figure 7). In all cases, each row represents a gene and each column represents the mean value for each strain and timing. The relative gene is expression is color-coded, being red, white and blue colors expression levels above mean, mean or lower than mean expression of a gene across all samples, respectively. The more intense the color (either red or blue), the higher the difference (fold induction) is.

Taking advantage of the NIH based DAVID software (DAVID v2.1, http://david.abcc.ncifcrf.gov/) (Dennis et al. 2003) a functional classification for all the genes selected in our array was performed (Supplementary table II). This approach confirmed that many of the genes up-regulated by cryolesion can be classified as genes involved in responses to stimulus, to wounding or to inflammation, whereas many of the genes down-regulated at 1dpl can be classified as genes involved in neuronal differentiation and those down-regulated at 4dpl are mainly involved in microtubule stability. This classification enables a better understanding of the different responses elicited by cryolesion, showing that in agreement with previous results, this model is able to elicit an inflammatory response at the gene level (Penkowa et al. 2006; Poulsen et al. 2005; Quintana et al. 2005).

It is obvious that it is not possible to undertake a throughout discussion for all these genes, and only some of them will be discussed as representative of the overall response. Instead of discussing the response to the lesion itself, we have rather focused

in analyzing the specific changes derived of either TNFR1 or TNFR2 deficiency. Full information is provided by Figures 2-7 and the two Supplementary files.

Impairment of TNF signaling affects early transcription factor activation

The first stages of the response to cryolesion involve the activation of many cell signaling and transcription factors. This activation depends on the diverse stimuli that the cell is receiving and will dictate the fate of the cells surrounding the affected area. *NFκBi*, *C/EBP*, *c-jun* and *c-fos* were found among the genes promptly up-regulated by cryolesion (Figure 2). Although not reaching statistical significance, all these factors tended to be down-regulated in animals lacking any of TNF receptors. C-fos and c-jun can dimerize to constitute AP-1, which is also induced by NFκB. *Gadd45β* and *Gadd45γ*, which are genes downstream NFκB pathway presented a lower expression both in TNFR1KO and TNFR2KO mice compared to wild type mice (Figure 2). These results would suggest a reduced *NFκB* activation, which can be considered as a consequence of TNF impairment, as TNF has been extensively described to be a major activator of NFκB pathway. This is in agreement with our previous work (Quintana et al. 2005) where we described many NFκB activated genes to present a lower induction in TNFR1KO mice after cryolesion. It has to be taken into account that although many animals were used (for microarray standards), the number may still not be high enough for statistical purposes alone, therefore further effort has been placed to confirm array results by other means (see below).

TNFR1, but not TNFR2, is involved in early inflammatory response

In our previous work (Quintana et al. 2005) we described from a histopathological view that only animals lacking TNFR1 presented fewer inflammatory related markers both at 3 and 7dpl, and a simplified gene expression analysis allowed us to observe that this was visible up to 24hpl, leading us to propose the TNFR1 pathway as the one involved in early inflammatory response. Nevertheless, TNFR2 KO mice were not evaluated in that regard. Herewith we extend the gene expression analysis up to 4dpl in both TNFR1 and TNFR2 KO mice, and by using global gene expression analyses by Affymetrix microarrays. In agreement with our previous study, TNFR1KO mice showed a reduced inflammatory response at 1dpl, as many inflammation related genes downstream proinflammatory cytokines signalling pathways, such as *interleukin-*

1 receptor, interleukin-6 driven transcription factor signal transducer and activator of transcription-3 (*stat-3*), or the silencer of cytokine signaling 3 (*socs3*) were down-regulated in TNFR1KO compared to TNFR2KO or WT mice (Figure 2). Also chemokines such as *ccl3, ccl6, cc10* and *ccl12* followed the same pattern (Figure 2-4), and so did genes involved in the inflammatory response such as *metallothionein 1* and *2* (Figure 2), which are anti-inflammatory factors induced as a result of tissue injury, glial fibrillary acidic protein (*gfap*) (Figure 3) or tissue inhibitor of metalloproteinase 1 (*timp1*) expression at 1dpl (Figure 2). However, no apparent changes were found in expression of these genes at 4dpl between strains (Figure 4), suggesting that the role of TNFR1 in inflammatory response is limited to the first phases whereas other factors may compensate its action at latter stages.

