Trabajo 4

La sobreexpresión de interleuquina-6 en el sistema nervioso central incrementa la respuesta inflamatoria después de criolesión pero reduce el daño oxidativo y la apoptosis.

Effect of Astrocyte-targeted production of Il-6 on traumatic brain injury and its impacto n the cortical transcriptome

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Effect Of Astrocyte-targeted Production Of IL-6 On Traumatic Brain Injury And Its Impact On The Cortical Transcriptome

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ABSTRACT Traumatic brain injury is one of the leading causes of incapacity and death among young people. The cascade of events elicited by injury is orchestrated by a number of growth factors and cytokines. Among these, interleukin-6 (IL-6) is one of the key players. IL-6 has been widely described as having pro-inflammatory roles as well as promoting cell survival. We have described previously that astrocyte-driven production of IL-6 (GFAP-IL6) in transgenic mice, although causing spontaneous neuroinflammation and long term damage, is beneficial after an acute lesion (freeze) injury in the cortex, increasing healing and decreasing oxidative stress and apoptosis. To determine the transcriptional basis for these responses here we analyzed the global gene expression profile of the cortex, at 0 (unlesioned), 1 or 4 days post lesion (dpl), in both GFAP-IL6 mice and their control littermates. In GFAP-IL6 mice, there was an increase in genes associated with the inflammatory response both at 1dpl (*Ifitm1, Endod1*) and 4dpl (*Gfap, C4b*), decreased expression of pro-apoptotic genes (i.e. *Gadd45b, Clic4, p21*) as well as reduced expression of genes involved in the control of oxidative stress (*Atf4*). Furthermore, the presence of IL-6 altered the expression of genes involved in hemostasis (*Vwf*), cell migration and proliferation (*Cap2*), and synaptic activity (*Vamp2*). All these changes in gene expression could underlie the phenotype of the GFAP-IL6 mice after injury, but many other possible factors were also identified in this study, highlighting the utility of this approach for deciphering new pathways orchestrated by cytokines such as IL-6 after injury.

Traumatic brain injury is one of the leading causes of injury-related death and disability, especially in young people (see (Giza and Prins, 2006) for review). Therefore, it is essential to elucidate all factors involved. The general response of the brain to traumatic injury is well established, comprising blood-brain barrier disruption and the infiltration and activation of inflammatory leukocytes and microglia, which generate proinflammatory factors, tissue digesting enzymes, adhesion/costimulatory molecules, complement, arachidonic acid metabolites, and reactive oxygen species (ROS) (Lucas et al., 2006; Seifert et al., 2006).

Cytokines, which are low molecular weight proteins, constitute one of the most important mediators of the inflammatory response. Among them, tumor necrosis factor (TNF)-α , interleukin (IL)-1, and IL-6 have been classically considered to be major components in the response to injury (for review; (Rothwell and Hopkins, 1995)), acting on various cell types and producing secondary damaging effects that in turn lead to lymphocyte recruitment and activation and may cause neuronal cell death (Kopf et al., 1994; Lee and Benveniste, 1999; Raivich et al., 1999; Yun et al., 2002; Hansson and Ronnback, 2003). Interleukin-6 is a cytokine that has important roles in normal brain function, including control of thermogenesis (Chai et al., 1996), body weight (Wallenius et al., 2002) and emotional behavior (Armario et al., 1998). However, it is after injury that IL-6 has been described to be an essential factor. Depending on the injury model, IL6 KO animals show a compromised inflammatory response, gliosis and specific neuronal survival (Kopf et al., 1994; Penkowa et al., 1999; Murphy et al., 2000; Swartz et al., 2001; Poulsen et al., 2005). On the other hand, transgenic production of IL-6 under the control of the glial fibrillary acidic protein (GFAP) gene promoter (GFAP-IL6 mice) induces neuroinflammation (Campbell et al., 1993; Chiang et al., 1994; Brett et al., 1995; Giralt et al., 2002), and learning impairments (Steffensen et al., 1994; Heyser et al., 1997). However, when an acute injury is superimposed, elevated IL-6 levels lead to increased CNS repair with decreased cell death (Swartz et al., 2001; Penkowa et al., 2003).

Thus, IL-6 exerts both proinflammatory and neuroprotective roles in the injured CNS. In recent years, our laboratories have extensively studied the role of IL-6 in a freeze injury model (cryolesion), by means of IL-6 KO mice and using standard methodologies (Penkowa et al., 2000a) as well as by means of microarray technology (Poulsen et al., 2005). The GFAP-IL6 mice show a somewhat opposite pattern of responses to the IL-6 KO mice as evaluated by these standard methods (Penkowa et al., 2003), but much remains to be understood. Therefore, the main aim of the current study was to further characterize the role of IL-6 in the normal brain and after an injury examining the overall gene expression patterns in the cortex of wild type and GFAP-IL6 mice by taking advantage of DNA microarray technology.

MATERIAL AND METHODS

Animals. Construction and characterization of the GFAP-IL6 transgenic mice was described previously (Campbell et al., 1993). Briefly, an expression vector derived from the murine glial fibrillary acidic protein

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(GFAP) gene was used to target expression of IL-6 to astrocytes. Heterozygous GFAP-IL6 mice on a C57Bl/6J background were crossed with C57Bl6/J breeders. The offspring were genotyped to identify the GFAP-IL6+/- (hereafter GFAP-IL6) and the GFAP-IL6-/- as previously described (Penkowa et al., 2000b). As both groups share the same genetic background we refer to the GFAP-IL6-/- animals as the wild type (WT) mice. Age and gender were matched for both groups. 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.

Experimental procedures. Twelve-month-old male mice were anesthetized 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., 1999; Penkowa et al., 2000b) using a 0.4cm diameter dry ice pellet for 30 seconds. Animals were allowed to recover and returned to the animal room, and were subsequently killed at 1or 4 days post lesion (dpl) for microarray studies (n=3 animals for each genotype and time point). Unlesioned wild type and GFAP-IL6 mice were used as controls. The ipsilateral cortex were dissected, frozen 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 (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 genotype 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.

RESULTS AND DISCUSSION

Gene selection and functional analysis.

As we have previously described (Poulsen et al., 2005) we used dChip software (http://www.dchip.org for more details), to generate a combined gene list, comparing the genes up- and down-regulated at each time point for each genotype with the unlesioned WT mice. We set a 1.2 fold change (that is, a 20% increase or decrease) and an absolute change in expression values of 100 expression units. With this procedure a list of 461 IDs (corresponding to 369 genes) was produced, therefore being comparable to previous experiments with the same model performed in our lab (Poulsen et al., 2005; Penkowa et al., 2006; Quintana et al., 2006).

It has been suggested that the best approach for analyzing microarray data is the combination of a fold-change selection plus a p-value rank ordering (Guo et al., 2006). Thus, given the experimental design of this experiment, with two different mouse

genotypes and with different time points after injury to be compared, a two-way ANOVA statistical analysis of the data was required. The expression values were exported to a standard statistical software, SPSS v13.00 (SPSS, Chicago, IL, USA), where a conventional two-way ANOVA was performed ($p < 0.05$). However, it should be mentioned that the number of animals used in the array (18 in total, n=3 for each time point and genotype, although being remarkable for a microarray experiment, is still reduced to ensure that a two-way ANOVA is able to pick up all affected genes as some of them may only change in a single timing. Some of the genes selected were found to be statistically affected by IL-6 production and many of them, although not reaching statistical significance, presented a clear trend. The fact that some of these genes (commented below) have been described to be affected by IL-6 led us to further realize the shortcomings of the ANOVA test in detecting all significantly affected genes, albeit, as commented, it was the best approach in our opinion.

This statistical analysis reduced the number of genes from 369 to 329 (89% of the genes selected by fold change) suggesting that the first selection was highly reliable. From these 329 genes, 300 genes were significantly affected by treatment only, 6 by the genotype only and 23 by both. Among these genes, 31 showed a positive interaction between both factors, which we normally interpret as genes that either change substantially between genotypes (i.e. it increases in one but decreases in other), or change only in a single timing (Supplementary table I). By means of the high-level analysis "Hierarchical clustering" utility of dChip, genes were grouped according to the similarity of their patterns of expression (Figure 1 to see the general overview). In this kind of figure, each row represents a gene and each column represents the mean value of the mice used for each group. 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. Finally, the intensity of the color (either red or blue) directly correlates with the fold induction.

As previously described in this model (Poulsen et al., 2005; Penkowa et al., 2006; Quintana et al., 2006), a clear pattern of induction was observed, with genes up-regulated by the cryolesion at 1 day post lesion (dpl) (Fig. 2), both at 1-4dpl (Fig. 3), or at 4dpl (Fig 4), those down-regulated either at 1dpl (Fig. 5) or at 1-4dpl (Fig. 6) and those only affected by central IL-6 production (Fig. 7). Taking advantage of the NIH based DAVID soft-

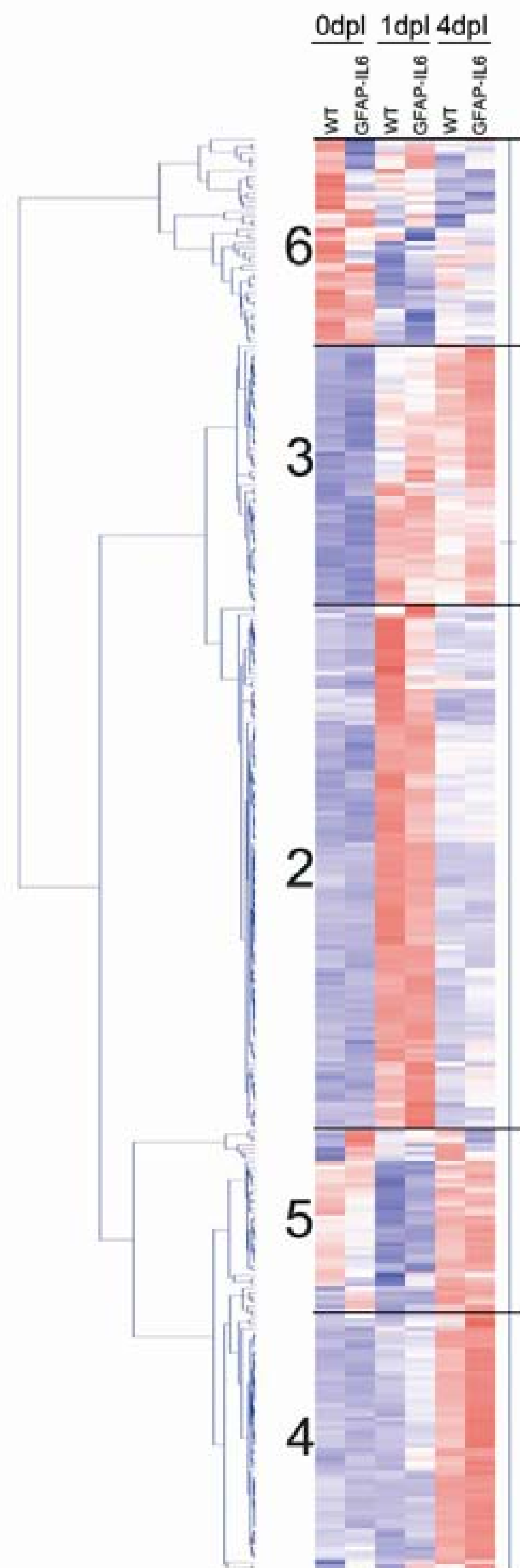

Figure 1. Hierarchical clustering of the 329 genes identified to be significantly ($p<0.05$) affected by the cryolesion only. Each row represents a gene and each column represents the mean value of the three mice for each strain and timing. The clustering was split into several parts as shown in order to show the name of the genes (see Figures 2-6) (see also Supplementary Table I).

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ware (DAVID v2.1, http://david.abcc.ncifcrf.gov/) a functional classification using Gene Ontology was performed as described previously (Dennis et al., 2003), enabling us to identify the categories over-represented among the genes selected in our array. Cryolesion caused dramatic changes in gene expression at 1 day post lesion (Fig 2), at 1 and 4 dpl (Fig. 3) or only at 4dpl (Fig. 4). Genes up-regulated at 1dpl constituted the largest cluster in our study, comprising 117 genes (35% of the total), 58 genes (17% of the total) comprised the cluster of genes up-regulated at both time points, and 57 (17% of the total) were found at 4dpl. Hence, the majority (69%) of the genes selected belong to genes that are up-regulated by injury. As expected, many of theses genes related to cell signaling and transcription factors, mostly classified as involved in the defense response/inflammatory response/ response to wounding. On the other hand, genes down-regulated at 1dpl (42 genes, 13% of the total – Fig. 5-) or both at 1 and 4dpl (46 genes, 14% of the total – Fig. 6-) were classified as being involved in neuronal differentiation, nervous system development and transport (Supplementary table II).

Therefore, cryolesion is a model that elicits an inflammatory response at the gene level, in agreement with previous results (Quintana et al., 2005; Penkowa et al., 2006). The effect of cryolesion at a transcriptome level has been already described by our group in animals lacking IL-6 (Poulsen et al., 2005), metallothioneins 1 and 2 (Penkowa et al., 2006) and tumor necrosis factor (TNF)-α receptor 1 or receptor 2 (Quintana et al., 2006). Furthermore, as the effect of a traumatic brain injury on gene expression has been extensively addressed by many groups (Gebicke-Haerter, 2005; von Gertten et al., 2005), we decided to focus on the specific changes caused by central IL-6 production that lead to neuroprotection, with increased healing process and a decrease in oxidative stress and apoptosis after injury (Penkowa et al., 2003).

Astrocyte-targeted IL-6 production enhances inflammatory response after injury.

IL-6 has been widely described as a pro-inflammatory cytokine (Munoz-Fernandez and Fresno, 1998; Van Wagoner and Benveniste, 1999) and in this regard GFAP-IL6 mice have been extensively described to produce spontaneous neuroinflammation (Campbell et al., 1993; Giralt et al., 2002). Activation of microglia/macrophages is part of the phenotype of the GFAP-IL6 mice (Campbell et al., 1993), and it has been shown to be higher than in control mice up to 6 days after cryolesion (Penkowa et al., 2003). On the other hand the inflammatory response has been shown to be clearly reduced in IL-6 knock-out mice during different pathological conditions of the CNS (Eugster et al., 1998; Raivich et al., 1999)

In agreement with these results, several genes expressed in immune cells were found to be increased at 1dpl in GFAP-IL6 animals (Fig. 2). Interferon induced transmembrane protein 3-like,(*Ifitm2*) and RIKEN cDNA 1110036C17 gene - Fragilis2 - (*Ifitm1*) are genes found in macrophages and described to be up-regulated in inflammation (Johnson et al., 2006) and involved of the control of leukocyte proliferation (Sato et al., 1997). Also, C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 8 (*Clec4d*), a gene expressed in activated monocytes and macrophages that is up-regulated by IL-6, TNF-α, IFN-γ and IL-10 (Arce et al., 2004) tended to be increased in GFAP-IL6 mice. Riken 1210067E08 - endonuclease domain containing 1 – (*Endod1*), a gene which is expressed in platelets (O'Neill et al., 2002), was found to be up-regulated in GFAP-IL6 mice at 1dpl. Platelet activation is the most important mechanism to cope with traumatic hemorrhage, which is one of the complications of brain injuries, leading to shock and death (Stein and Dutton, 2004).

This first wave of activation, found at 1dpl paves the way to a number of genes that are found to be up-regulated at both 1 and 4dpl (Fig. 3) or only at 4dpl (Fig. 4). Following the pattern observed at 1 dpl, inflammatory response-related genes were also significantly up-regulated in GFAP-IL6 mice at these time points. In the cluster representing genes affected both at 1 and 4dpl (Fig. 3) we found Fc receptor IgG, low affinity IIb (*Fcgr2b*), which is expressed in leukocytes and has been described to be up-regulated in inflammatory processes. It has been suggested to be involved in the modulation of B-cell activation and in the regulation of autoimmunity and inflammation (Hamaguchi et al., 2006). Furthermore, Fc receptor IgG, low affinity III (*Fcgr3*) which is involved in the strengthening of the inflammatory response by increasing the synthesis of chemoattractant proteins (Radeke et al., 2002) presented a similar trend, although not reaching statistical significance; but further supporting the view that inflammatory response is increased in GFAP-IL6 mice.

Reactive astrogliosis, characterized histologically by cell hypertrophy, which leads to an increase of glial fibrillary acidic protein (*Gfap*) mRNA and

protein is one of the most prominent features following injury in the CNS (see (Eng et al., 2000) for review). Although no differences in the level of GFAP protein have been found in the cortex of GFAP-IL6 mice compared to wild-type animals (Chiang et al., 1994), increased numbers of reactive astrocytes have been described in GFAP-IL6 mice after cryolesion from 1 to 10dpl, peaking at 3dpl (Penkowa et al., 2003). Accordingly, our results show increased expression of *Gfap* mRNA was only found to be differentially increased in GFAP-IL6 mice at 4dpl, but not at early time points. Again, the fact that expressions differ only at a single time point may have caused that ANOVA was not able to pick this gene up.

As described above, many inflammatory response-related genes were up-regulated by cryolesion at 4dpl (Fig. 4), and although a high percentage of them presented an increased expression in GFAP-IL6 compared to wild type mice, only two were found to have statistical significance. Histocompatibility 2, class II Ag E α (*H2Ea*) belongs to the MHC-II complex and it is mostly expressed in antigen-presenting cells (APCs). Its expression is increased in inflammatory processes (Cresswell, 1996), therefore suggesting an increase either in the cell numbers or in the activation of APCs in GFAP-IL6 mice after injury. Complement component 4 (*C4b*) is involved in the classical pathway, and acts inducing vasodilatation, capillary permeability and expression of leukocyte adhesion molecules in inflammatory processes (Petersen et al., 1988). Furthermore, chronic complement 3 (*C3*) expression has been described in GFAP-IL6 mice (Barnum et al., 1996).

Hence, it can be concluded that the increase in the inflammatory response described histologically in GFAP-IL6 after cryolesion (Penkowa et al., 2003) is confirmed and extended from a transcriptional point of view and that this response is orchestrated by diverse genes and pathways.