Gene expression is widely affected by TNFR2 deficiency

A great number of genes were found to be affected by TNFR2 deficiency in unlesioned mice, and remained basically unchanged after the lesion. Most of them are found in Figures 6 and 7. Diverse physiologically important genes were strikingly down-regulated in TNFR2KO mice, such as the beta subunit of cAMP protein kinase (*prkacb*), phospholipase c (*plc*) and calcineurin B, type I (*Cnb1*), which may play a role in learning and memory processes. Another important group of genes are involved in microtubule formation and stabilization, contributing to neuronal development, maintenance and even cell survival: these include microtubule-associated protein 1B (*mtap1B*), microtubule-associated protein 2 (*map2*), and kinesin family member 5C (*Kif5c*).

TNFR2 deficiency also down-regulated the expression of genes involved in synaptic formation and stabilization and the control of membrane excitability, such as synaptotagmin (*syt1*), dynamin-1 like (*dpl1*), SH3-domain GRB2-like 2 (*Sh3gl2*) and potassium voltage-gated channel, shaker-related subfamily, beta member 1 *(kcnab1)* (Leicher et al. 1996). Other interesting genes down-regulated in TNFR2KO mice are related to the tyroid-hormone pathway, namely, tyrotroph embryonic factor (*tef*), the tyroid-hormone receptor alpha gene (*thra*) and the PFTAIRE protein kinase 1 (*pftk1*). This family of genes has been described to be important factors in neuronal and oligodendrocyte development.

On the other hand, TNFR2 deficiency increased the expression of genes important for neuronal development and maturation but also for neuronal survival after an injury, such as glial cell line derived neurotrophic factor (*gdnf*).

Microarray results are confirmed by other approaches

There are little doubts that microarray results are reliable, yet it is always important to confirm their results independently; we have done this by means of Ribonuclease Protection assay (RPA) and in situ hybridization. By means of RPA, we confirmed that *gfap* gene expression was induced by the cryolesion at 2-6dpl and that its expression (along with another inflammation-related gene such as the acute phase gene *ebb22/5*) was remarkably reduced at 2dpl in TNFR1KO mice (Figure 8a,b) but not in TNFR2 KO mice (Figure 9a,b). *gfap* expression judged by RPA was essentially similar in the three strains at 3-6dpl, which again is in accordance with the microarray results.

No differences were found for Mac1 or ICAM expression at any of the time points studied other than the effect of the cryolesion itself, although remarkably at 6dpl the two KO strains showed opposite tendencies (Figures 8,9).

It is noteworthy that no differences were observed between naïve and SHAM animals, neither regarding the expression of analyzed genes (Figure 10) nor in the horizontal ladder beam behavioral test (not shown).

Finally, by in situ hybridization we also confirm that TNFR1 but not TNFR2 deficiency decreases *metallothionein-1* expression (Figure 11).

DISCUSSION

TNFR1/TNFR2 signaling is involved in locomotor activity and exploratory behavior

Although the role of TNFα in different injuries in the CNS has been extensively described (for review, (Muñoz-Fernández and Fresno 1998), much less work has been performed in describing the effect of TNFα signaling in animal behavior. Taking advantage of mice knocked-out either for TNFR2 or TNFR2 we first studied the behavioral pattern of these animals prior to any insult to the CNS. For this purpose we used two very well established behavioral tests such as the *hole-board* and the *elevated plus maze*. The nature of each test is different, being the first a test that mostly evaluates exploratory behavior and locomotor activity (File and Wardill 1975) and the latter basically a test for anxiety (Pellow and File 1986). However, it must be kept in mind that any behavior observed in mice performing these tests is never exclusively due to one factor or the other, but to a mix of different components.

Lack of either receptor alone was capable of increasing the locomotor/exploratory activity of mice, as observed by the increase in the number of total ambulations, which was due to an increase in the number of outer quadrant ambulations. In agreement with these results, TNF-α over-expressing mice presented a decreased locomotor activity (Fiore et al. 1998). However, inner quadrant ambulations did not differ to WT mice. This fact, together with a lack of effect of TNFR deficiencies on the number of defecations, does not support a role of these receptors in the control of anxiety. In accordance, no differences between the three strains were observed with a more sensitive anxiety test, the elevated plus maze. Thus, unlike other pro-inflammatory cytokines like IL-6 (Armario et al. 1998) or IL-2 (Pawlak et al. 2003), TNF-α does not seem to play a role in anxiety behavior. Since lymphotoxin-α may signal through the TNFR1 (Bossen et al. 2006), these results also may apply to this cytokine.