IL-6 production decreases the expression of pro-apoptotic genes after cryolesion

In addition to the pro-inflammatory role described before, IL-6 also belongs to the neuropoietin family of cytokines (Bauer et al., 2007), and has neurotrophic effects related to the regulation of neuronal protection (Murphy et al., 2000; Swartz et al., 2001;

Figure 2. Hierarchical clustering of a subset of genes whose expression was up-regulated at 1dpl (see also Figure 1). Green dots: WT vs. GFAP-IL6 ($p<0.05$). Black dots: Significant interaction between cryolesion and strain ($p<0.05$)

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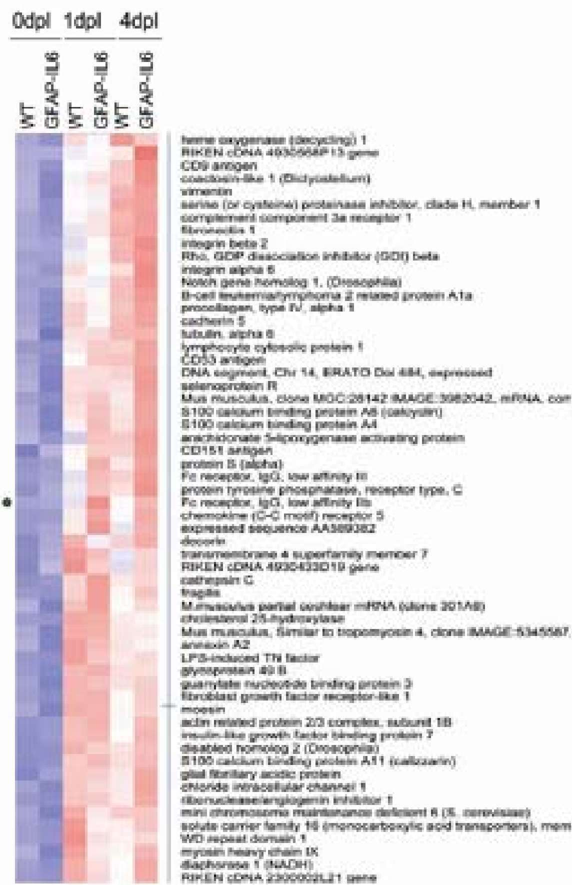

Figure 3. Hierarchical clustering of a subset of genes whose expression was up-regulated at 1-4dpl (see also Figure 1). Green dots: WT vs. GFAP-IL6 ($p<0.05$)

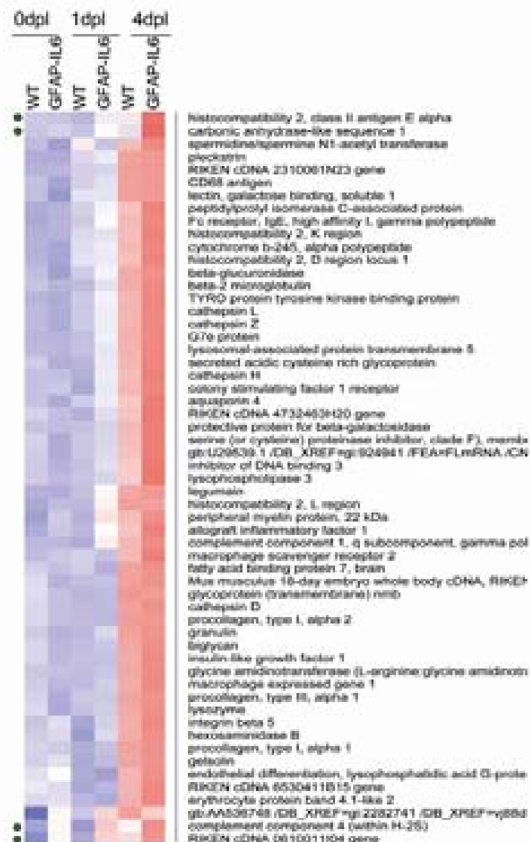

Figure 4. Hierarchical clustering of a subset of genes whose expression was up-regulated at 4dpl (see also Figure 1). Green dots: WT vs. GFAP-IL6 ($p<0.05$).

Westberg et al., 2007). IL-6-null animals subjected to cryolesion presented higher numbers of TUNEL+ cells (mostly neurons and some astrocytes) than their control littermates (Penkowa et al., 2000a), being these results confirmed at the transcriptional level in a microarray study (Poulsen et al., 2005). Conversely, GFAP-IL6 animals presented fewer apoptotic cells in the same paradigm (Penkowa et al., 2003).

In the present work we have been able to detect several genes involved in cell growth/apoptosis that may account for the protective role of IL-6. At 1dpl (Fig. 2) several apoptotic-related genes were down-regulated in GFAP-IL6 mice. Chloride intracellular channel 4 (mitochondrial) (*Clic4*) is a direct response gene for p53 transactivation involved in TNF-α and p53-mediated signaling pathways. It is induced in response to stress and it has been described that its overexpression induces apoptosis associated with loss of mitochondrial membrane potential, cytochrome c release, and caspase activation suggesting that apoptosis is mediated by mitochondrial dysfunction (Suh et al., 2004). A decreased oxidative status in GFAP-IL6 mice after cryolesion has been already described (Penkowa et al., 2003) and the opposite is found in IL6KO mice (Penkowa et al., 2000a). Accordingly, a clear trend was found in the expression of activating transcription factor 4 (*Atf4*), a transcription factor that is involved in the integrated reticular stress response and resistance to oxidative stress mediated apoptosis (Harding et al., 2003), that tended to be reduced in GFAP-IL6, although not reaching statistical significance.

Caveolin 2 expression was also reduced in GFAP-IL6 mice. Caveolins were discovered as the basic constituent of the caveolae or "small caves", which are 50- to 100-nm flask-shaped, non-clathrin-coated invaginations of the plasma. However, its functions have widened from their initial role in endocytosis and potocytosis, and new roles in signal transduction and apoptosis have emerged (Williams and Lisanti, 2004). In this regard, Caveolin 2 (*Cav2*) is considered to modulate caveolin 1 function and to have tumor supressor properties. PPARγ, a gene induced in inflammatory situations induces caveolins and p21, inducing apoptosis in bladder tumor cells (Kassouf et al., 2006). Interestingly, *p21* expression tended to be reduced in GFAP-IL6 in our experiment. P21 is involved in cell cycle and a very well known tumor suppressor gene involved in

apoptosis in many cancer cell types as several antitumoral treatments actions are mediated by this factor (Svechnikova et al., 2007). Furthermore, growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*) also showed a trend to be decreased in GFAP-IL6 mice. GADD45 family proteins are stress and cytokine-inducible and are specific activators of MTK1 (MEKK4), a MAPK kinase kinase upstream in the p38 pathway, and induce apoptosis. GADD45β participates in TGF-β-induced apoptosis by acting upstream of p38 activation (Yoo et al., 2003).
On the other hand, calmodulin binding protein 1 (also known as abnormal spindle-like microcephaly associated - *Aspm*-) was found to be up-regulated in GFAP-IL6 compared to wild type animals. Aspm has been suggested to control the so-called S-M checkpoint, important for the proper timing of mitosis (Craig and Norbury, 1998). It has been recently described that *Aspm* is involved in the proliferation of glioblastoma and that its inhibition decreases both tumoral and neural stem cell proliferation (Horvath et al., 2006).

The number of genes related to apoptosis detected decreased upon time, suggesting a key role of pro-apoptotic signals in the first wave-response after injury. Only at 4dpl (Fig. 4), RIKEN cDNA 0610011I04 gene (transmembrane protein 167a -*Tmem167a* –) was found to be up-regulated in GFAP-IL6 mice. Little is known about this gene, but given the fact that it is also known as hepatocellular carcinoma-associated antigen 112 (**http://www.ncbi.nlm.nih.gov/entrez**) it gives rise to the idea that it might be involved in the regulation of proliferation and cell survival in the same way as Aspm.

Thus, the highlighted genes in this work may underlie some of the neuroprotective roles described for IL-6 and might mediate the reduced cell death described in GFAP-IL6 mice after cryolesion (Penkowa et al., 2003).

Neuronal function is altered by cryolesion and modulated by IL-6

As described above, only a minor part of the genes selected were found to be down-regulated by cryolesion. These genes can be seen in figures 5 and 6 and in the supplementary tables. The functional classification of these genes comprised neuronal differentiation, nervous system development and transport. It has been widely described a decrease in neuronal function after an injury. It is caused by the primary death of neurons but contributed by the downshift in the activity of the area surrounding the

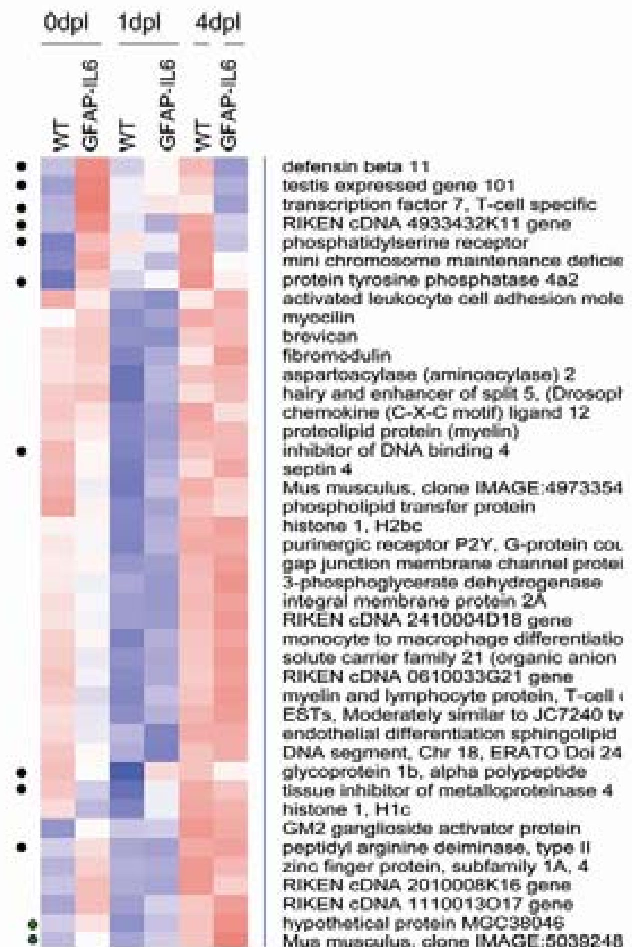

Figure 5. Hierarchical clustering of a subset of genes whose expression was down-regulated at 1dpl (see also Figure 1). Green dots: WT vs. GFAP-IL6 ($p<0.05$). Black dots: Significant interaction between cryolesion and strain ($p<0.05$)

lesion core (penumbra) that survives the primary damage (Wieloch and Nikolich, 2006).

Only two genes were significantly affected by IL-6 production at 1dpl (Fig. 5), hypothetical protein MGC38046, also known as transmembrane protein 119 (*Tmem119*) with no information available, and Mus musculus, clone IMAGE:5039248, mRNA (phosphofurin acidic cluster sorting protein 2 – *Pacs2*-). PACS-2 is a protein involved in the apposition of mitochondria with the ER, allowing the communication between both organelles, the exchange of calcium, and also is a key player in the translocation of Bid to mitochondria (Simmen et al., 2005). Both sequences were slightly but significantly up-regulated in GFAP-IL6 mice at all time points.
When looking at the cluster of genes mostly down-regulated both at 1 and 4dpl (Fig. 6), several genes were found to differ in GFAP-IL6 animals. Interestingly, many of them are involved in calcium signaling. Altered calcium homeostasis is one of the features of traumatic injury, leading to excitotoxicity

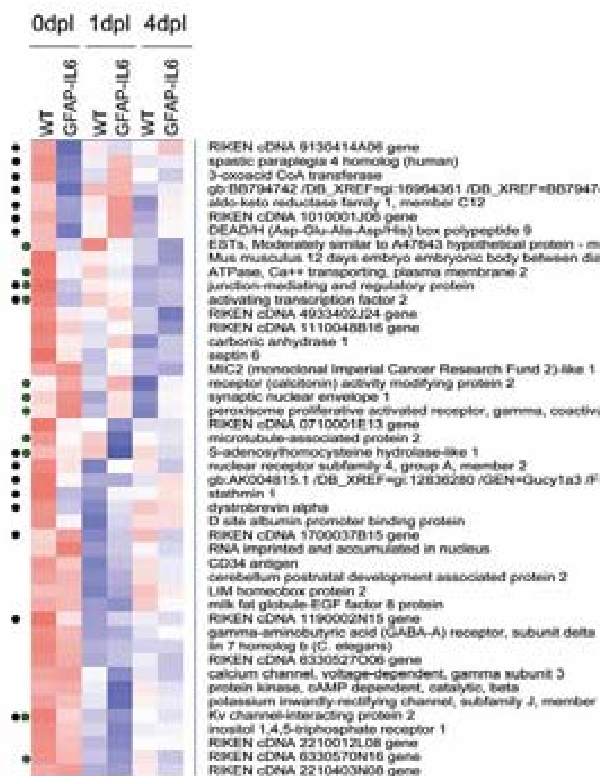

Figure 6. Hierarchical clustering of a subset of genes whose expression was down-regulated at 1-4dpl (see also Figure 1). Green dots: WT vs. GFAP-IL6 ($p<0.05$). Black dots: Significant interaction between cryolesion and strain ($p<0.05$)

and cell death, both by necrotic and apoptotic mechanisms as well as learning impairment after traumatic injuries (see (Won et al., 2002) for review). Furthermore, it is not surprising to find that calcium-related genes are down-regulated after cryolesion as this has been described previously both at at the protein and mRNA level for Calcium-calmodulin dependent protein kinase II (CamKII) after an ischemic insult (Uemura et al., 2002), epilepsy (Liang and Jones, 1997) and in an excitotoxic model in vitro (Churn et al., 1993). In this regard, the gene codifying for the isoform beta from CaMKII (EST, moderately similar to A47643 hypothetical protein – mouse, also known as calcium/calmodulin-dependent protein kinase II, beta – *Camk2b*-), although slightly increased in wild type mice at 1dpl was remarkably down-regulated in GFAP-IL6 mice at all time points. Since CaMKII is one of the most prominent factors involved in synaptic plasticity and memory (Colbran and Brown, 2004) this may lead to decreased synaptic plasticity in GFAP-IL6 mice. Accordingly, CaMK kinase 2 (RIKEN cDNA 6330570N16 gene – *Camkk2*-), also involved in calcium signaling and long term potentiation (LTP) (Peters et al., 2003) was also decreased in GFAP-IL6 mice. In this regard, deficits in hippocampal activity (Steffensen et al., 1994) and impaired learning (Heyser et al., 1997) have been described in GFAP-IL6 as part of their phenotype. The role of IL-6 in the inhibition of synaptic plasticity and memory has been described elsewhere (Balschun et al., 2004).

Another key player in calcium homeostasis, such as ATPase, Ca2+ transporting, plasma membrane 2 (*Atp2b2* or *Pmca2*), involved in the extrusion of calcium from neurons, was downregulated in GFAP-IL6 mice. It has been described that PMCA2 immunoreactivity decreases after glutamate excitotoxicity in rat hippocampal neurons (Pottorf et al., 2006) and after an spinal cord injury model (Kurnellas et al., 2005), however, its exact role after an injury is not clear. Kv channel-interacting protein 2 (*Kcnip2*), belongs to a subfamily of Neuronal Calcium sensor (NCS) proteins, that have been described to play important roles in modulation of the neuronal function (Xiong et al., 2004) was down-regulated by IL-6. Furthermore, S-adenosylhomocysteine hydrolase-like 1 (*Ahcyl1*), also known as inositol 1,4,5-trisphosphate (IP3) receptor-binding protein (IRBIT) which has been described to be involved in the IP3 pathway signaling (Ando et al., 2003) and calcium release (Cooper et al., 2006) was also down-regulated in GFAP-IL6 mice. Moreover, chronic administration of IL-6 to Purkinje cerebellar neurons has been described to alter calcium signaling involving calcium release from intracellular stores (Nelson et al., 2004), further confirming that calcium signaling pathway is affected by IL-6 over-expression.

Other genes found to be down-regulated by cryolesion and to present lower levels of expression in GFAP-IL6 mice were Junction-mediating and regulatory protein (*Jmy*), activating factor 2 (*Atf2*) and Microtubule-associated protein 2 (*Mtap2*). JMY is a transcription cofactor of p53 signaling (Coutts et al., 2007). ATF2 is a constituent of the AP-1 transcription factor, along with c-jun and c-fos. A decrease in ATF-2 has been suggested to be involved in apoptosis through facilitating the formation of c-jun/c-fos heterodimers (Bhoumik et al., 2002). Another gene affected in the same way is Mtap2, which is involved in neural plasticity via stabilization of microtubules in dendrites, being needed for neurite growth and PKA signaling (Harada et al., 2002). Decreases on MAP2 immunoreactivity in cortex and hippocampus have been described after a mild cortical impact (Huh et al., 2003) and a negative role of IL-6 in the expression of MAP2 has been described in vitro (Marx et al., 2001).

On the other hand, some genes presented

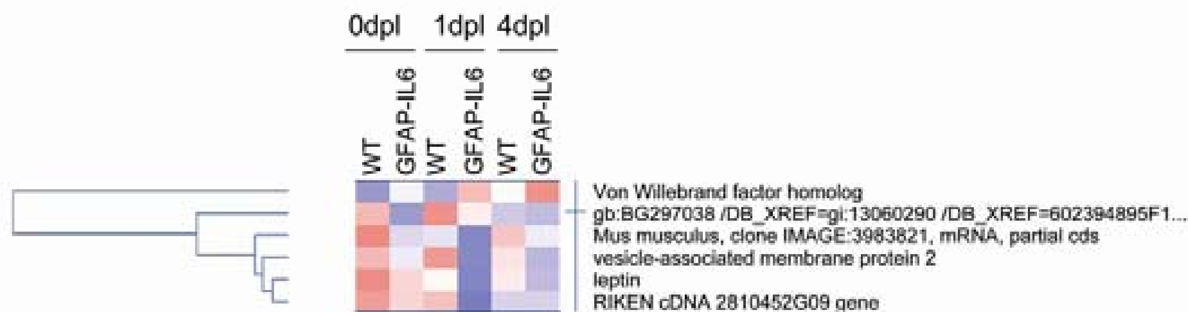

Figure 7. Hierarchical clustering of the 6 genes identified to be significantly ($p<0.05$) affected by the strain only.

higher transcription levels in GFAP-IL6 mice even though being down-regulated by cryolesion at 1 or 4dpl (Fig. 6). Receptor (calcitonin) activity modifying protein 2 (*Ramp2*), which encodes a receptor of adrenomodullin, therefore having vasodilating and angiogenic properties (Abe et al., 2003) which is in agreement with the angiogenic phenotype described in GFAP-IL6 mice (Brett et al., 1995). Synaptic nuclear envelope 1 (*Syne1*) is a gene present in neuromuscular junctions that has also been implicated in some forms of cerebellar ataxia (Gros-Louis et al., 2007), also a brain-specific splicing variant, CPG2, has been suggested to intervene in the postsynaptic plasticity of excitatory synapses (Cottrell et al., 2004). Finally, peroxisome proliferative activated receptor, gamma, coactivator 1 (*Pgc1a*) was also increased in GFAP-IL6 mice compared to wild type. PGC-1α is a gene involved in boosting mitochondrial respiration that has been suggested to be needed for the induction of several anti-oxidant enzymes (St-Pierre et al., 2006).