We were also able to demonstrate that lack of TNFR2 is able to increase exploratory activity, as shown by the increased number of head dippings and rearings in the hole board test, suggesting a different behavioral pattern in these mice. The microarray results clearly show a remarkable effect of TNFR2 deficiency on the expression of many genes involved in development stages as well as synaptic activity (supplementary tables and II). This suggests that TNFR2 signaling may act during developmental stages changing the pattern of genes expressed and that this, in turn, will

end up in the effects we observed in adult mice in these behavioral traits, although activational effects cannot be ruled out. It is noteworthy to mention that TNF-α has recently been described to play a role in synaptic scaling (Stellwagen and Malenka 2006), so it may well fit with our hypothesis.

TNF receptors signaling significantly affects the response to brain injury.

As stated above, traumatic brain injury is one of the main causes of disability and death, especially among young people. In order to study the role of TNFR1 and TNFR2 in the response elicited by the injury, we studied TNFR1KO and TNFR2KO mice as well as wildtype mice in the cryolesion model. This model of injury of the cortex has extensively been used in our lab, and has allowed us to demonstrate a role for IL-6 (Penkowa et al. 2003; Penkowa et al. 1999b; Poulsen et al. 2005) as well as preliminary roles for TNF receptors (Quintana et al. 2005). In the latter study, we described that TNFR1, but not TNFR2, was involved in the establishment of the inflammatory response to a cryolesion of the cortex, suggesting a detrimental role for TNFR1 but not TNFR2 after an injury. However, the role of TNF-α/lymphotoxin-α and their receptors remain conflicting, with many studies showing both neuroprotective and neuropathological effects depending on the type of cell, production of other cytokines, oxidative stress, experimental paradigm, timing, etc. A neuroprotective role in the CNS has been described in mice lacking both TNF receptors in ischemia and kainic acid-induced seizures (Bruce et al. 1996; Gary et al. 1998). Opposing roles, in contrast, have been described for both receptors in a multiple sclerosis animal paradigm, having TNFR1 a detrimental effect and the opposite for TNFR2 (Eugster et al. 1999; Suvannavejh et al. 2000). However, no effect on damage following deletion of either TNFR1 or TNFR2, alone, is observed in the axotomized facial motor nucleus model (Raivich et al. 2002), but if both receptors are missing an almost complete prevention of cell death is seen (Raivich et al. 2002). It is thus clear that further analyses of these mice are needed.

The clinical effect of a lesion to the cortex was readily observed in the horizontal ladder beam. Thus, the cryolesion increased the number of errors made by mice of all strains. Confirming our previous neuropathological results (Quintana et al. 2005), TNFR1KO animals performed better in this test, suggesting a decreased brain damage from 4 to 6 days after the lesion. This in principle fits well with the general description of TNFR1 signaling pathway as an inflammatory pathway. However, to our surprise,

animals lacking receptor 2 also presented a better outcome in the horizontal ladder beam, suggesting that both receptors in fact may be considered detrimental pathways during brain injury despite enormous differences in inflammation-related gene expression and neuropathology compared to WT mice (Quintana et al. 2005). To give insight into the roles of TNFR1 and TNFR2 signaling pathways, we have studied the expression of more than 20000 genes by means of microarray technology at 0, 1 or 4 days post-lesion.

We were able to identify many genes that were affected by cryolesion, by strain or by both (see Figures 2-7 and the supplementary files). Transcription factors such as *c/ebp*, *c-jun* and *c-fos* tended to be down-regulated in animals lacking any of the TNF receptors. Of special relevance is *NFκB*, as it is considered a key factor in TNF-α signaling. Decreased NFκB signaling in TNF deficient animals has been suggested in many studies (Kim et al. 2001; Quintana et al. 2005; Sullivan et al. 1999), although the final outcome varied depending on the downstream genes induced by this factor. NFκB can induce both inflammatory and anti-inflammatory genes (for review see (Hallenbeck 2002), so depending on this balance the final outcome can be one or another. Further evidence of decreased NFκB activity may be the lower expression observed for *c-jun*, *c-fos*, *Gadd45β* and *Gadd45γ*, which are in the NFκB downstream pathway.

When looking for inflammatory related genes, microarray confirmed our previous results (Quintana et al. 2005), as only TNFR1KO animals presented a tendency for lower gene expression of genes involved in pro-inflammatory cytokine signaling at 1dpl, namely *interleukin 1 receptor I* which is responsible for the effects of the proinflammatory cytokine IL-1 (Rothwell and Hopkins 1995), signal transducer and activator of transcription- 3 (*stat-3*), a main effector of the IL-6 cascade, or the silencer of cytokine signaling 3 (*socs-3*), involved in the regulation of cytokine signaling. Gene expression for the chemokines *ccl3, ccl6, cc10* and *ccl12*, which play important roles in attracting immune cells (for review, (Wang et al. 2002), was also decreased. All these effects are consistent to what is known of the phenotype of these mice. The anti-inflammatory, antioxidant proteins *metallothionein* 1 and 2 were found to be reduced in TNFR1KO mice at 1dpl. These proteins have been extensively described as anti-inflammatory and anti-apoptotic factors by our group (Hidalgo et al. 2002; Penkowa 2006), and have been described to be induced by IL-6 via STAT-3 signaling in liver (Lee et al. 1999). *Timp1*, a gene that counteracts the negative effects of matrix

metalloproteinases was also downregulated at 1dpl and so was glial fibrillary acidic acid expression which expression was decreased only in TNFR1KO mice at 1dpl. However, levels remained similar in all strains at 4dpl.