Hence, IL-6 production alters in a diverse fashion, affecting mostly genes involved in synaptic plasticity but also in many other systems, therefore highlighting the important roles of this cytokine.

IL-6 production affects normal cortical gene expression pattern

As described above, 6 genes were found to be significantly affected by IL-6 production but not by cryolesion (Fig. 7). However, a couple of considerations have to be taken into account. First of all, as stated above, the use of the two-way ANOVA test may have masked an effect of cryolesion at only one time point in some cases, but may have also overlooked genes different only between genotype at one time point (i.e. unlesioned mice) but that behave in a similar fashion afterwards. Secondly, it has been described that IL-6 transgenic expression in the cerebral cortex of GFAP-IL6 mice is rather mild compared to other areas such as cerebellum (Chiang et al., 1994), which may explain why no differences on IL-6 expression were detected between GFAP-IL6 and wild type mice. However, we have previously described that an effect of IL-6 production is visible in the cortex even though transgenic IL-6 mRNA is very low (Penkowa et al., 2003).

Von Willebrand factor homolog (*Vwf*) was the only gene found to be increased in GFAP-IL6 at all times analyzed. This is consistent with an earlier study documenting increased vWF in the cerebellum of the GFAP-IL6 mice (Campbell et al., 1993). VWF has been extensively described to take part in hemostasis, playing regulating the adhesion of platelets and their subsequent activation (Andrews et al., 1997). However, it has been recently described that it is involved in the formation of Weibel-Palade bodies of endothelial cells, where it is usually stored, recruiting different proteins, such as secretory organelle membrane protein vesicle-associated membrane protein-2 (*Vamp2*) (Blagoveshchenskaya et al., 2002). Interestingly, *Vamp2* (also known as synaptobrevin2) was found as well among the genes affected by IL-6 over-expression, being down-regulated in GFAP-IL6 mice. *Vamp2* is involved in synaptic transmission, being described to mediate Ca2+-triggered exocytosis (Deak et al., 2006). It has also been described to be present in gliosomes, which contain glutamate-accumulating vesicles co-expressing the vesicular glutamate transporter type 1 and can release the amino acid by a process resembling neuronal exocytosis (Stigliani et al., 2006).

Another gene down-regulated in GFAP-IL6 mice was RIKEN cDNA 2810452G09 gene- CAP, adenylate cyclase-associated protein, 2 (yeast) (*Cap2*) is strongly expressed in nearly all cells of developing and adult mice and in specific areas of the central nervous system, being involved in the regulation of actin dynamics and playing important roles in processes such as morphogenesis, polarization, migration, and endocytosis (Bertling et al., 2004). It

has been suggested that CAP2 might be related to cell proliferation and tumor formation in liver (Shibata et al., 2006).

Leptin (*Lep*) is a cytokine extensively described as a key player in the regulation of energy balance in the body. Although it is mainly expressed and secreted in adipocytes it is now clear that many other tissues are able to express leptin. Its expression in different neuron populations throughout the brain has been recently described (Ur et al., 2002). In this regard, many possible actions in the CNS apart from regulating energy balance are being highlighted, for instance, it has been described that leptin can alter the excitability of hippocampal neurons (Harvey, 2007). Furthermore, a role for leptin in immune response has been suggested, being leptin plasmatic levels increased by many cytokines such as IL-6 (see (Otero et al., 2005) for review). However, we have observed a decrease in leptin expression in GFAP-IL6 mice, which might be explained by different regulatory pathways of systemic versus central leptin pools, therefore warranting future studies on this regard.

It was surprising to find that gb:BG297038, also known as RIKEN cDNA 1300007C21 gene - gag protein, murine leukemia retrovirus (*1300007C21Rik*), which belongs to an integrated sequence from a retroviral origin (Murine Leukemia Virus – MuLV-) was expressed. However, almost all inbred mice strains have been described to have integrated MuLV sequences and its expression varies among tissue and strain (Stoye and Coffin, 1987). Interestingly, the expression of this sequence was decreased in GFAP-IL6, suggesting a role for IL-6 in the regulation of this viral sequence. In this regard, IL6-null mice have shown significantly enhanced virus replication after acute MuLV infection (Strestik et al., 2001).

The last sequence found to be affected in the cortex of the GFAP-IL6 mice was Mus musculus, clone IMAGE:3983821, mRNA, partial cds, that was down-regulated by IL-6 production. No information is available on this sequence but interestingly, it was found to be up-regulated in IL6 KO mice after cryolesion (Poulsen et al., 2005), hence, suggesting that its expression may be IL-6 mediated.

Genes presenting a significant interaction between genotype and cryolesion

Several genes showing significant interaction were found throughout the different clusters, which are indicated with a black dot in the figures and also pointed out in the supplementary table I. These genes may represent both sequences that differ fer only at one time point between strains or (many of them) that show a different pattern of expression between GFAP-IL6 and control mice. As many of them just confirm the views expressed throughout this work we decided, for the sake of clarity, not to describe them and to leave them accessible in the supplementary table I.

In conclusion, the present work demonstrates a major role of IL-6 in the response of the cortex to the lesion, but also in basal conditions, and again highlights the usefulness of the microarray technology for identifying the pathways involved.

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Trabajo 5

Las metalotioneínas, inducidas por IL-6, actúan como factores antiinflamatorios y antioxidantes en la criolesión, así como ayudan en procesos de regeneración tisular y en la neurogénesis tras lesión.

Novel roles for metallothionein-I + II (MTI+II) in defense responses, neurogenesis, and tissue restoration after traumatic brain injury: Insights from global gene expresión profiling in wild-type and MT-I + II knockout mice.

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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 Wild-Type and MT-I + II Knockout Mice

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Traumatic injury to the brain is one of the leading causes of injury-related death or disability, especially among young people. Inflammatory processes and oxidative stress likely underlie much of the damage elicited by injury, but the full repertoire of responses involved is not well known. A genomic approach, such as the use of microarrays, provides much insight in this regard, especially if combined with the use of gene-targeted animals. We report here the results of one of these studies comparing wild-type and metallothionein-I + II knockout mice subjected to a cryolesion of the somatosensorial cortex and killed at 0, 1, 4, 8, and 16 days postlesion (dpl) using Affymetrix genechips/oligonucleotide arrays interrogating ~10,000 different murine genes (MG_U74Av2). Hierarchical clustering analysis of these genes readily shows an orderly pattern of gene responses at specific times consistent with the processes involved in the initial tissue injury and later regeneration of the parenchyma, as well as a prominent effect of MT-I + II deficiency. The results thoroughly confirmed the importance of the antioxidant proteins MT-I + II in the response of the brain to injury and opened new avenues that were confirmed by immunohistochemistry. Data in KO, MT-I-overexpressing, and MT-II-injected mice strongly suggest a role of these proteins in postlesional activation of neural stem cells. **© 2006 Wiley-Liss, Inc.**

Key words: traumatic brain injury; metallothionein-I + II deficiency; murine genome; Affymetrix microarrays

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Traumatic brain injury is one of the leading causes of injury-related death and disability, especially among young people (for review see Prins and Hovda, 2003), so continued efforts to characterize all factors involved are essential. Over the last years, the general response of the brain to traumatic injury has been well described by using conventional methods: infiltration and activation of inflammatory leukocytes and microglia, which generate proinflammatory factors, such as cytokines, tissue digesting enzymes, adhesion/costimulatory molecules, complement, arachidonic acid metabolites, and reactive oxygen species (ROS), and, along with this, the blood brain barrier is disrupted (Lucas et al., 2006; Seifert et al., 2006). Although inflammation is necessary for brain tissue restoration, it also mediates collateral and delayed (secondary) damage. In general, ROS-mediated oxidative stress is responsible for secondary brain damage, including neurodegeneration and ultimately apoptotic cell death, which can be prominent after a primary neurotrauma (Mhatre et al., 2004; Lucas et al., 2006; Potashkin and Meredith, 2006). In the brain, ROS are particularly toxic compared with other tissues, because the cerebral microenvironment is characterized by low antioxidant capacity with low activity of catalase, superoxide dismutase, and glutathione peroxidase; also, the brain is rich in polyunsaturated fatty acids (myelin) that are readily oxidized and/or peroxidized and iron, which generates the highly reactive hydroxyl radical by the Fenton reaction. Accordingly, increased oxidative stress is involved in the pathogenesis of several neurological diseases, as demonstrated in animal models as well as in a number of human brain diseases (Halliwell, 1992; Coyle and Puttfarcken, 1993; Olanow, 1993).

However, the brain may mount compensatory, anti-inflammatory responses, which consist mainly of the astroglial responses, including increases in antioxidants and growth/trophic factors, angiogenesis, and neurogenesis. It is now well-established that metallothioneins (MTs) are important factors in the brain. MTs are small, cysteine-rich proteins that bind heavy metals such as zinc and copper with high affinity. Four closely linked *Mt* genes are present in rodents (Palmiter et al., 1992; Quaife et al., 1994). *Mt1* and *Mt2* (*Mt1+2*) are preferentially expressed in astrocytes and activated microglia/macrophages and vascular endothelium, in which they are induced by practically any inflammatory or pathological mediator (Yagle and Palmiter, 1985; Blaauwgeers et al., 1993; Holloway et al., 1997; Vela et al., 1997; Acarin et al., 1999; Penkowa et al., 1999a; van Lookeren Campagne et al., 2000). MTI + II are seen in the cytoplasm and subcellular organelles such as mitochondria and lysosomes. Depending on the cell cycle progression and level of differentiation and/or during pathology, MTI + II are rapidly translocated to the nucleus, as seen during early S-phase and during oxidative stress (Cherian and Apostolova, 2000; Takahashi et al., 2005). *Mt3* is expressed predominantly in neurons and is relatively unresponsive to inflammatory factors compared with MT-I + II (Masters et al., 1994b; Carrasco et al., 1998a,b, 2000a; Uchida, 1999; Giralt et al., 2001). However, *Mt3* is also expressed in astrocytes under some conditions and has been suggested to be involved in the ethiology of Alzheimer's disease (Uchida et al., 1991; Tsuji et al., 1992; Kobayashi et al., 1993; Masters et al., 1994b). *Mt4* has not been shown in the brain (Quaife et al., 1994).

Previous work has demonstrated that *Mt1+2* genes protect the CNS in response to pathology, including the cryoinjury used in this report. *Mt1+2*-null mice display impaired brain parenchyma recovery following injury, with a prolonged inflammatory response and a significant increase of oxidative stress and apoptosis, whereas transgenic *Mt1* overexpression and exogenous administration of MT-II lead to quite opposite responses, with less damage and improved outcome (Penkowa et al., 1999a, 2000, 2001b; Giralt et al., 2002b). A neuroprotective role of MT-I + II has also been shown in other models of CNS injury, such as experimental autoimmune encephalomyelitis (Penkowa and Hidalgo, 2000, 2001; Penkowa et al., 2001a), kainic acid-induced seizures (Carrasco et al., 2000b), traumatic injury (Chung et al., 2003; Natale et al., 2004), transgenic mice with a familial amyotrophic lateral sclerosis-linked mutation of the SOD1 gene (Nagano et al., 2001; Puttaparthi et al., 2002), ischemia and reperfusion (van Lookeren Campagne et al., 1999; Trendelenburg et al., 2002), and transgenic IL-6-induced neuropathology (Giralt et al., 2002a; Molinero et al., 2003; Penkowa et al., 2003a). The significance of MT-III is less clear, but MT-III regulation, expression, and functions clearly differ from those of MT-I + II (for review see Hidalgo et al., 2001; Chung and West, 2004; Hozumi et al., 2004).

Despite the sound evidence of MT-I + II as potent neuroprotective agents, the mechanisms through which these proteins mediate these effects are poorly understood. Microarray technology is a state-of-the-art methodology expected to have a great impact in neurobiology (Insel et al., 2004; Mirnics and Pevsner, 2004). One of the main aims of the present study is to gain insight into the role of MT-I + II by comparing wild-type mice with *Mt1+2* KO mice in a traumatic brain injury model, cryolesion, which we have previously studied in considerable detail using conventional approaches and that is generally considered a good animal model (Chan et al., 1991; Cook et al., 1998; Knerlich et al., 1999; Murakami et al., 1999). The data presented improve and enlarge our understanding of MT-I + II in system biology as well as providing essential information on the brain response to injury.

MATERIALS AND METHODS

Experimental Design for Array Analysis

Breeding pairs of *Mt1+2* KO mice (Masters et al., 1994a) as well as of wild-type mice (129/SvJ) were obtained from The Jackson Laboratory, and colonies were raised in Barcelona. Both strains are maintained in the installations of Harlan Ibérica, S.L. Mice have been confirmed to be KO by Southern blotting (Masters et al., 1994a), in situ hybridization (Carrasco et al., 1998b), and radioimmunoassay (Gasull et al., 1993).

Wild-type and *Mt1+2* KO mice were subjected to a cryolesion of the somatosensorial cortex as previously de-

scribed (Penkowa and Moos, 1995; Penkowa et al., 1999b) and were killed at four time points after cryolesion (1, 4, 8, and 16 days). Briefly, the mice were lesioned while under tribromethanol anesthesia, and the skull over the right frontoparietal cortex was exposed and a focal cryoinjury produced on the surface of the brain by applying dry ice for 60 sec. The animals were returned to the animal room and were killed by decapitation at the different times stated. The ipsilateral cortex was immediately dissected, frozen in liquid nitrogen, and stored afterward at −80°C. Unlesioned control and *Mt1+2* KO mice were also killed. For this study, we used n = 3 for most groups, except for unlesioned WT and 8 dpl *Mt1+2* mice, fow which n = 2. In total, therefore, we used 28 mice and thus 28 arrays.

Experimental Design for Immunohistochemistry Analysis

For validation and further insight on results obtained in the microarrays, we performed immunohistochemistry analyses (see below for further details) in separate animals that were sacrificed at different time points after the cryolesion. In addition to *Mt1+2* KO mice and their corresponding wild-type strain, we also studied transgenic *Mt1*-overexpressing mice. Homozygous TgMT* mice used in this study were kindly provided by Dr. Glen K. Andrews (Department of Biochemistry and Molecular Biology, University of Kansas Medical Center). These mice carry 56 copies of a minimally mutated *Mt1* gene, and their background is B6/SJL F1 (Palmiter et al., 1993), outbred to CD-1 females (Dalton et al., 1996). For experiments, and to use the proper controls, male TgMTI* mice were crossed with CD-1 females, and the F1 offspring were crossed with each other to obtain the F2 offspring. From these, we identified by Southern blot (Masters et al., 1994a) the 25% of homozygous transgenic mice and the 25% of nontransgenic mice, which were used as the control animals in the TgMTI*-related experiments.

Some of the wild-type and *Mt1+2* KO mice were divided into two groups receiving either saline (placebo) treatment or exogenous MT-II protein (Sigma code M9542) administered i.p. (twice each day, starting on the day of the cryolesion, at a dosage of 5 μg MT/10 g body weight) as described by Giralt et al. (2002b). All experiments were carried out in a humane manner and were approved by the proper ethical committees.

DNA Microarray Analysis

Total RNA was isolated from each hemisphere cortex sample with Trizol reagent (Invitrogen, San Diego, CA) and further purified with RNeasy1 kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was used to synthesize double-stranded cDNA with the Superscript Choice System (Invitrogen) with an oligo-dT primer containing a T7 RNA polymerase promoter (GenSet). 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). 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 hr to Affymetrix MG-U74Av2 arrays (Affymetrix Inc., Santa Clara, CA), which contain ~12,400 mouse probe sets corresponding half to expressed sequences tags (ESTs) and half to characterized genes. The arrays were washed and stained with phycoerytrin streptavidin (SAPE) using the Affymetrix Fluidics Station 400. The arrays were scanned in the Affymetrix GeneArray 2500 scanner, as described in the Affymetrix GeneChip protocol, to generate fluorescent images.

The image files were imported into the software package DNA-Chip Analyzer (dChip) developed by C. Li and W.H. Wong (2001), which is freware available at 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 nondifferentially 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 by Two-Way ANOVA and Functional Analysis of Gene Expression

Several analysis methods for filtering the genes being up- or down-regulated under the different conditions can be followed, but for this type of study we have used a conservative but robust strategy, namely, a standard statistical assay, two-way ANOVA with strain and cryolesion as main factors. In a previous study, we did this by transferring generated lists of genes with other type of analysis to standard statistical software in a quite time-consuming manner (Poulsen et al., 2005), but for this study we have had the advantage of using the built-in ANOVA analysis of dChip1.3.

After the genes changing significantly were identified by the two-way ANOVA (generating three different lists of genes: those affected only by the lesion, only by the strain, or by both factors), a high-level analysis was carried out by means of the hierarchical clustering utility of dChip, which identifies patterns of gene expression. Functional classification in gene-ontology categories of the genes belonging to each of the clusters identified was done with the program EASE 2.0 (Hosack et al., 2003). All GO categories with three or more genes and a Fisher exact probability of less than 0.05 were selected.

Immunohistochemical Evaluation and Validation

Mice were deeply anesthetized with 10 mg/100 g body weight of Brietal (Methohexital 10 mg/ml; Eli Lilly) and were transcardially perfused with 0.9% saline with 0.3% heparin (15,000 IU/liter) for 3–5 min, followed by perfusion with Zamboni's fixative, pH 7.4, for 8–10 min. Afterward, all the brains were fixed by immersion in Zamboni's fixative, pH 7.4, for 4 hr at room temperature. Brains were dehydrated according to standard procedures, embedded in paraffin, and cut into serial coronal 5-μm-thick sections. Sections were rehydrated, and, for heat-induced antigen retrieval, sections were boiled in citrate buffer, pH 9.1 or pH 6.0, in a microwave oven for 10 min. After cooling to room temperature, the sections were incubated in 1.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40; Sigma-Aldrich, St. Louis, MO; code N-6507) for 15 min at room temperature to

quench endogenous peroxidase. Afterward, sections were incubated with 10% goat serum (In Vitro, Denmark; code 04009-1B) or donkey serum (The Binding Site, Cambridge, United Kingdom; code BP 005.1) in TBS/Nonidet for 30 min at room temperature to block nonspecific binding.