We confirmed these results by looking at gene expression of selected genes such as *gfap* and *metallothionein-1* by other means such as RPA or *in situ* hybridization, techniques more sensitive than microarray. TNFR1KO mice showed a reduced *gfap* expression, an effect more obvious at 2dpl than latter, and also metallothionein-I expression was decreased in TNFR1 but not TNFR2 KO mice. These results are in line with those obtained in the microarray analyses. The expression of the acute phase-gene *eb22/5* (Campbell et al. 1994) was also decreased at 2dpl in the TNFR1 KO mice, an effect compatible with the overall decreased inflammatory response of these mice. All in all, we were able to confirm a role for TNFR1 in early stages of inflammatory response, which likely underlies, at least in part, the observed lower functional and neuropathological damage, in agreement with previous results suggesting a detrimental role for TNFR1 (Eugster et al. 1999; Quintana et al. 2005; Suvannavejh et al. 2000) in different paradigms.

As discussed above, it was totally unexpected that the TNFR2KO would manifest lower symptoms of brain damage as demonstrated by the functional test horizontal ladder beam. These mice do not differ from wildtype regarding inflammation and prototypical proinflammatory gene expression, as the TNFR1 KO mice do. It is thus obvious that the mechanisms underlying such a better functional performance differ dramatically in both KO mouse models. Hence, the microarrays study proven to be a valuable and unique tool for elucidating this differential action between both receptors. It was striking to observe the large number of genes that were constitutively affected in TNFR2KO mice in comparison to either WT or TNFR1 KO mice. As stated briefly in the results section, many of these genes are involved in development, memory processes, synaptic plasticity and microtubule stabilization. It is impossible to discuss all genes identified, and thus the reader is invited to see Figures 2-7 as well as the supplementary tables. Further studies are warranted for a complete understanding of the contribution of each TNF receptor in brain physiology.

Traumatic brain injury is one of leading causes of transient and permanent disabilities, and thus it is essential to gain insight into the mechanisms involved. Microarray technology provides a powerful tool in this regard, which has been used successfully in a number of models of brain injury by microarrays (Dhodda et al. 2004;

Faden et al. 2005; Kury et al. 2004; Long et al. 2003; Lu et al. 2004; Natale et al. 2003; Natale et al. 2004; Rao et al. 2003; Read et al. 2001 ; Roth et al. 2003; Tang et al. 2002). See also (Dash et al. 2004; Gebicke-Haerter 2005) for review. While this is a significantly well-known scenario, the comparison of wildtype animals with genetically modified ones add a further layer of complexity to the analyses that provides great insight. Our group has previously described the effect of cryolesion in animals lacking interleukin 6 (Poulsen et al. 2005) or metallothioneins 1 and 2 (Penkowa et al. 2006), and in this study we have analyzed the role(s) of the TNF receptors with the same approach. This opens new venues for understanding the importance of this major cytokine in brain physiology.

Acknowledgements.

These studies were supported by Ministerio de Ciencia y Tecnología and Feder SAF2005-00671 and European Comission FP6 Integrated Project Exgenesis (Ref. LSHM-CT-2004-005272) (JH); by NIH grants NS36979 and MH62231 (ILC); and by The Lundbeck Foundation, Hørslev-fonden, Novo Nordisk, The Danish Medical Research Council, Direktør Ib Henriksens Fond, The Danish Medical Association Research Fund- The Wacherhausens Legat, Grosserer Johan Quentin og Hustrus Legat, Marshalls Fond, Kathrine og Vigo Skovgaards Fond, Dir. Jacob Madsen´s Fond, Gerda og Aage Haensch´s Fond, Eva og Henry Frænkels Mindefond, Scleroseforeningen, Dir. Ejnar Jonasson´s Fond, Warwara Larsens Fond, Karen A. Tolstrups Fond, Dansk Parkinsonforening, Fru Lily Benthine Lunds Fond, Hans og Nora Buchards Fond, Holger Rabitz Mindelegat, Ragnhild Ibsens Legat for Medicinsk Forskning, Grosserer Vald. Foersom og Hustrus Fond, Hestehandler Ole Jacobsens Mindelegat, Asta Florida Boldings Mindelegat´s Fond (MP).