Sections prepared for incubation with monoclonal mouse-derived antibodies were in addition incubated with Blocking Solutions A + B from HistoMouse-SP Kit (Zymed, South San Francisco, CA; code 95-9544) to quench endogenous mouse IgG. Sections were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-rat aquaporin 2 1:100 (Chemicon, Temecula, CA; catalog No. AB3066), mouse anti-RAGE 1:60 (Chemicon catalog No. MAB5328), sheep anti-human catalase 1:50 (The Binding Site catalog No. PC 136), and rabbit anti-frizzled-9 1:100 (AbCam, Cambridge, United Kingdom;0 catalog No. ab13000). The primary antibodies were detected by using biotinylated goat anti-mouse IgG 1:200 (Sigma-Aldrich code B8774), biotinylated mouse anti-rabbit IgG 1:400 (Sigma-Aldrich code B3275), or biotinylated donkey anti-sheep/goat IgG 1:20 (Amersham, Amersham, United Kingdom; code RPN 1025) for 30 min at room temperature, followed by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP; Dako, Glostrup, Denmark; code K377) prepared at manufacturer's recommended dilutions for 30 min at room temperature. Afterward, sections were incubated with biotinylated tyramide and streptavidin-peroxidase complex (NEN, Life Science Products, Boston, MA; code NEL700A) prepared following manufacturer's recommendations. Finally, the immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine-tetrahydrochloride (DAB)/TBS for 10 min at room temperature. In case of rabbit anti-frizzled-9, the primary antibodies were detected using fluorescein-linked goat anti-rabbit IgG 1:50 (Jackson Immunoresearch, West Grove, PA; 111-095-144).

To evaluate the extent of nonspecific binding of the antisera in the immunohistochemical experiments, the primary antibody step was omitted. Results were considered only if these controls were negative.

RESULTS AND DISCUSSION

Gene Selection and Functional Analysis

In an experiment like this, in which two different mouse strains are being compared and in which different times following a cryolesion are analyzed, it is obvious that a statistical analysis of the data such as two-way ANOVA is imperative. dChip1.3 allows such possibility and in addition uses it as a method for filtering genes in the arrays, a superb utility in this software, preferable to filtering genes by -fold change or by similar means. Nevertheless caution is warranted, in that the number of mice per group is not as high as one would use under normal circumstances. However, we used 28 arrays in total, one of the largest numbers ever used in similar arrays studies.

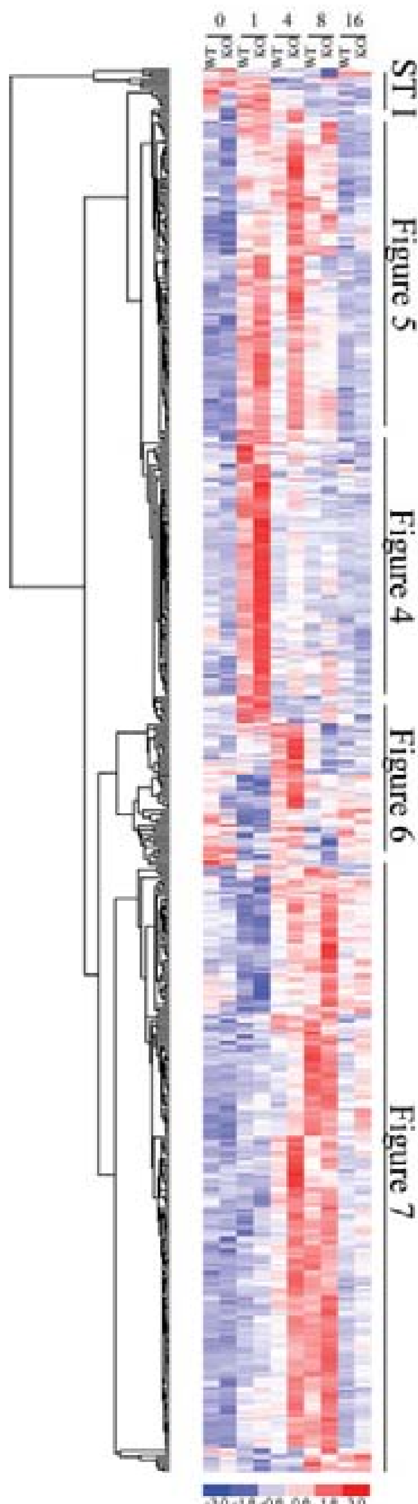
By analyzing all animals simultaneously, ANOVA can identify many genes changing even relatively moderately if they do so in several timings that otherwise would not be detected. Thus, a large number of genes were readily identified to change significantly (Table I).

TABLE I. Significant Changes of in Gene Expression According to Two-Way ANOVA

Significance	Cryolesion	Strain	Both
$P < 0.05$	1,042	470	800
$P < 0.01$	459	73	150
$P < 0.001$	190	5	22

Obviously, the higher the significance level, the more robust and reliable is the gene selection. To be conservative but not excessively restrictive, we focused our discussion on those genes changing with a significance of $P < 0.01$: 459 genes were significantly affected by the cryolesion only, 73 by the strain only, and 150 by both. The pattern of expression of these genes was analyzed by using the high-level analysis "Hierarchical clustering" utility of dChip (see Figs. 1–3, respectively). In the clustering picture, each row represents a gene, and each column represents the mean value of the mice used for each group. In Figure 1, the names of the genes are not shown, because there are too many to be readable, theredore, this figure is split into several to show the names (Figs. 4–7). The color scale on the bottom represents the relative expression level, the red, white, and blue colors corresponding to expression level above mean, at mean, and lower than mean expression of a gene across all samples, respectively (see www.dchip.org for further details). This high-level analysis detects clusters of genes that behave similarly, i.e., those genes that are down-regulated or up-regulated by the cryolesion at specific timings, etc., and by looking at Figures 1–3 it is obvious that a number of clusters are readily identified and that both cryolesion and MT-I + II deficiency show clear-cut effects. Thus, Figure 1 clearly shows that there are genes that are up-regulated at 1dpl, others at 4dpl, etc. Figure 2 shows genes that are different in the *Mt1+2* KO mice compared with WT mice, with one cluster showing those genes whose expression is decreased and a second cluster showing the opposite, throughout all time periods. Figure 3 shows genes affected by both the cryolesion and the strain, which typically means those genes whose response to cryolesion is different in the two strains, for instance, one gene that is down-regulated in WT mice whereas it is up-regulated in *Mt1+2* KO mice. It is likely that some genes stated to be affected by the "cryolesion only" are in fact affected by MT-I + II deficiency too, and vice versa, but that they are not identified as such because gene expression is being affected in a single timing or not prominently or because the number of mice is not high. Despite these shortcomings, the use of two-way ANOVA is still the most appropriate method for an experiment such as this, and we hope that the overall quality of the sets of genes identified is reasonable.

In Supplementary Table I, we provide the Affymetrix probe set numbers and the corresponding full names of the genes for each figure, arranged alphabetically. Considering the number of genes significantly affected in



this experimental paradigm, it becomes obvious that they cannot be discussed one by one in detail. Thus, we carried out a functional analysis by using gene ontology as described previously (Hosack et al., 2003) to identify the categories overrepresented among the genes belonging to each of the ANOVA sets of genes shown in Figures 1–3 (Supplementary Tables II–IV). For this study, we will rather be focusing on those genes affected by MT-I + II deficiency, and comment only briefly on those genes up- or down-regulated by the cryolesion itself. One potential source of concern is the putative variability in the knockout samples compared with the control samples; this was measured by the standard deviation and coefficient of variation for all values across each sample, respectively. The values are shown in Supplementary Table V: there is no significant difference in the variation of the microarrays for the two strains.

Genes Significant for Strain Only

Figure 2 shows the hierarchical clustering analysis of those genes identified by the two-way ANOVA as being statistically different between WT and *Mt1+2* KO mice but that were not affected by the lesion ("strain only"). As mentioned above, it is likely that some of these genes are indeed affected by the lesion, but the effect is too small (or occurs just in a single timing) to provide statistical significance in the ANOVA. Rather, what is being detected here are genes whose expression is normally significantly different between strains, and, because there are many animals being analyzed, this method is superior to using just the unlesioned animals. A brief inspection of the hierarchical clustering already shows that there are two groups of genes, one whose expression is lower in *Mt1+2* KO mice than in WT mice and other with the opposite pattern (Fig. 2).

Genes lower in MTKO. Obviously, in this group we found the *Mt1* gene, which was absent in *Mt1+2* KO mice; somewhat surprisingly, *Mt2* was not here but was picked up by the ANOVA as one of the genes being affected by the lesion at 1 dpl (see Fig. 4) but not by the strain. This apparent discrepancy highlights how important is the design of the oligoprobes (as well as the perfect match method). The mutations done for *Mt2* and *Mt1* result in peptides of 15 and 9 amino acids for MT-II and MT-I, respectively (Masters et al., 1994a); whereas the array did discriminate true (native) *Mt1* from mutated *Mt1* (it does not if the perfect match method is not used), it could not do so with *Mt2*

Fig. 1. Hierarchical clustering of the 459 genes (including redundant probe sets and genes of unknown function) identified to be significantly ($P < 0.01$) affected by the cryolesion only. Each row represents a gene and each column represents the mean value of the two (unlesioned) or three (lesioned) mice for each strain and timing. The clustering was split into several parts (a few genes are shown only in Supplementary Table I) in order to show the name of the genes (see Figs. 4–7). Figure can be viewed in color online via www.interscience.wiley.com.

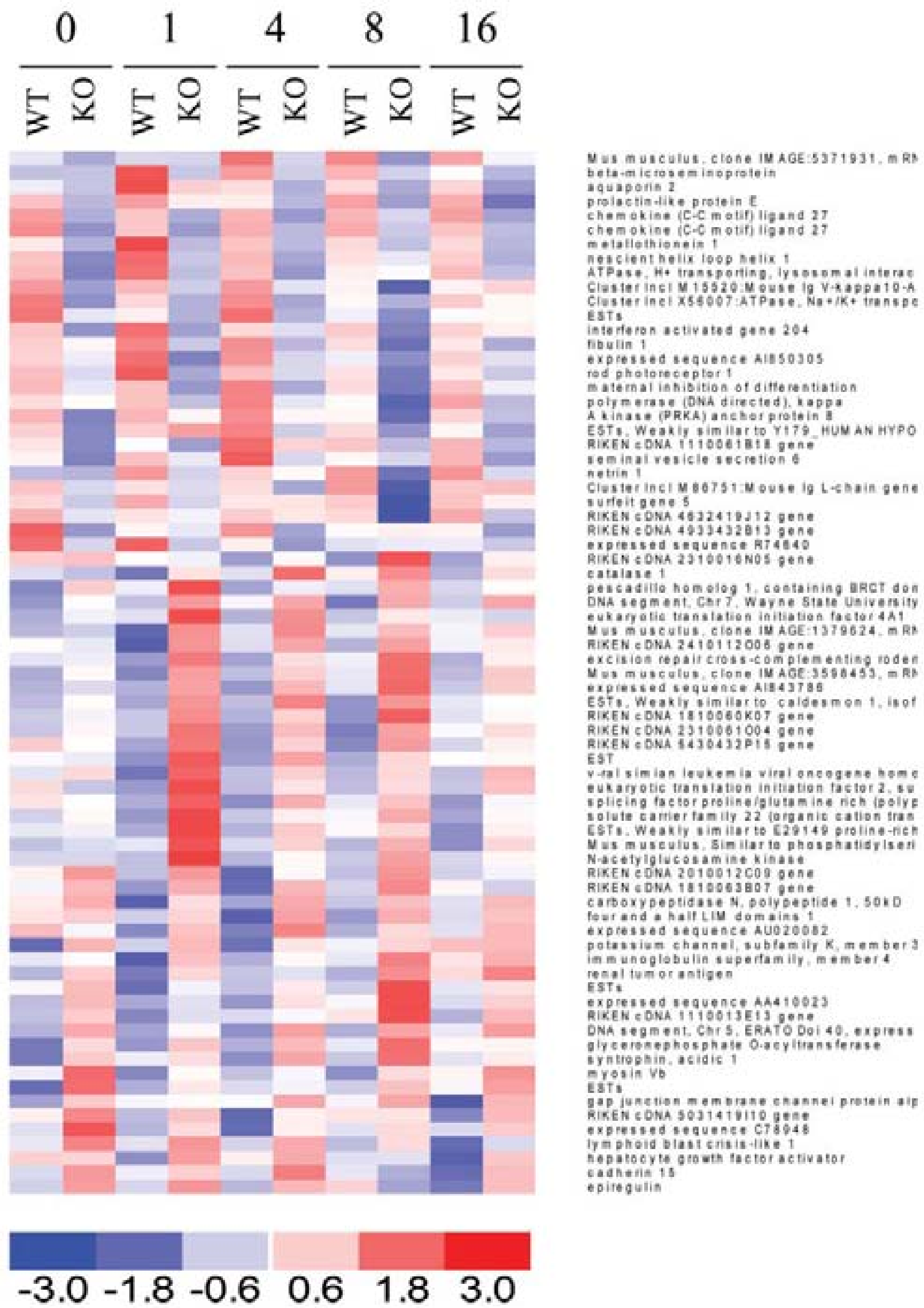


Fig. 2. Hierarchical clustering of the 73 genes (including redundant probe sets and genes of unknown function) identified to be significantly ($P < 0.01$) affected by the strain only. Figure can be viewed in color online via www.interscience.wiley.com.

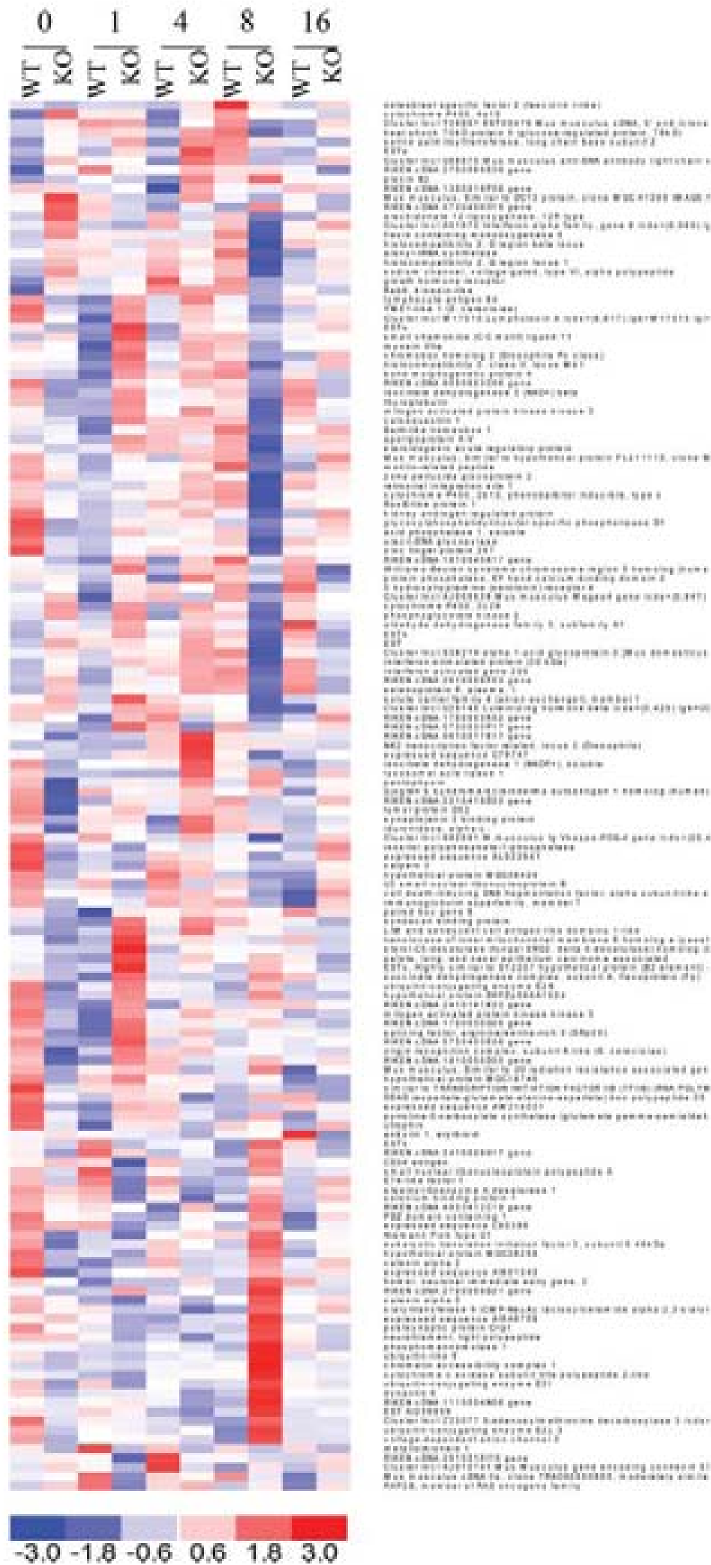


Fig. 3. Hierarchical clustering of the 150 genes (including redundant probe sets and genes of unknown function) identified to be significantly ($P < 0.01$) affected by both factors (cryolesion and strain). Figure can be viewed in color online via www.interscience.wiley.com.

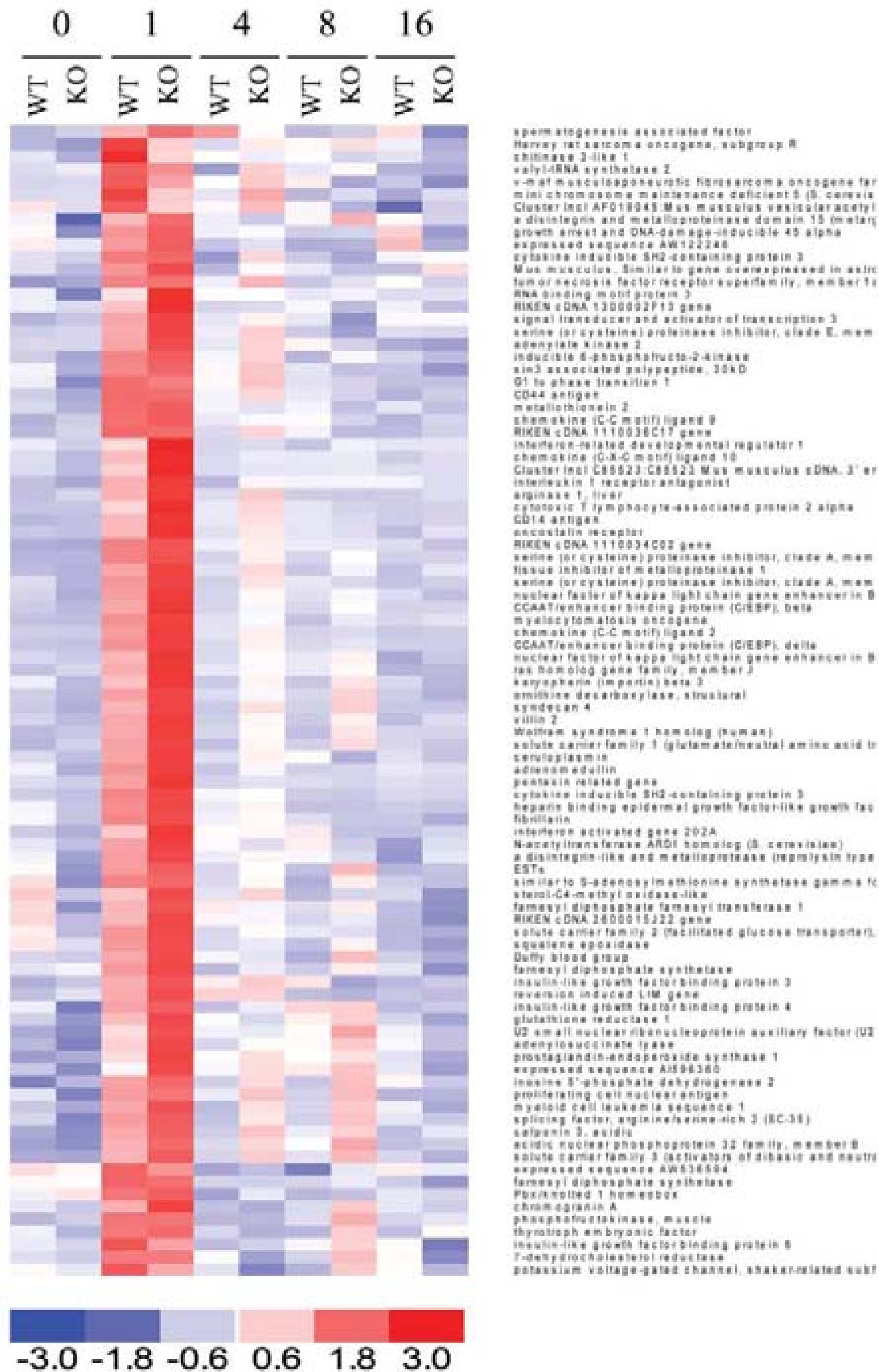


Fig. 4. Hierarchical clustering of a subset of genes whose expression changes basically at 1 dpl (see also Fig. 1). Figure can be viewed in color online via www.interscience.wiley.com.

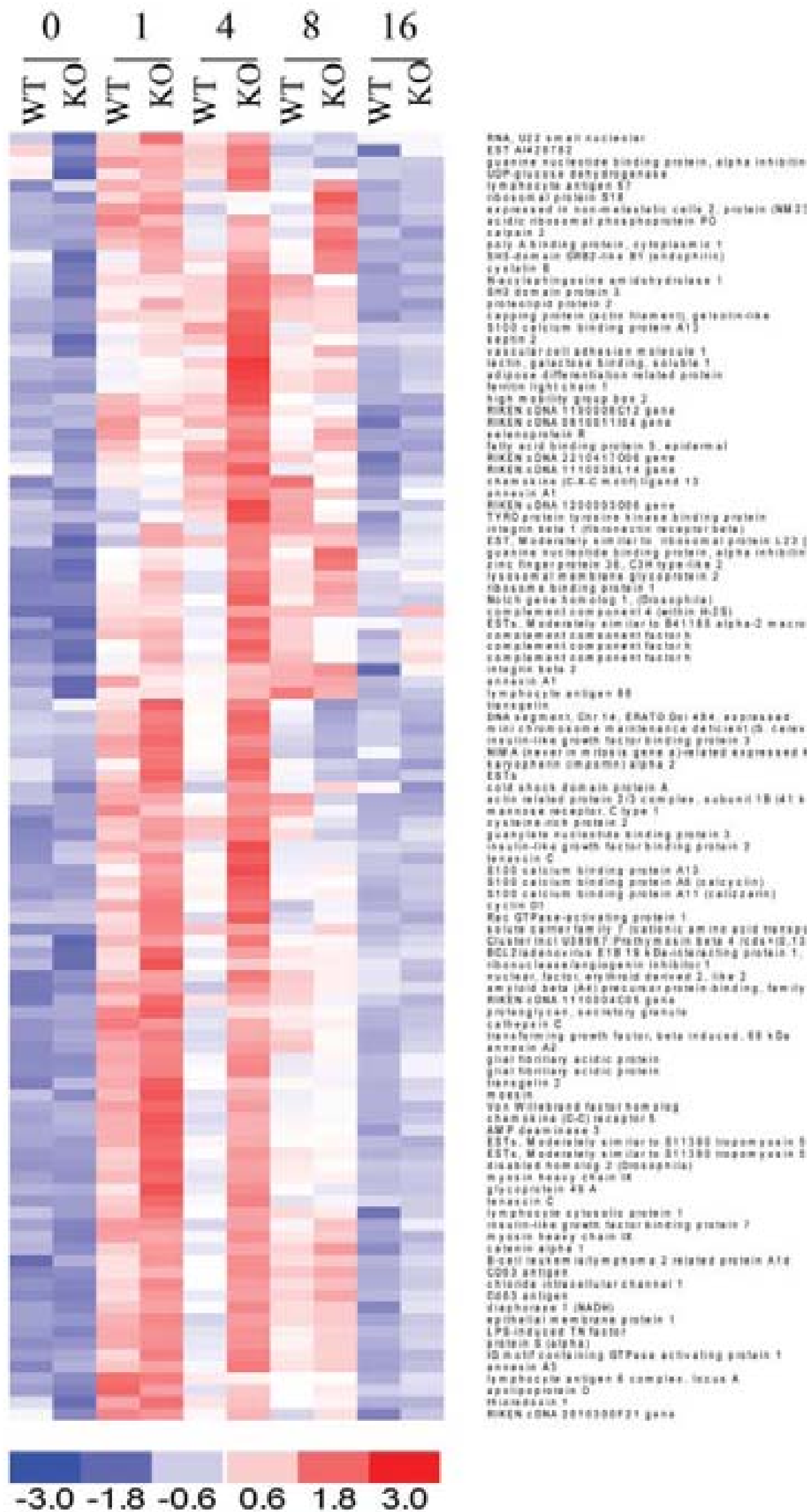


Fig. 5. Hierarchical clustering of a subset of genes whose expression changes at 1–8 dpl (see also Fig. 1). Figure can be viewed in color online via www.interscience.wiley.com.

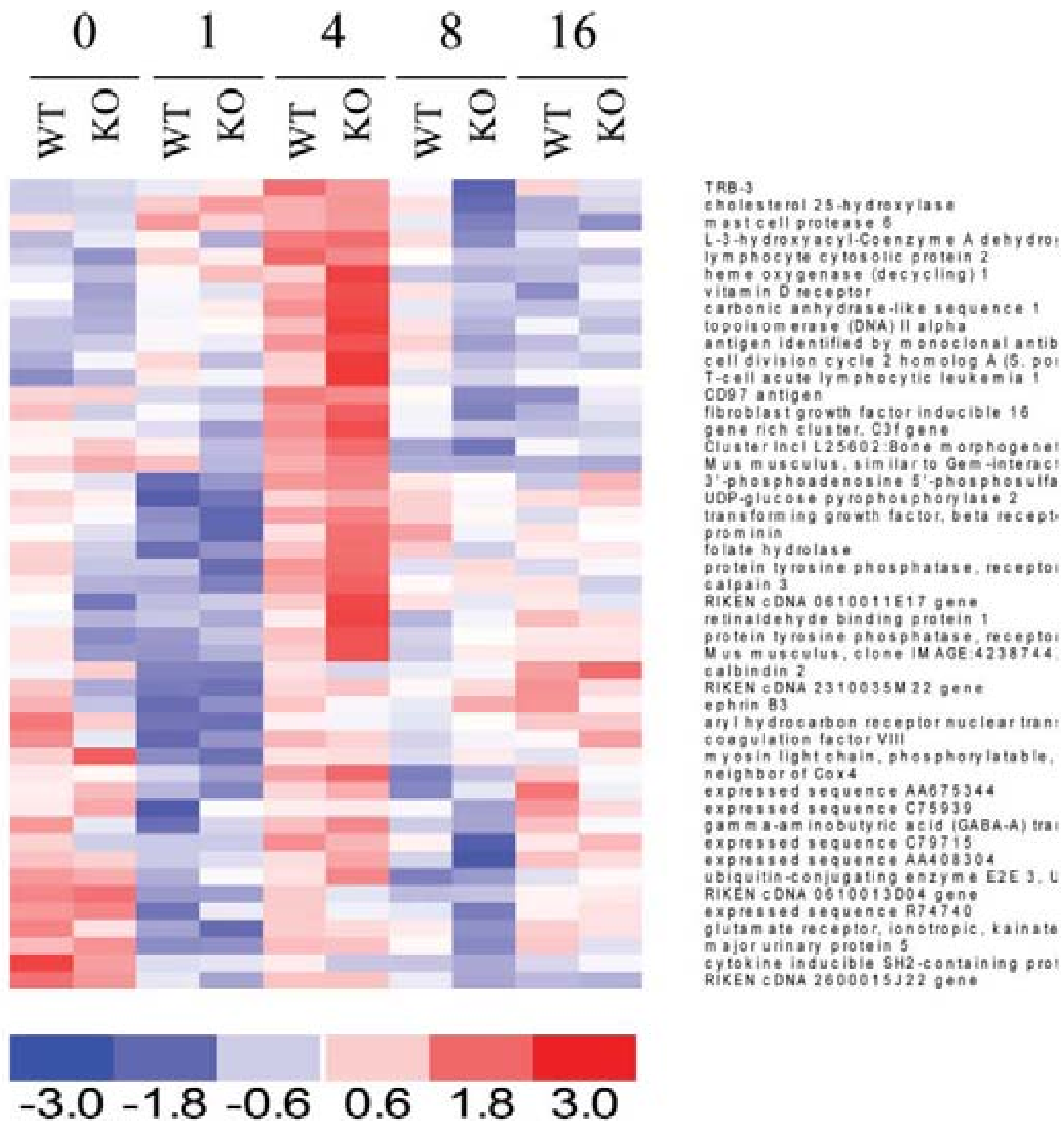
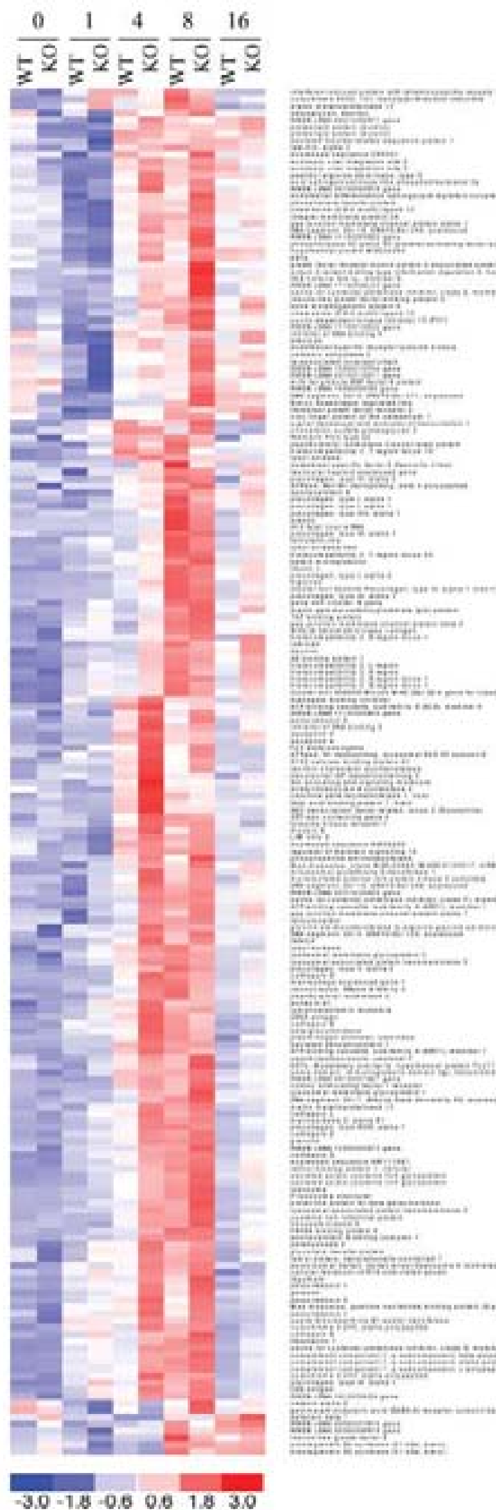


Fig. 6. Hierarchical clustering of a subset of genes whose expression changes basically at 4 dpl (see also Fig. 1). Figure can be viewed in color online via www.interscience.wiley.com.

because too much of the mRNA is still present in the mutated *Mt2*. A closer inspection of the results clearly shows that *Mt1* expression is in fact increased at 1 dpl in WT mice, very much in line with many previous studies (for review see Hidalgo et al., 2001), but, because no change is seen at other timings, such an effect is not detected by the two-way ANOVA, again highlighting the limitations inherent to this method when few animals per group are being used. The most logical result would be *Mt1* being detected as belonging to the strain + cryolesion set of genes, which indeed was the case for other *Mt1* probe set (see Fig. 3, Supplementary Table I).

One major result of this study, readily detected also by the GO analysis, is the finding that monoamine oxi-



dase A (MaoA; Riken 1110061B18) was decreased in the *Mt1+2* KO mice. MaoA is a well-known enzyme that degrades serotonin and norepinephrine and therefore has a major importance in a wide range of CNS functions, including behavior (Cases et al., 1995). Furthermore, the 5-hydroxytryptamine (serotonin) receptor 4 was also significantly affected by MT-I + II deficiency (Fig. 3). Thus, it is interesting that a recent study with the same mouse model has identified a learning deficit in the eight-arm radial maze (Levin et al., 2006). In a different genetic background, MT-I + II deficiency also has been shown to produce behavioral changes (Yoshida et al., 2006). Whether this deficiency in MaoA underlies these neurobiological effects remains to be established. Indeed, other genes known to affect CNS functions were also significantly affected in the *Mt1+2* KO mice.

The GO analysis (Supplentary Table III) also revealed expression changes in several extracellular matrix genes, including decreased expression of fibulin 1 and netrin 1. Fibulin 1 is an extracellular matrix calcium binding glycoprotein controlled by Sp1 and Sp3, expressed in neurons only, and appears to be important in the control in a number of cell processes (Kostka et al., 2001). Netrin-1 is expressed during development but also in the adult by multiple types of neurons and by myelinating glia and has dramatic importance in axon guidance, cell migration, cell survival, and likely axon regeneration following injury (Manitt and Kennedy, 2002). Several of these features are consistent with the phenotype of the *Mt1+2* KO mice during injury.

Although not detected as overrepresented categories by the GO analysis, other genes were found to be affected by MT-I + II deficiency by dChip that could be expected to be important in the CNS. Some are transcription factors, such as nescient helix-loop-helix 1 (Hen1, Nscl, Tal2; Cogliati et al., 2002); maternal inhibition of differentiation (Maid, SSEC-8), a cyclin D-type binding-protein 1 that contains a conserved HLH motif without a basic DNA binding domain and that has been suggested to function as a negative regulator of bHLH (Terai et al., 2000); interferon-activated gene 204 (IFI16), a member of the hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats (HIN-200) family of transcription factors known to suppress cell growth and that has recently been shown to be involved in p53-mediated transmission of DNA damage signals and apoptosis (Aglipay et al., 2003); chemokine (C-C motif) ligand 27 (Ccl27), which by alternate splicing can produce a secretable form that interacts with its cognate receptor, CC chemokine receptor 10 (CCR10), and a nonsecreted form that is targeted to the nucleus, PESKY, which is able to modulate transcription and alter cell morphology (Nibbs and Graham, 2003); and ankyrin repeat and KH domain containing 1 (MASK; Rik 4933432B13), which

Fig. 7. Hierarchical clustering of a subset of genes whose expression changes basically at 4–8 dpl (see also Fig. 1). Figure can be viewed in color online via www.interscience.wiley.com.

belongs to the group of genes established by Kazusa cDNA sequencing project (KIAA), but of unknown function (Kikuno et al., 2004). It is noteworthy that MT-I + II deficiency also caused significant decreases in genes known to affect gene transcription by other means: polymerase (DNA directed) kappa (DNA damage-inducible protein b; Dinb1) is a member of the Y family of DNA polymerases involved in tolerance to DNA damage by translesion DNA synthesis (Guo et al., 2003), which is consistent with the known susceptibility of these mice to DNA damage; and A kinase anchor protein 8 (AKAP8, AKAP95), which binds cAMP-dependent protein kinase as well as DNA in the nuclear matrix but also other proteins such as p68 RNA helicase and D-type cyclins and that has been suggested to be a scaffold for coordinating the assembly of transcriptionally active protein complexes (Akileswaran et al., 2001). Interestingly, AKAP8 has also been shown to be a partner of the minichromosome maintenance (MCM) 2 protein, a component of the prereplication complex, thus suggesting a role on DNA replication (Eide et al., 2003).