REFERENCES

Allan SM, Rothwell NJ. 2003. Inflammation in central nervous system injury. Philos Trans R Soc Lond B Biol Sci 358:1669-1677.

Armario A, Hernández J, Bluethmann H, Hidalgo J. 1998. IL-6 deficiency leads to increased emotionality in mice: evidence in transgenic mice carrying a null mutation for IL-6. J Neuroimmunol 92:160-169.

Bluethmann H, Rothe J, Schultze N, Tkachuk M, Koebel P. 1994. Establishment of the role of IL-6 and TNF receptor using gene knockout mice. J Leukoc Biol 56:565-570.

Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P. 2006. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. J Biol Chem 281:13964-13971.

Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP. 1996. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nat Med 2:788-794.

Campbell IL. 1998. Transgenic mice and cytokine actions in the brain: bridging the gap between structural and functional neuropathology. Brain Res Rev 26:327-336.

Campbell IL, Hobbs MV, Kemper P, Oldstone MBA. 1994. Cerebral expression of multiple cytokine genes in mice with lymphocytic choriomeningitis. J Immunol 152:716-723.

Carrasco J, Hernández J, González B, Campbell IL, Hidalgo J. 1998. Localization of metallothionein-I and -III expression in the CNS of transgenic mice with astrocyte-targeted expression of interleukin 6. Exp Neurol 153(2):184-194.

Carrasco J, Penkowa M, Hadberg H, Molinero A, Hidalgo J. 2000. Enhanced seizures and hippocampal neurodegeneration following kainic acid induced seizures in metallothionein-I+II deficient mice. Eur J Neurosci 12:2311-2322.

Cook JL, Marcheselli V, Alam J, Deininger PL, Bazan NG. 1998. Temporal changes in gene expression following cryogenic rat brain injury. Mol Brain Res 55:9-19.

Chiang C-S, Stalder A, Samimi A, Campbell IL. 1994. Reactive gliosis as a consequence of interleukin 6 expression in the brain: studies in transgenic mice. Dev Neurosci 16:212-221.

Dash PK, Kobori N, Moore AN. 2004. A molecular description of brain trauma pathophysiology using microarray technology: an overview. Neurochem Res 29:1275-1286.

Debatin KM. 2004. Apoptosis pathways in cancer and cancer therapy. Cancer Immunol Immunother 53:153-159.

Dennis GJ, Sherman BT, Hosack DA, Yang J, Baseler MW, Lane HC, Lempicki RA. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biology 4:P3.

Dhodda VK, Sailor KA, Bowen KK, Vemuganti R. 2004. Putative endogenous mediators of preconditioning-induced ischemic tolerance in rat brain identified by genomic and proteomic analysis. J Neurochem 89:73-89.

Eugster HP, Frei K, Bachmann R, Bluethmann H, Lassmann H, Fontana A. 1999. Severity of symptoms and demyelination in MOG-induced EAE depends on TNFR1. Eur J Immunol 29:626-632.

Faden AI, Movsesyan VA, Knoblach SM, Ahmed F, Cernak I. 2005. Neuroprotective effects of novel small peptides in vitro and after brain injury. Neuropharmacology 49:410-424.