Other genes of known function affected were: rod photoreceptor 1 (phosducin), a rod-abundant protein known to interact with the beta gamma subunits of G proteins (transducin), which is also present in other tissues, including the brain, where it inhibits receptor-stimulated adenylyl cyclase activity in cell membranes by about 50% (Danner and Lohse, 1996); ATPase, H^+ transporting, lysosomal accessory protein 1, which is involved in the generation of a proton gradient across membranes (Schoonderwoert and Martens, 2002); Na/K-ATPase beta 2 subunit, also known as adhesion molecule on glia (AMOG), which is part of an Na,K-ATPase complex expressed preferentially by astrocytes in mouse brain (Pagliusi et al., 1990); serpina-12 (Riken 4632419J12), which belongs to the serine (or cysteine) proteinase inhibitor superfamily (serpins) functioning within a delicate system involved in many CNS functions both during development (cell migration, axon outgrowth, and synapse elimination) and in the adult state (neuropeptide processing, regulation of neuronal survival and structural plasticity; Molinari et al., 2003); OTU domain, ubiquitin aldehyde binding 1 (Otubain 1; sxpressed sequence AI850305), which belongs to a new family of specific ubiquitin isopeptidases with no sequence homology to known deubiquitylating enzymes (Balakirev et al., 2003); and genes related to the Ig family (Ig V-kappa10-Ars-A kappa chain gene, Ig L-chain gene variable region). The other genes shown in Figure 2 to be decreased in the *Mt1+2* KO mice have not been normally shown to be expressed in the brain and/or are of unknown function (see also Supplementary Table I). Aquaporin-2 (AQP2), for instance, has normally been considered not to be expressed in the brain, yet it was detected in the array and, furthermore, also by immunohistochemistry (see below; Fig. 8); aquaporins are a family of membrane-channel proteins essential for movement of water through cell membranes (King et al., 2004), also in the brain, where some of them (e.g., AQP4) might have a significant role in the control of brain edema following trauma (Gunnarson et al., 2004).

Genes higher in MTKO. The hierarchical clustering also detected a large group of genes showing a higher expression in the *Mt1+2* KO compared with the WT mice. The difference could eventually be more marked at a specific time in some genes, but for most cases this tendency was general for all the timings, so they were classified as being in this ANOVA category by dChip.

Remarkably, many of the genes up-regulated in the *Mt1+2* KO mice can be linked to protein synthesis by acting at several levels, and the translation initiation factor activity was overrepresented in the GO analysis (Supplementary Table III). First, some of these genes are involved in general transcription/splicing processes: exosome component 5 (Exosc5) is a member of the exosome, a complex consisting almost exclusively of exoribonucleolytic proteins that is involved in maintaining correct RNA levels (Raijmakers et al., 2004); DEAD (Asp-Glu-Ala-Asp) box polypeptide 51 (Ddx51; Riken 2310061O04) is one of several ATP-using enzymes containing the characteristic DExD sequence motif that is required for the assembly, remodeling, and disassembly of the spliceosome, a complex molecular machine responsible for pre-mRNA splicing (Staley and Guthrie, 1998); splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated; Sfpq; PSF), also essential for pre-mRNA splicing; and excision repair cross-complementing rodent repair deficiency, complementation group 3 (Ercc3), which is one of the nine subunits of the general transcription factor IIH (TFIIH) family and thus is essential for controlling RNA PolII and gene transcription (Fukuda et al., 2002). Ercc3 also belongs to the nucleotide excision repair pathway, which removes injury-induced DNA adducts and appears to be involved in p53-induced apoptosis (Wang et al., 1996), which is compatible with the known phenotype of the *Mt1+2* KO mice. Second, some of these genes were in fact transcription factors, such as four and a half LIM domains 1 (FHL1; Lee et al., 1998) and armadillo repeat-containing 1 (Armc1; Riken 2310016N05), which may also mediate protein–protein interactions in diverse cellular processes, including cell junction assembly and nuclear transport (Andrade et al., 2001). Third, other genes are involved in ribosome function in several ways: pescadillo homolog 1 (Pes1, mouse homolog of zebrafish pescadillo) is essential for ribosome biogenesis and nucleologenesis and disruption of its function results in cell cycle arrest (Lerch-Gaggl et al., 2002); eukaryotic translation initiation factor 4A1 (Eif4a1) is one of the protein synthesis initiation factors involved in the binding of mRNA to the ribosome (Nielsen and Trachsel, 1988); and eukaryotic translation initiation factor 2, subunit 2 (beta; Eif2s2), that forms part of the trimeric complex eIF2 (α, β, γ subunits) and whose phosphorylation has been recognized as a mechanism for suppressing global protein synthesis, and, together with some downstream targets (eIF4E binding protein-1, S6K1, eIF4G) of a signaling pathway that includes the protein kinase target of rapamycin (TOR), constitute the essential inte-

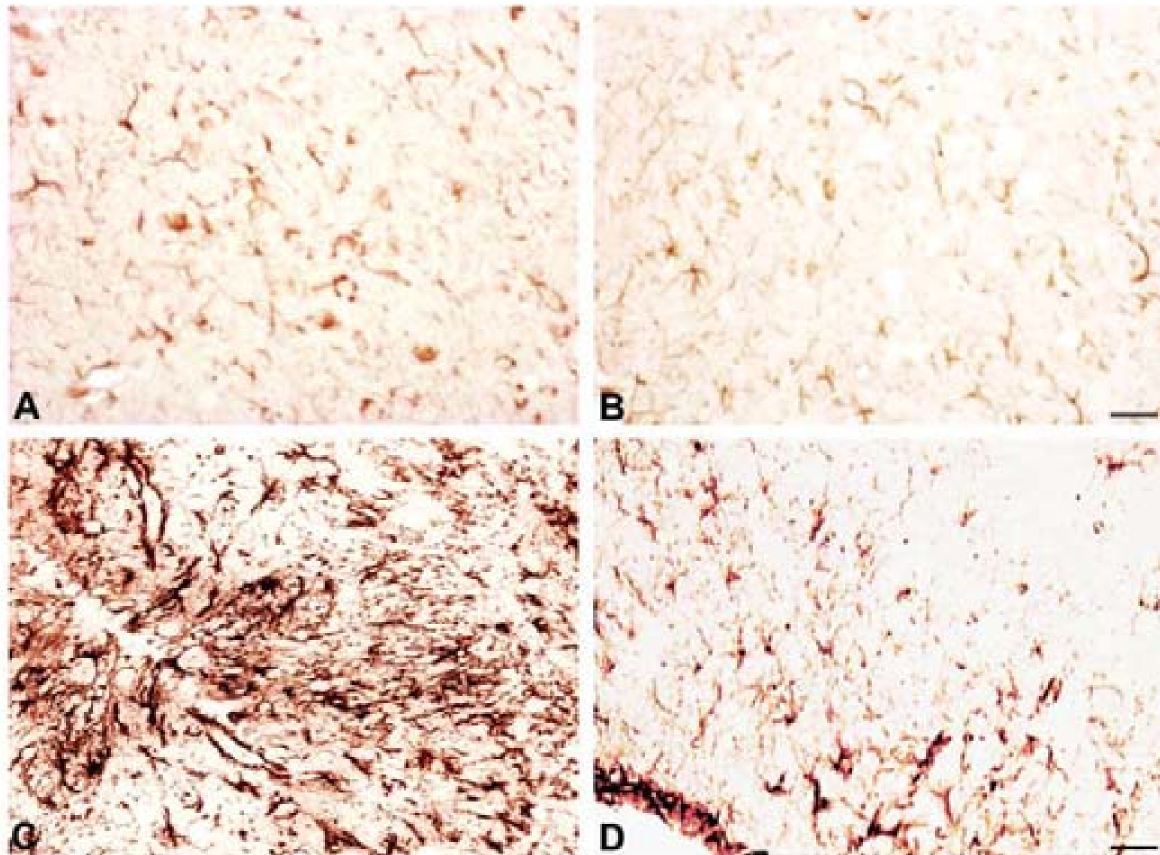


Fig. 8. Aquaporin 2 expression in brains of unlesioned mice (**A,B**) and lesioned mice at 10 dpl (**C,D**). Both wild type mice (A,C) and *Mt1+2* KO mice (B,D) mice are shown. MT I + II deficiency reduced aquaporin 2, which is seen mainly in astrocytes. Scale bars = 90 µm. Figure can be viewed in color online via www.interscience.wiley.com.

grating mechanisms coordinating nutrient availability with cell growth and proliferation (Jefferson and Kimball, 2003). Interestingly, both Eif4a1 and Eif2s2 were also significant in the GO analysis. Fourth, genes affecting mitochondrial functions are also altered: the translational initiation factor 2 (Mtif2; Riken 2410112O06), also shown as significant by the GO analysis, is nuclear-encoded but functions in the mitochondria to initiate the translation of proteins encoded by the mitochondrial genome, which, leaving aside the genes encoding for rRNA and transfer RNA, encodes for 13 protein subunits of the oxidation phosphorylation system (Overman et al., 2003); and the translocase of outer mitochondrial membrane 20 homolog (yeast; Tom20, Riken 1810060K07) belonging to the TOM complex and functioning to recognize initially mitochondrial preproteins with N-terminal presequence (whereas Tom70 recognizes preproteins with internal targeting signals, such as inner membrane carrier proteins; Yano et al., 2004). Other genes affecting mitochondria were differentially expressed in the KO mice (see below for the set of genes being affected by "cryolesion and strain").

Other genes picked up by dChip in this set could be related to the control of cell cycle and to cell signaling: cyclin-dependent kinase inhibitor 1B (P27, p27kip1, Cdkn1b, expressed sequence AI843786), which is a central regulator of the cell cycle (Fero et al., 1996); hepatocyte growth factor activator is a serine proteinase that activates the precursor form of hepatocyte growth factor (HGF; Yamada et al., 1998); and epiregulin is a member of the epidermal growth factor (EGF) superfamily (Shirasawa et al., 2004); v-ral simian leukemia viral oncogene homolog A (ras related; RalA, Ral, Rasl 1) belongs to the small G protein superfamily of GTPases, which are involved in intracellular trafficking, secretion, and vesicular transport (Polzin et al., 2002); Gem-interacting protein (Riken 5031419I10) is a Rho GTPase-activating protein (GAP) that interacts with Gem (Aresta et al., 2002); and rho/rac guanine nucleotide exchange factor

(GEF) 2 (lymphoid blast crisis-like 1) activates some Rho-family GTPases (Rossman et al., 2005). Many Rho GEFs and Rho GAPs are used in the CNS to activate specific Rho GTPase family members, regulating neuronal shape, growth, and plasticity (Rabiner et al., 2005). Also, myosin Vb and Rab11a (small GTP-binding proteins) are in this set and are proteins that function in plasma membrane recycling systems and have been shown to be critical regulators of the M_4 muscarinic acetylcholine (ACh) G-protein-coupled receptor return to the plasma membrane in the brain (Volpicelli et al., 2002).

Other important genes were DnaJ (Hsp40) homolog, subfamily C, member 9 (Dnajc9; expressed sequence AU020082), which belongs to an ancient superfamily of proteins, the so-called heat-shock proteins, which involved in the control of protein folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations (Ohtsuka and Suzuki, 2000); cell division cycle 26 (cdc26, Riken 2010012C09), that forms part of the anaphase-promoting complex (APC), a cell cycle-regulated ubiquitin-protein ligase that targets cyclin B, securin, and other destruction box-containing proteins for proteolysis (Gmachl et al., 2000); lipocalin-interacting membrane receptor (Riken 1110013E13, DNA segment, Chr 15, ERATO Doi 735, expressed), which likely mediates the internalization of lipocalins, ubiquitous extracellular carriers of lipophilic compounds involved in a variety of physiological processes including inflammation, modulation of growth and metabolism, apoptosis, and even behavior processes (Wojnar et al., 2003); potassium channel, subfamily K, member 3 (Kcnk3; TASK), which belongs to the two-pore domain-type K^+ channels that are believed to be essential in setting the resting membrane potential (Lesage and Lazdunski, 2000); syntrophin, acidic 1 (also significant in the GO analysis), which mediates the association of the inwardly rectifying potassium channel Kir4.1 to the dystrophin-glycoprotein complex in glial cells, which is essential for controlling an optimal potassium concentration in the extracellular environment (Connors et al., 2004); gap junction membrane channel protein alpha 7 (connexin 45), important in some electrical synapse between murine neurons (Sohl et al., 2005); solute carrier family 22 (organic cation transporter), member 4 (Slc22a4; OCTN1), which is an efficient polytopic transmembrane sodium-dependent carnitine and sodium-independent organic cation transporter (Peltekova et al., 2004); N-acetylglucosamine kinase, which enters N-acetylglucosamine (normally produced by the endogenous degradation of glycoconjugates and by the degradation of dietary glycoconjugates by glycosidases) into the pathways of aminosugar metabolism (Hinderlich et al., 2000); glyceronephosphate O-acyltransferase, which functions in lipid biosynthesis; huntingtin-interacting protein 1 related (Hip1r; expressed sequence AA410023), which participates in the clathrin trafficking network (Hyun et al., 2004); carboxypeptidase N, polypeptide 1 (CPN1), forming part of a tetramer comprising two heterodimers, each consisting of a CPN1 and a CPN2 subunit, with a number of important substrates (kinines such as bradykinin and kallidin, complement anaphylatoxins—C3a, C4a, and C5a—or creatine kinase), thus affecting the control of vascular permeability (Matthews et al., 2004); and SynCAM (immunoglobulin superfamily, member 4A), a synaptic adhesion molecule that drives synapse assembly (Biederer et al., 2002). Interestingly, cadherin 15 (M-cadherin), in fact, was also increased in the *Mt1+2* KO mice.

Finally, it is noteworthy that some genes in this set affected in the *Mt1+2* KO mice readily denote the well-known phenotype of these animals: increased oxidative stress and inflammation. Thus, catalase 1, which has a major role in defending cells and tissues against oxidative stress in the injured brain (Ho et al., 2004), was increased, as was the receptor of advanced glycation endproducts (RAGE; renal tumor antigen; MOK; MAPK/MAK/MRK overlapping kinase), a protein that belongs to the immunoglobulin superfamily and is a membrane-bound receptor of several ligands, including amphoterin, S100b, and sugar-derived oxidation products known as advanced glycation endproducts (AGE); upon activation, RAGE activates microglia and produces a number of cytokines, such as IL-1, IL-6, TNF, and M-CSF, and clear symptoms of inflammation and oxidative stress, likely at least in part through the activation of the transcription factor NF-κB (Stuchbury and Munch, 2005). This is totally consistent with the phenotype of *Mt1+2* KO mice. Interestingly, the results for catalase and RAGE were confirmed by immunohistochemistry (see Figs. 9 and 10, respectively).

Genes Significant for Strain and Cryolesion

Figure 3 shows genes significantly affected by both the strain and the cryolesion ($P < 0.01$; see Supplementary Table I for full description and Supplementary Table IV for GO analysis). As stated above, the ANOVA is identifying those genes that respond to the brain injury but for which MT-I + II deficiency is making a difference. The hierarchical clustering is complex, insofar as such an effect may occur at a specific timing, and, moreover, MT-I + II deficiency increases the response in some cases but decreases it in others. There are many genes in this set, so we will discuss only some of them. The reader is referred to Figure 3 to appreciate the when and how of effect of both the lesion and MT-I + II deficiency.

In the set of genes being affected by "strain only," we noticed a number of genes affecting mitochondrial functions (see Fig. 2): the translational initiation factor 2 and the translocase of outer mitochondrial membrane 20 homolog. Interestingly, in the present set many other genes related to mitochondria were also involved, which were significant in the GO analysis. This is noteworthy in that a number of studies suggest that MT-I + II could have a significant role in mitochondrial functions (Ye et al., 2001; El-Assal et al., 2004; Feng et al., 2005), which might be occurring in part through some of these genes. Moreover, there is a tendency to obesity in

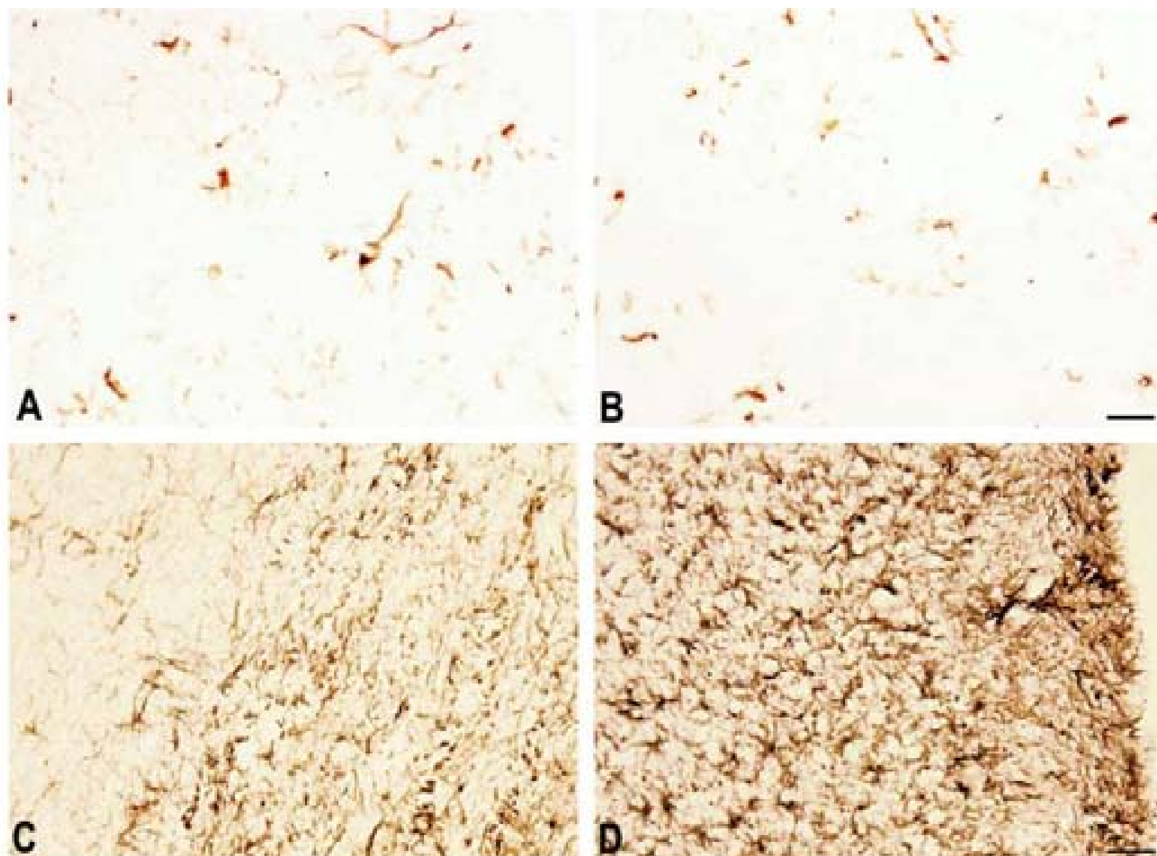


Fig. 9. Catalase in brains of unlesioned (**A**,**B**) mice and lesioned mice (**C**,**D**) brains at 3 dpl. Wild type control (A,C) and *Mt1+2* KO (B,D) mice are shown, and the lesioned *Mt1+2* KO mice showed fewer catalase positive cells relative to wild type controls. Scale bars = 90 μm. Figure can be viewed in color online via www.interscience.wiley.com.