File SE, Wardill AG. 1975. Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacologia 44:53–59.
Fiore M, Alleva E, Probert L, Kollias G, Angelucci F, Aloe L. 1998. Exploratory and displacement behavior in transgenic mice expressing high levels of brain TNF-alpha. Physiol Behav 63:571-576.
Gary DS, Bruce-Keller AJ, Kindy MS, Mattson MP. 1998. Ischemic and excitotoxic brain injury is enhanced in mice lacking the p55 tumor necrosis factor receptor. J Cereb Blood Flow Metab 18:1283-1287.
Gebicke-Haerter P, J. . 2005 Microarrays and expression profiling in microglia research and in inflammatory brain disorders. J Neurosci Res 81:327-341.
Gilks CB, Price K, Wright JL, Churg A. 1998. Antioxidant gene expression in rat lung after exposure to cigarette smoke. Am J Pathol 152(1):269-278.
Gregersen R, Lambertsen K, Finsen B. 2000. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. . J Cereb Blood Flow Metab 20:53-65.
Hallenbeck JM. 2002. The many faces of tumor necrosis factor in stroke. Nat Med 8:1363-1368.
Hansson E, Ronnback L. 2003. Glial neuronal signaling in the central nervous system. . Faseb J 17 341-348.
Hicks RR, Li C, Zhang L, Dhillon HS, Prasad MR, Seroogy KB. 1999. Alterations in BDNF and trkB mRNA levels in the cerebral cortex following experimental brain trauma in rats. J Neurotrauma 16:501-510.
Hidalgo J, Penkowa M, Giralt M, Carrasco J, Molinero A. 2002. Metallothionein expression and oxidative stress in the brain. Methods Enzymol 348:238-249.
Kim GM, Xu J, Xu J, Song SK, Yan P, Ku G, Xu XM, Hsu CY. 2001. Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury. J Neurosci 21:6617-6625.
Kury P, Schroeter M, Jander S. 2004. Transcriptional response to circumscribed cortical brain ischemia: spatiotemporal patterns in ischemic vs. remote non-ischemic cortex. Eur J Neurosci 19:1708-1720.
Lee DK, Carrasco J, Hidalgo J, Andrews GK. 1999. Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression. Biochem J 337(Pt 1)():59-65.
Lee SJ, Benveniste EN. 1999. Adhesion molecule expression and regulation on cells of the central nervous system. . J Neuroimmunol 98:77-88.
Lee TS, Chau LY. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 8:240-246.
Leicher T, Roeper J, Weber K, Wang X, Pongs O. 1996. Structural and functional characterization of human potassium channel subunit β1 (KCNA1B). Neuropharmacology 35:787-795.
Li C, Wong WH. 2001. Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. Proc Natl Acad Sci USA 98:31-36.
Long Y, Zou L, Liu H, Lu H, Yuan X, Robertson CS, Yang K. 2003. Altered expression of randomly selected genes in mouse hippocampus after traumatic brain injury. J Neurosci Res 71:710-720.
Lu XC, Williams AJ, Yao C, Berti R, Hartings JA, Whipple R, Vahey MT, Polavarapu RG, Woller KL, Tortella FC, Dave JR. 2004. Microarray analysis of acute and

delayed gene expression profile in rats after focal ischemic brain injury and reperfusion. J Neurosci Res 77:843-857.
MacEwan DJ. 2002a. TNF ligands and receptors-a matter of life and death. Br J Pharmacol 135:855-875.
MacEwan DJ. 2002b. TNF receptor subtype signalling: differences and cellular consequences. Cell Signal 14:477-492.
Muñoz-Fernández MA, Fresno M. 1998. The role of tumour necrosis factor, interleukin 6, interferon-gamma and inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol 56:307-340.
Natale JE, Ahmed F, Cernak I, Stoica B, Faden AI. 2003. Gene expression profile changes are commonly modulated across models and species after traumatic brain injury. J Neurotrauma 20:907-927.
Natale JE, Knight JB, Cheng Y, Rome JE, Gallo V. 2004. Metallothionein I and II mitigate age-dependent secondary brain injury. J Neurosci Res 78:303-314.
Olanow C. 1993. A radical hypothesis for neurodegeneration. Trends Neurosci 16:439-444.
Pawlak CR, Ho YJ, Schwarting RK, Bauhofer A. 2003. Relationship between striatal levels of interleukin-2 mRNA and plus-maze behaviour in the rat. Neurosci Lett 341:205-208.
Pellow S, File SE. 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat Pharmacol Biochem Behav 24:525–529.
Penkowa M. 2006. Metallothioneins are multipurpose neuroprotectants during brain pathology. FEBS J 273:1857-1870.
Penkowa M, Cáceres M, Borup R, Nielsen FC, Poulsen CB, Quintana A, Molinero A, Carrasco J, Florit S, Giralt M, Hidalgo J. 2006. Novel roles for Metallothionein-I+II (MT-I+II) in defense responses, neurogenesis and tissue restoration after traumatic brain injury: insights from global gene expression profiling in wildtype and MT-I+II knockout mice. J Neurosci Res in press.
Penkowa M, Carrasco J, Giralt M, Moos T, Hidalgo J. 1999a. CNS wound healing is severely depressed in metallothionein I- and II-deficient mice. J Neurosci 19(7):2535-2545.
Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernández J, Molinero A, Campbell IL, Hidalgo J. 2003. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp Neurol 181:130-148.
Penkowa M, Moos T, Carrasco J, Hadberg H, Molinero A, Bluethmann H, Hidalgo J. 1999b. Strongly compromised inflammatory response to brain injury in interleukin-6-deficient mice. Glia 25(4):343-357.
Poulsen CB, Penkowa M, Borup R, Nielsen FC, Caceres M, Quintana A, Molinero A, Carrasco J, Giralt M, Hidalgo J. 2005. Brain response to traumatic brain injury in wild-type and interleukin-6 knockout mice: a microarray analysis. J Neurochem 92:417-432.
Prins ML, Hovda DA. 2003. Developing experimental models to address traumatic brain injury in children. J Neurotrauma 20:123-137.
Quintana A, Giralt M, Rojas S, Penkowa M, Campbell IL, Hidalgo J, Molinero A. 2005. Differential role of tumor necrosis factor receptors in mouse brain inflammatory responses in cryolesion brain injury. J Neurosci Res 82:701-716.
Raivich G, Bohatschek M, Werner A, Jones LL, Galiano M, Kloss CU, Zhu XZ, Pfeffer K, Liu ZQ. 2003. Lymphocyte infiltration in the injured brain: role of proinflammatory cytokines. J Neurosci Res 72:726-733.

Raivich G, Liu ZQ, Kloss CU, Labow M, Bluethmann H, Bohatschek M. 2002. Cytotoxic potential of proinflammatory cytokines: combined deletion of TNF receptors TNFR1 and TNFR2 prevents motoneuron cell death after facial axotomy in adult mouse. Exp Neurol 178:186-193.

Rao VLR, Dhodda VK, Song G, Bowen KK, Dempsey RJ. 2003. Traumatic brain injury-induced acute gene expression changes in rat cerebral cortex identified by genechip analysis. J Neurosci Res 71:208-219.

Read SJ, Parsons AA, Harrison DC, Philpott K, Kabnick K, O' Brien S, Clark S, Brawner M, Bates S, Gloger I, Legos JJ, Barone FC. 2001 Stroke genomics: approaches to identify, validate, and understand ischemic stroke gene expression. J Cereb Blood Flow Metab 21:755-778.

Roth A, Gill R, Certa U. 2003. Temporal and spatial gene expression patterns after experimental stroke in a rat model and characterization of PC4, a potential regulator of transcription. Mol Cell Neurosci 22:353-364.

Rothwell NJ, Hopkins SJ. 1995. Cytokines and the nervous system II: actions and mechanisms of action. Trends Neurol Sci 18:130-136.

Sayer FT, Oudega M, Hagg T. 2002. Neurotrophins reduce degeneration of injured ascending sensory and corticospinal motor axons in adult rat spinal cord. Exp Neurol 175:282-296.

Shohami E, Bait-Yannal E, Horowitz M, Kohen R. 1997. Oxidative stress in closed-head injury: brain antioxidant capacity as an indicator of functional outcome. J Cereb Blood Flow Metab 17:1007-1019.

Shohami E, Ginis I, Hallenbeck JM. 1999. Dual role of tumor necrosis factor alpha in brain injury. Cytokine Growth Factor Rev 10:119-130.

Stellwagen D, Malenka RC. 2006. Synaptic scaling mediated by glial TNF-alpha. . Nature 440:1054-1059.

Sullivan PG, Bruce-Keller AJ, Rabchevsky AG, Christakos S, Clair DK, Mattson MP, Scheff SW. 1999. Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury. J Neurosci 19:6248-6256.

Suvannavejh GC, Lee HO, Padilla J, Dal Canto MC, Barrett TA, Miller SD. 2000. Divergent roles for p55 and p75 tumor necrosis factor receptors in the pathogenesis of MOG(35-55)-induced experimental autoimmune encephalomyelitis. Cell Immunol 205:24-33.

Tang Y, Lu A, Aronow BJ, Wagner KR, Sharp FR. 2002. Genomic responses of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. Eur J Neurosci 15:1937-1952.

Wang J, Asensio VC, Campbell IL. 2002. Cytokines and chemokines as mediators of protection and injury in the central nervous system assessed in transgenic mice. Curr Top Microbiol Immunol 265:23-48.

Yankner BA. 1996. Mechanisms of neuronal degeneration in Alzheimer's disease. Neuron 16:921-932.

FIGURE LEGENDS

Figure 1. Hole board (top), elevated plus maze (middle) and horizontal ladder beam (bottom) results obtained in wild-type (C57), TNFR1 knockout mice (TNFR1KO) and TNFR2 knock-out mice (TNFR2KO). For the two first tests, n=8, 13 and 9 for WT, TNFR1KO and TNFR2 KO, respectively. For the latter, n=5, 7 and 4. Results are mean ± SEM. * $p<0.05$, ** $p<0.01$ vs respective WT group. ♦♦ $p< 0.01$ vs unlesioned mice.