Mt1+2 KO mice (Beattie et al., 1998). Thus, the present results open a new dimension in this regard. In this set of genes, among others, some are involved in transport: translocase of the inner mitochondrial membrane 14 (Riken 1810055D05; Dnaj (Hsp40) homolog, subfamily C, member 19; Ohtsuka and Suzuki, 2000), translocase of inner mitochondrial membrane 8 homolog a (Timm8a; Tranebjaerg et al., 2001), steroidogenic acute regulatory protein (Star) that mediates the intramitochondrial transport of cholesterol (Kim et al., 2004), and solute carrier family 25, member 1 (Slc25a1, Riken 1300019P08; also called citrate transport protein, or CTP), responsible for the movement of citrate across the mitochondrial inner membrane (Kaplan et al., 1993); energy production: cytochrome c oxidase subunit VIIa polypeptide 2-like (Cox7a2l), a rate-limiting enzyme in oxidative phosphorylation (Sakata et al., 2005), succinate dehydrogenase complex, subunit A, involved in the oxidation of succinate (Pawlu et al., 2005), and isocitrate dehydrogenase 3 (NAD^+) beta (Idh3b), which catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate (Mootha et al., 2003); and DNA repair: uracil-DNA glycosylase (Ung), which is involved in base excision repair of aberrant uracil residues in nuclear and mitochondrial DNA, and its deficiency has been shown to have a dramatic effect in the brain following ischemia (Endres et al., 2004; see also Supplementary Tables I and IV for a complete list).

Interestingly, MT-I + II deficiency clearly affects the control of protein synthesis at several levels, including the mRNA splicing process, insofar as, in addition to those already stated above, other genes important in this regard were readily identified in this set of genes (also by GO analysis): small nuclear ribonucleoprotein polypeptide A (U1 snRNP) and U2 small nuclear ribonucleoprotein B (Snrpb2), both involved in the mRNA splicing process (Meister et al., 2001); DEAD box polypeptide 25 (Ddx25), important in the control of spliceo-

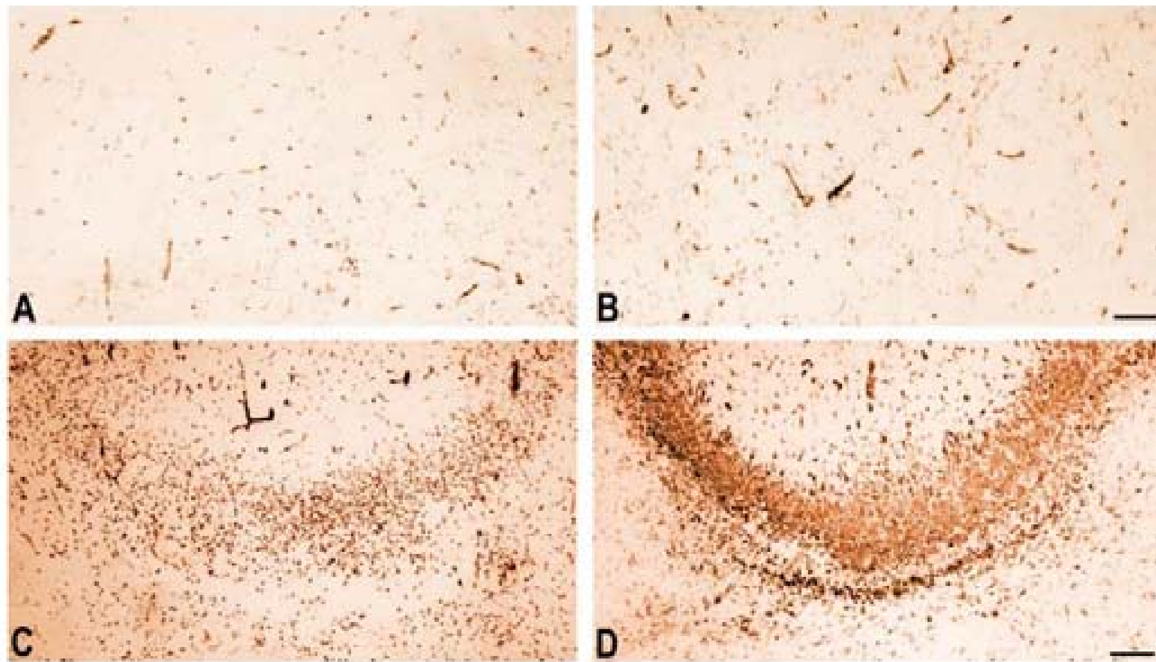


Fig. 10. RAGE expression in unlesioned brains of wild ype mice (A) and *Mt1+2* KO mice (B) and in lesioned brains at 10 dpl of wild type controls (C) and *Mt1+2* KO mice (D). MT I + II deficiency resulted in increased RAGE expression, as seen mainly in macrophages and activated microglia. Scale bars = 182 μm. Figure can be viewed in color online via www.interscience.wiley.com.

somes (Staley and Guthrie, 1998); and splicing factor, arginine/serine-rich 3 (SRp20), which promotes the nucleocytoplasmic export of intronless mRNA (Huang and Steitz, 2001). As could be expected, some genes involved in the control of gene transcription were present in this set of genes: general transcription factor IIB, required by RNA polymerase II to form a preinitiation complex (Elsby and Roberts, 2004); chromatin accessibility complex 1 (Chrac1), a histone-fold protein that interacts with other histone-fold proteins to bind DNA in a sequence-independent manner, which combine within larger enzymatic complexes for DNA transcription, replication, and packaging (Bolognese et al., 2000); chromobox homolog 2 (Cbx2), which might play a role in defining access to retinoic acid response elements localized in the regulatory regions of several Hox genes (Core et al., 1997); proline-rich nuclear receptor coactivator 2, which interacts with nuclear receptors using a proline-rich sequence (with orphan receptors SF1 and estrogen receptor-related receptor α1 in a ligand-independent manner and with the ligand-binding domains of ER, GR, PR, TR, RAR, and RXR in a ligand-dependent manner; Zhou and Chen, 2001); E74-like factor 1 (Elf1, myeloid ELF1-like factor), a member of the ETS family of transcription factors that is expressed in hematopoietic cells (Hedvat et al., 2004); and interferon-activated gene 205 (Ifi205; Aglipay et al., 2003), among others. Genes important for the translation process could also be observed in this set: eukaryotic translation initiation factor 3, subunit 6, one of the multisubunit protein complex that plays a central role in the pathway of initiation by promoting the binding of both methionyl-tRNAi and mRNA to the 40S ribosomal subunit (Bommer et al., 1991); eukaryotic translation termination factor 1 (Etf1; hypothetical protein MGC18745); eukaryotic translation initiation factor 4E member 2 (Eif4e2; Riken 2700069E09); and, in line with other genes identified in Figure 2, the ribosomal protein S6 kinase, polypeptide 1 (Rps6kb1; Riken 2610318I15), which is downstream of mTOR, a protein kinase activated by nutrients and insulin-like growth factors (Jefferson and Kimball, 2003).

As could be expected from what is known of the phenotype of the *Mt1+2* KO mice, several genes involved in the inflammatory response were selected in this list of genes: several members of the histocompatibility system [histocompatibility 2, class II, locus Mb1 (H2-DMb1), H2-Q1, and H2-Ob]; CD34 antigen, expressed in immature cells that could participate in neovascularization of ischemic brain (Taguchi et al., 2004); heat shock 70-kD protein 5 (glucose-regulated protein; Hspa5), a stress protein found in endoplasmic reticulum

and on the cell surface that is essential for α2-macroglobulin-induced signal transduction in macrophages (Misra et al., 2002); Cd300C antigen (Immunoglobulin superfamily, member 7, dendritic cell-derived immunoglobulin receptor 1), which plays an important role in the immune response (Clark et al., 2000); lymphocyte antigen 64 YM E1 (Ly64), a B-cell surface molecule that transmits a growth-promoting signal and is implicated in the life/death decision of B cells (Miura et al., 1996); and small chemokine (C-C motif) ligand 11 (Ccl11, eotaxin), which acts by attracting eosinophils, basophils, and Th2 lymphocytes (Ogilvie et al., 2004). Less expected was the finding that many lipogenic enzymes, signalling factors, and ubiquitin system factors were clearly identified by dChip and the GO analysis (see Supplementary Table IV).

Genes Significant for Cryolesion Only

Figure 4 shows genes dramatically up-regulated at 1 dpl. As expected, many of these genes are related to lipid metabolism, cell signaling, transcription factors, and inflammatory and neuroprotective factors. Figure 5 shows those genes expressed mostly at 1–8 dpl. Presumably, these genes are more related to sustained brain functions following injury than an immediate and transient response; thus, although some signaling factors are in these clusters, genes involved in cell adhesion/ECM and cytoskeleton were abundant. Figure 6 shows two clusters, one representing a second wave of increased gene expression at 4 dpl and another showing genes down-regulated at 1 and 8 dpl. Finally, Figure 7 shows the genes up-regulated mostly at 4–8 dpl, the largest group of genes being affected by the cryolesion. According to the functional GO classification (Supplementary Table II), many of these genes are related to the cellular response to damage, tissue reconstruction, and immune response, as expected.

As stated above, the number of mice used in this study constrains the chances of finding statistical significances in some cases. Although the overall response to the lesion is robust, and the hierarchical clustering readily identifies specific patterns of responses, we observed for some of the genes a clear tendency to be affected by MT-I + II deficiency, consistent with other genes and/or previous studies, but which nevertheless are classified into these clusters. For instance, *Mt1+2* KO mice normally show signs of more neuroinflammation, with increased proinflammatory cytokine expression, T-cell recruitment, gliosis, and increased oxidative stress and apoptosis. A typical gene marker of astrogliosis is glial fibrillary acidic protein (GFAP) expression, and, several days following the lesion, the *Mt1+2* KO mice show increased GFAP immunoreactivity compared with WT mice: this is exactly the pattern observed in this study (Fig. 7), confirming the reliability of the present results. CD14 is a prototypical marker of monocytes/macrophages and is up-regulated by injury in the CNS associated with an increase in NF-κB activity and proinflammatory factors (Zekki et al., 2002), and, as expected, its expression tended to be higher in the *Mt1+2* KO mice (Fig. 4). Also, the cytotoxic T-lymphocyte-associated protein 2α and the chemokine (C-C motif) ligand 2 (also known as monocyte chemoattractant protein; MCP-1), which is recognized to mediate extravasation of mononuclear leukocytes into the brain (Song and Pachter, 2004), were higher in the *Mt1+2* KO mice (Fig. 4). Genes related to increased oxidative stress increased robustly in the injured animals and tended also to be higher in *Mt1+2* KO mice: examples are heme oxygenase (decycling) 1 (HO-1; Otterbein and Choi, 2000; Fig. 6) and peroxiredoxin 5 (Wang et al., 2003; Fig. 7). Finally, that these mice are more prone to engage in apoptosis following injury is also denoted by changes in genes such as CCAAT/enhancer binding protein (C/EBP), a transcription factor that potentiaties neuronal apoptosis induced by N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx (Marshall et al., 2003; Fig. 4), and Gadd45α (growth arrest and DNA damage-inducible) that together with Gadd45β and Gadd45γ constitutes a family of evolutionarily conserved, small, acidic, nuclear proteins that respond to brain injury and have been implicated in terminal differentiation, growth suppression, and apoptosis (Chen et al., 1998; Fig. 4). Other major genes likely underlying in part the increased apoptosis observed in the *Mt1+2* KO mice are cyclin-dependent kinase inhibitor p21 1A (Cip1/WAF1; not shown) and p21 1C (Fig. 7), which are under p53 control (Tanaka et al., 2002), although much remains to be understood (for review see Liu et al., 2001).

Validation of the Results

Although microarrays are considered a reliable technique (Stears et al., 2003; Dumur et al., 2004; Abruzzo et al., 2005), it is always important to validate the results. Many of the genes discussed above have been shown to be affected in accordance with previously published reports; also, and regarding the main aim of this study, many of those changes were consistent with the phenotype of the *Mt1+2* KO mice as described from conventional techniques. To validate the microarray data further and to determine whether the expressed mRNA was translated into the corresponding proteins, we examined protein expression changes in brains by using immunohistochemistry. As shown in Figures 8–10, the cryolesion increased the immunostaining of AQP-2, catalase and RAGE, and the deficiency of MT-I + II decreased this response for aquaporin while increasing that of catalase and RAGE. These results are in line with those obtained in the microarrays; also, the results for catalase and RAGE are fully consistent with the phenotype of the *Mt1+2* KO mice, i.e., increased oxidative stress and inflammation during injury. The results for AQP2 are novel, but, should this protein play roles similar to those of AQP4, then its deficiency could also contribute to the phenotype of the *Mt1+2* KO mice following cryoin-

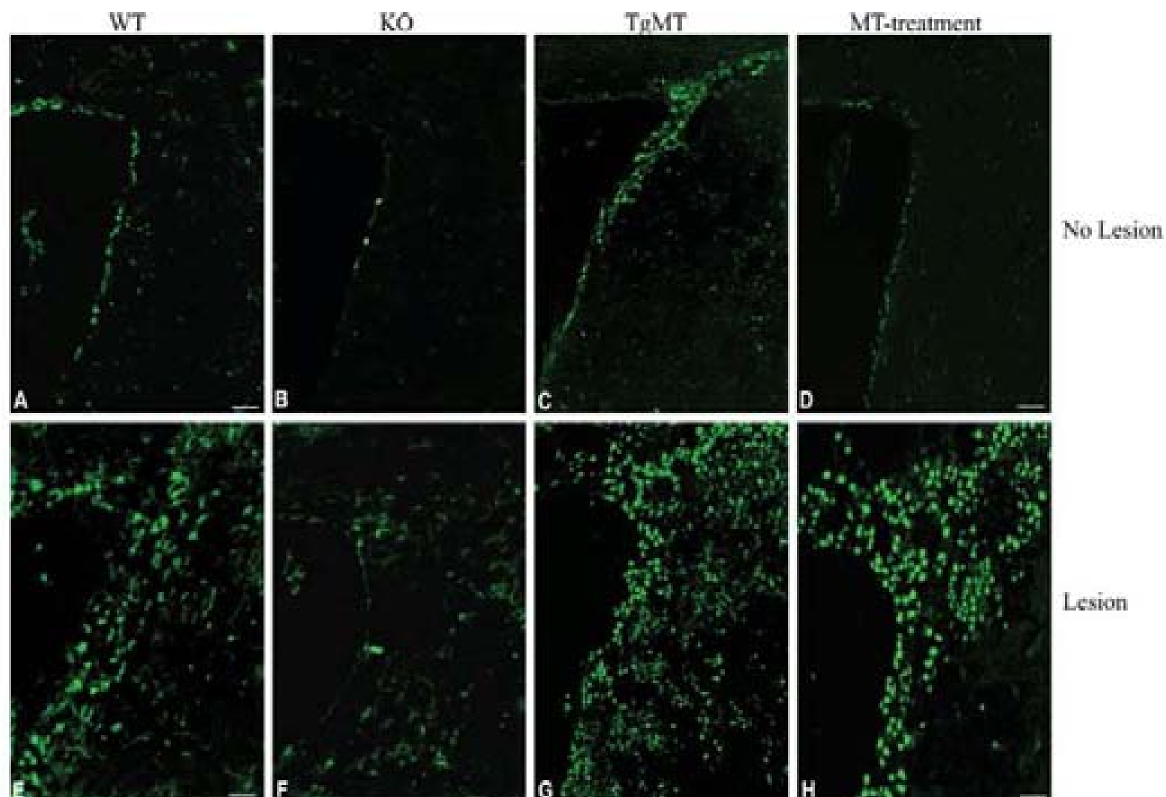


Fig. 11. Immunofluorescence stainings of frizzled 9, a marker of NSCs seen in the subventricular zone of the lateral ventricles of unlesioned brains (**A–D**) and lesioned brains (**E–H**; lesioned brains are shown at 10 dpl). Brains of wild type controls (A,E), *Mt1+2* KO mice (B,F), TgMT mice (C,G), and MT II treated mice (D,H). As shown, MT I + II had a stimulating effect on frizzled 9 positive cells; *Mt1+2* KO mice showed reduced and TgMT mice showed increased frizzled 9 positive cells relative to wild type mice, as seen in the subventricular zone of the lateral ventricles of both unlesioned and lesioned brains. The numbers of frizzled 9 positive cells in unlesioned MT II treated mice (D) were comparable to those of wild type controls (A), whereas, after the lesion, the MT II treatment clearly increased the NSC activation. As shown, the frizzled 9 positive cells activated by exogenous MT II (H) are more round and show fewer signs of differentiation, such as ramification, compared with those of TgMT mice, and even moreso compared with those of wild type controls. Scale bars = 55 μm. Figure can be viewed in color online via www.interscience.wiley.com.

jury: AQP4 KO mice show brain swelling and clinical outcome worse than those in WT mice (Verkman et al., 2006).

As shown above, MT-I + II deficiency reduced the expression of netrin-1 (Fig. 2), which is important for CNS development, neuronal growth cones, and regeneration following injury (Manitt and Kennedy, 2002). Hence, we decided to evaluate the postlesional activation of neural stem/precursor cells (NSCs; for a recent review see Martino and Pluchino, 2006), as judged by using immunostaining for frizzled-9, which is a marker that distinguishes NSCs from other precursor cells as well as differentiated CNS cells (Cai et al., 2002). We show here that frizzled-9-expressing NSCs, which were up-regulated following brain injury as expected, were reduced in the subventricular zone (SVZ) of the lateral ventricles of unlesioned and lesioned *Mt1+2* KO mice compared with those of wild-type controls (Fig. 11). Moreover, transgenic mice overexpressing MT-I and mice injected (i.p.) with MT-II showed significant increases in the immunoreactivity for frizzled-9 in the SVZ. Also, frizzled-9-expressing cells were seen inside the brain parenchyma, likely indicating migration in the direction of the lesion cavity. Interestingly, this is in accordance with previous results obtained in the EAE model that showed an overall impairment of neuroglial stem cells, including NG2-positive stem/progenitor cells in *Mt1+2* KO mice (Penkowa et al., 2003b). Accordingly, MT-I + II are likely activating stem/progenitor cell populations derived from the stem cell niches in the brain, opening exciting perspectives regarding the putative therapeutical use of these proteins.