Figure 2. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by cryolesion and mainly up-regulated at 1dpl (names can also be found in supplementary table I). Green dots: Significant ($p<0.05$) effect of TNFR1 deficiency vs. WT. Red dots: Significant ($p<0.05$) effect of TNFR2 deficiency vs. WT.

Figure 3. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by cryolesion and mainly up-regulated at 1-4dpl.

Figure 4. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by cryolesion and mainly up-regulated at 4dpl. Green dots: Significant ($p<0.05$) effect of TNFR1 deficiency vs. WT. Red dots: Significant ($p<0.05$) effect of TNFR2 deficiency vs. WT. Black dots: Significantly ($p<0.05$) different TNFR1KO vs. TNFR2KO.

Figure 5. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by cryolesion and mainly down-regulated at 1dpl. Green dots: Significant ($p<0.05$) effect of TNFR1 deficiency vs. WT. Red dots: Significant ($p<0.05$) effect of TNFR2 deficiency vs. WT. Black dots: Significantly ($p<0.05$) different TNFR1KO vs. TNFR2KO.

Figure 6. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by cryolesion and mainly down-regulated at 4dpl. Green dots: Significant ($p<0.05$) effect of TNFR1 deficiency

vs. WT. Red dots: Significant ($p<0.05$) effect of TNFR2 deficiency vs. WT. Black dots: Significantly ($p<0.05$) different TNFR1KO vs. TNFR2KO.

Figure 7. Hierarchical clustering of the subset of genes (including genes of unknown function) identified to be significantly ($p<0.05$) affected by TNFR deficiencies. Green dots: Significant ($p<0.05$) effect of TNFR1 deficiency vs. WT. Red dots: Significant ($p<0.05$) effect of TNFR2 deficiency vs. WT. Black dots: Significantly ($p<0.05$) different TNFR1KO vs. TNFR2KO.

Figure 8. Analysis of host response gene expression. Wild type (C57Bl/6) and TNFR1knockout (TNFR1 KO) mice were subjected to a cryolesion of the left cortex and killed after 2, 3 or 6 days after the lesion (2dpl, 3dpl and 6dpl, respectively). Unlesioned mice from both strains were also killed (0dpl). mRNA levels of several host response genes were analyzed by Rnase protection assay (RPA) (**a**). mRNA levels were quantitated by phosphorimaging and normalized with L32 mRNA levels (**b**). Results (arbitrary units; mean ±SEM; *n*=3-5) were evaluated using two-way ANOVA using cryolesion and strain as the main factors. ♦♦ $p< 0.01$ vs unlesioned mice; *$p<0.05$; ** $p<0.01$ vs wild type mice. The image shown here is an autoradiographic film, while quantification was done by phosphorimaging.

Figure 9. Analysis of host response gene expression. Wild type (C57Bl/6) and TNFR2knockout (TNFR2 KO) mice were subjected to a cryolesion of the left cortex and killed after 2, 3 or 6 days after the lesion (2dpl, 3dpl and 6dpl, respectively). Unlesioned mice from both strains were also killed (0dpl). mRNA levels of several host response genes were analyzed by Rnase protection assay (RPA) (**a**). mRNA levels were quantitated by phosphorimaging and normalized with L32 mRNA levels (**b**). Results (arbitrary units; mean ±SEM; *n*=3-5) were evaluated using two-way ANOVA using cryolesion and strain as the main factors. ♦♦ $p< 0.01$ vs unlesioned mice;
** $p<0.01$ vs wild type mice. #$p=0.058$ vs. wild type mice. The image shown here is an autoradiographic film, while quantification was done by phosphorimaging.

Figure 10. Comparison of host response gene expression in naïve and SHAM operated animals sacrificed at 1, 2 or 3 days after surgery. No effect of the surgery is observed at any time point studied on gene expression (*n*=4 for all groups)

Figure 11. Analysis of *metallothionein 1* gene expression by *in situ* hybridization. Wild type (C57Bl/6), TNFR1knockout (TNFR1 KO) and TNFR2Knockout (TNFR2KO) mice were subjected to a cryolesion of the left cortex and killed after 8 or 24 hours after the lesion (8hpl and 24hpl, respectively). Unlesioned mice from all strains were also killed (0hpl). *Mt1* mRNA levels were analyzed. Autoradiographies from 6 slides/animal were quantified (arbitrary units; mean ±SEM; *n*=3) and evaluated using two-way ANOVA using cryolesion and strain as the main factors. ♦♦ $p< 0.01$ vs unlesioned mice; * $p<0.05$ vs wild type mice. Microautoradiographies were obtained as described in the methods section and showed here for the sake of clarity.