In conclusion, traumatic brain injury is one of leading causes of transient and permanent disabilities. This

study demonstrates a large number of genes being involved in the responses of the brain to damage. Many of them have also been shown in other models of brain injury by microarrays (Read et al., 2001; Tang et al., 2002; Roth et al., 2003; Long et al., 2003; Natale et al., 2003, 2004; Rao et al., 2003; Dhodda et al., 2004; Kury et al., 2004; Lu et al., 2004; Faden et al., 2005; for review see also Dash et al., 2004; Gebicke-Haerter, 2005). Many of the genes identified here are consistent with those found in our previous study (Poulsen et al., 2005), but it is also clear that wild-type mice (C57Bl6 vs. 129/SvJ) show differences worthy of further study. Nevertheless, it is beyond the scope of this study to search for the number of matches among different studies regarding the effect of brain injury. Previous studies, including our studies, have shown that *Mt1+2*-deficient mice have a clear phenotype following injury (see the introductory paragraphs), and the present study has identified for the first time many potential genes that could be underlying such a phenotype.

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Trabajo 6

La administración exógena de metalotioneína I+II nativas o recombinantes de diferentes especies muestra un papel neuroprotector tras criolesión, descartando a su vez que sus acciones se deban a interacciones proteína-proteína.

Specificity and divergente in the neurobiologic effect of different metallothioneins alter brain injury.

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Specificity and Divergence in the Neurobiologic Effects of Different Metallothioneins After Brain Injury

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Brain injury and neuroinflammation are pathophysiologic contributors to acute and chronic neurologic disorders, which are progressive diseases not fully understood. Mammalian metallothioneins I and II (MT-I&II) have significant neuroprotective functions, but the precise mechanisms underlying these effects are still unknown. To gain insight in this regard, we have evaluated whether a distant, most likely single-domain MT (*Drosophila* MTN) functions similarly to mammalian MT-I&II (recombinant mouse MT-I and human MT-IIa and native rabbit MT-II) after cryogenic injury to the cortex in *Mt1&2* KO mice. All the recombinant proteins showed similar neuroprotective properties to native MT-II, significantly reducing brain inflammation (macrophages, T cells, and pro-inflammatory cytokines), oxidative stress, neurodegeneration, and apoptosis. These results in principle do not support specific protein–protein interactions as the mechanism underlying the neuroprotective effects of these proteins because a nonhomologous and structurally unrelated MT such as *Drosophila* MTN functions similarly to mammalian MTs. We have also evaluated for the first time the neurobiologic effects of exogenous MT-III, a major CNS MT isoform. Human rMT-III, in contrast to human nMT-IIa, did not affect inflammation, oxidative stress, and apoptosis, and showed opposite effects on several growth factors, neurotrophins, and markers of synaptic growth and plasticity. Our data thus highlight specific and divergent roles of exogenous MT-III vs. the MT-I&II isoforms that are consistent with those attributed to the endogenous proteins, and confirm the suitability of recombinant synthesis for future therapeutic use that may become relevant to clinical neurology. © 2006 Wiley-Liss, Inc.

Key words: oxidative stress; neuroprotection; apoptosis; neurodegeneration; plasticity

Brain inflammation is rapidly induced by any neuropathologic agent and comprises reactive astrogliosis, inflammatory responses of macrophages and lymphocytes including induction of proinflammatory cytokines like IL-1, IL-6, IL-12, and TNF-α (Allan and Rothwell, 2003). Although inflammation is a host defense response, it also mediates cerebral cytotoxicity at least in part because of the formation of excess reactive oxygen species (ROS) that rapidly cause oxidative stress, a detrimental process leading to neurodegeneration and apoptotic cell death in the central nervous system (CNS) (Barnham et al., 2004). This cerebral tissue damage may be ameliorated, however, by protective agents like antioxidants, neurotrophins, and growth factors (Tabakman et al., 2004).

Among neuroprotective factors, mammalian metallothionein-I and -II (MT-I&II) are significant therapeutic candidates (Hidalgo et al., 2001; Chung and West, 2004). MT-I+II are low molecular-weight, cysteine-rich proteins with significant antiinflammatory, antioxidant, and neuro-

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protective roles during neuropathologic conditions. According to this, various CNS disorders like traumatic brain injury, ischemia, epilepsy, experimental autoimmune encephalomyelitis (EAE), experimental amyotrophic lateral sclerosis (ALS), and cytokine-induced encephalopathy are all more severe in MT-I+II deficient mice (*Mt1&2* KO mice) and less severe in MT-I overexpressing mice (Penkowa et al., 1999; van Lookeren Campagne et al., 1999; Carrasco et al., 2000; Penkowa et al., 2000; Nagano et al., 2001; Asanuma et al., 2002; Giralt et al., 2002a,b; Puttaparthi et al., 2002; Trendelenburg et al., 2002; Molinero et al., 2003). Interestingly, it is now clear that exogenously administered rabbit and human MT-II can also induce neuroprotection in a number of injury models (Penkowa and Hidalgo, 2000; Giralt et al., 2002b; Chung et al., 2003; Xie et al., 2004). The MTs injected in these experiments were Zn-MT complexes, but equimolar amounts of zinc alone did not show CNS therapeutic effects per se either in the cryo injury (Giralt et al., 2002b) or the EAE (Penkowa and Hidalgo, 2000) models.

MT-III expression has been shown to be altered (up- or downregulated) in Alzheimer disease (Uchida et al., 1991; Erickson et al., 1994; Carrasco et al., 1999; Yu et al., 2001), Down syndrome (Arai et al., 1997), Creutzfeldt-Jakob disease (Kawashima et al., 2000), pontosubicular necrosis (Isumi et al., 2000), Parkinson disease, meningitis, and amyotrophic lateral sclerosis (Uchida, 1994). A similar trend (up- or downregulation depending on the model, time, etc.) has been observed in animal models of brain injury (Hidalgo et al., 2001; Hozumi et al., 2004). Results in transgenic mice suggest strongly that MT-III has a neuroprotective role in some experimental paradigms (Erickson et al., 1997; Puttaparthi et al., 2002; Lee et al., 2003), but it seems equally clear that the opposite is true for other models of injury (Carrasco et al., 2003; Ceballos et al., 2003) or depending on the brain area (Lee et al., 2003). This complexity is also evident in in vitro work, with both neuroprotective (Montoliu et al., 2000; Ren et al., 2001; Chen et al., 2002; Uchida et al., 2002) and detrimental (Uchida et al., 1991; Erickson et al., 1994; Sewell et al., 1995; Chung et al., 2002) roles of MT-III being shown.

Consequently, in the mouse brain non-murine exogenous MT-II (rabbit, human) seem to induce neuroprotection after injury that is similar to that of murine MTs, and suggests that extracellular MT has the capability of reaching the same targets (whether within the CNS only or peripherally or both remains to be established) than the endogenously expressed protein. Whether this is mediated by specific protein-protein interaction(s) including MT receptors or transporters is unknown. To gain insight in this regard, we have compared the non-structurally related MT protein *Drosophila* MTN with mammalian MTs in the cryolesion model. Interfering effects of endogenous MT-I+II were ruled out by using *Mt1&2* KO mice. This therapeutic approach has never been attempted for MT-III, and this study also aimed to ascertain whether or not exogenously administered MT-III is capable of inducing any neurobiologic effects.

MATERIALS AND METHODS

Animals

Male mice (3–5 months old) of wild-type (129/SvJ) and homozygous MT-I+II deficient genotypes (*Mt1&2* knock-out, *Mt1&2* KO mice) (Masters et al., 1994) were used in this study. The mice were initially obtained from Jackson Laboratories (Bar Harbor, MA), and colonies have been established at the Autonomous University of Barcelona. We used five animals per group in all cases.

Preparation of the MT proteins

In the first part of this work we tested the therapeutic effectiveness of different native and recombinant MT exogenous proteins in *Mt1&2* KO mice, to determine if there distinct effects were produced when using: 1) MTs from different species, either very close (mammalian) or distant (insect); and 2) native vs. recombinant MT proteins. The MTs used included rabbit native MT-II (nMT-II), mouse recombinant MT-I (rMTI), human recombinant MT-IIa (rMTII), and *Drosophila* recombinant MTN (rMTN). The *Drosophila* MTN is a non-vertebrate MT that is non-homologous to the mammalian MT-I+II forms. It contains only 40 amino acids including 10 cysteines (Lastowski-Perry et al., 1985), whereas mammalian MT-I+II contain 61 amino acids including 20 cysteine residues (Hidalgo et al., 2001) (Table I). All the MTs were used as fully-loaded Zn-complexes.

The three recombinant MT proteins were synthesized in *E. coli* after a GST-fusion strategy that, after thrombin cleavage, renders homogeneous MT preparations. In this case, because the bacterial cultures were supplemented with 300 µM $ZnCl_2$, the corresponding mouse Zn_7-MTI (Cols et al., 1997), Zn_3,Zn_4-MTN (Valls et al., 2000), and human Zn_7-MTII (this study) were recovered. The human MTIIa cDNA (kindly provided by Dr. Richards, Australian National University, as a pUC13 clone) (Karin and Richards, 1982) was subcloned in the pGEX-4T-1 expression vector by means of a PCR amplification that introduced 5′ BamH*I* and 3′ Sal*I* restriction sites at the ends of the coding region, suitable for cloning purposes. The oligonucleotides 5′CCCGGATCCA TGGACCCCAACTG3′ and 5′CGGCGCGTCGACTCAG GCGCAGCA3′ were used as upstream and downstream PCR primers, respectively. Finally, the correct DNA sequence was confirmed by automatic DNA sequencing (ABI 370 Perkin Elmer). All further experimental steps were carried out as for mouse MTI (Cols et al., 1997). It is worth noting that recombinant synthesis of hMTIIa required, under the same experimental conditions, four times culture volume, because the mean yield was 25% that of other MTs (Table I). MT concentration and metal-to-protein ratios, which also confirmed major protein non-oxidation, were assessed by atomic absorption ICP-AES measurements of S, Zn, Cd, and Cu sample content, as described in Cols et al. (1997). The rabbit native MT-II protein was commercially available (Sigma-Aldrich, St. Louis, MO; code M9542).

TABLE I. Recombinant Metallothioneins Used in this Study

Metallothionein	Cys[a]	[Prot][b]	Zn/prot[c]	Vol[d]	Protein sequence
mMT1	20	2.35	6.86	6	MDPNCSCSTGGSCTCTSSCACKNCKCTSC KKSCCSCCPVGCSKCAQGCVCKGAADKCTCCA
hMTII	20	0.53	6.47	6	MDPNCSCAAGDSCTCAGSCKCKECKCTSCKK SCCSCCPVGCAKCAGGCICKGASDKCSCCA
		0.32	6.25	10	
		0.40	6.27	10	
hMTIII[e]	20	—	—	—	MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSC CPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
MTN	10	2.17	3.23	6	MPCPCGSGCKCASQATKGSCNCGSDCKCGGDKKSACGCSK

[a]Number of cysteine residues in the MT protein.
[b]Concentration of the MT preparations calculated from the ICP-AES results M × 10^{-4}.
[c]Zn-to-MT ratio, obtained from the ICP-AES data.
[d]Volume of the bacterial cultures (in liters) grown to purify the corresponding MT.
[e]Prepared as in Faller et. al. 1999.

In the second part of this study the therapeutic effect of human rMT-III was investigated in front of that of human nMT-IIa. WT mice and *Mt1&2* KO mice were used. Human rMT-III was prepared according to Faller et al. (1999) and human nMT-IIa was purified from human liver (Vašák, 1991). The rMT-III obtained was found to be bioactive in an assay of neuronal survival (Hasler et al., 2000).

Mice Brain Injury and Post-Lesion MT Treatment

Mice were subjected to a focal traumatic brain injury under tribromethanol anesthesia. The skull over the right fronto-parietal cortex was exposed, and a cryo-induced focal lesion was produced by application of a piece of dry ice (of a consistent size for all animals) on the surface of the skull as described previously (Penkowa et al., 1999). This simple and reproducible method results in a cryogenic (freeze) lesion in the cerebral cortex with immediate necrosis and blood-brain barrier (BBB) disruption that are followed by inflammatory responses in the surrounding brain tissue areas. The animals were then followed for 3 days post-lesion (dpl), during which they received the corresponding treatment with exogenous MTs. We have determined previously the optimum MT treatment schedule during brain disorders and demonstrated that rabbit MT-II injected intraperitoneally (i.p.) reaches the CNS extracellular space in 15–45 min by passing through disruptions in the BBB in an EAE model (Penkowa and Hidalgo, 2000) and in the cryolesion model (Giralt et al., 2002b). Unlesioned (healthy) and freeze-lesioned *Mt1&2* KO mice received two injections daily with saline (control [placebo] treatment) or with an MT solution in a dose of 1.0 mg MT/kg/day i.p. for human rMT-IIa, mouse rMT-I, human rMT-IIa, and *Drosophila* rMTN. In the second experiment, human nMT-IIa and human rMT-III and were injected in a dose of 0.5 mg MT/kg/day. A dose of 0.5 mg /kg/day has been shown sufficient for mammalian MT to induce the neuroprotective assayed effects (Giralt et al., 2002b). By 3 dpl, the mice were deeply anesthetized with 10 mg/100 g body weight of Brietal (Methohexital 10 mg/ml, Eli Lilly) and were transcardially perfused with 0.9% saline with heparin (15,000 IU/liter) for 2–3 min followed by perfusion with Zamboni fixative for 8 min. The brain was then removed surgically for histopathologic analyses.

Tissue Processing

All the fixed brains were processed for paraffin embedding in the fully automatic Shandon Excelsior Tissue Processor Histokinette and serial, coronal 3-μm sections were cut and processed for histochemistry, immunohistochemistry, and TUNEL as described previously (Penkowa et al., 1999, 2000).

Sections were rehydrated and underwent heat-induced antigen retrieval in citrate buffer at pH 9.1 or pH 6.0 in a microwave oven for 10 min. Sections were incubated in 0.5% H_2O_2 in TBS/Nonidet P-40 to quench endogenous peroxidase followed by 10% goat serum (In Vitro, Fredensburg, Denmark, code 04009-1A) or donkey serum (The Binding Site, Birmingham, UK, code BP 005.1) to block nonspecific binding. Sections to be immunostained with monoclonal mouse-derived primary antibodies were also incubated with Blocking Solutions A+B from the HistoMouse-SP Kit (Zymed Laboratories, San Francisco, CA, code 95-9544) to quench endogenous mouse IgG.

Histochemistry

Hematoxylin and eosin (H&E) stainings of brain sections were carried out according to standard procedures. Biotinylated tomato lectin from the *Lycopersicon esculentum* (Sigma-Aldrich, code L9389) 1:500, was used as a marker for cells of the myelo-monocytic cell lineages, such as macrophages/microglia, as well as a marker for vessels. The lectin was developed using streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, Glostrup, Denmark; code K377) prepared according to the manufacturer's recommendations. The reaction product was visualized using DAB (0.015% H_2O_2 in DAB/TBS) for 10 min at room temperature.

Immunohistochemistry

Sections were incubated overnight at 4°C with antibodies or detection systems.

Detection of CNS Resident and Recruited Cells. Rabbit anti-cow GFAP 1:250 (Dakopatts, code Z334) (marks astrocytes); rabbit anti-human NSE 1:1000 (Calbiochem, La Jolla, CA, code PC237) (marks viable mature neurons); goat anti-

mouse CD14 1:30 (Santa Cruz Biotechnology, Santa Cruz, CA, code sc-6999) or rat anti-mouse F4/80 1:25 (Serotec, Oxford, UK, code MCA-497) (marking monocytes/macrophages); rat anti-mouse MOMA 1:50 (Serotec, code MCA-947) (marking only metallophilic macrophages from peripheral lymphoid tissues); mouse anti-rat CD3 1:50 (Serotec, code MCA-772) (as a marker for all T lymphocytes); mouse anti-rat CD4 1:50 (Serotec, code MCA-55R) (marker for T-helper [Th] cells); goat anti-mouse CD20 1:200 (Santa Cruz Biotechnology, code sc-7736) (marker for B cells); mouse anti-human IL-1β 1:50 (Biogenesis, Kingston, NH, code 5375-4329); rabbit anti-mouse TNF-α 1:100 (Biosource, Camarillo, CA, code AMC 3012).

Oxidative Stress, Neurodegeneration, and Apoptosis. Rabbit anti-NITT 1:100 (Alpha Diagnostics, San Antonio, TX, code NITT 12-A) (a marker for peroxynitrite-induced nitration of tyrosine residues); rabbit anti-MDA 1:100 (Alpha Diagnostics, San Antonio, TX, code MDA 11-S) (a marker for malondialdehyde [MDA] produced as a byproduct of fatty acid peroxidation); mouse anti-8-oxoguanine 1:100 (Chemicon, Temecula, CA, code MAB-3560) (marks oxidative DNA damage; rabbit anti-neurofibrillary tangles 1:200 (Chemicon, code AB-1518) (marks several of the insoluble proteins that form extra- and intracellular neurofibrillary tangles; abbreviated as neurotang in figures); goat anti-human amyloid precursor protein Frameshift Mutant (APP-FM) 1:50 (Chemicon, code AB-5342); rabbit anti-human (activated/cleaved) caspase-3 1:50 (Cell Signaling Technology, Danvers, MA, code 9661); mouse anti-human p53 1:50 (Dakopatts, DK, code M7001); goat anti-horse cytochrome-c 1:100 (Santa Cruz Biotechnology, code sc-7159).

Growth/Trophic Factors, Synaptic Plasticity, and Repair. Rabbit anti-human bFGF 1:100 (Santa Cruz Biotechnology, code sc-79); rat anti-mouse bFGF Receptor (FGF-R) 1:50 (Neomarkers, Fremont, CA, code RT-794-P0); goat anti-human NT-3 1:20 (R&D Systems, Abingdon, UK, code AF-267-NA); rabbit anti-human NT-4 1:50 (Santa Cruz Biotechnology, code sc-545); mouse anti-BDNF 1:50 (Oncogene Research Products, Darmstadt, Germany, code GF35L); rabbit anti-human TrkB (the tyrosin kinase receptor-B for NT-4 and BDNF) 1:200 (Calbiochem, code PC86); mouse anti-PSA-NCAM (polysialic acid neural cell adhesion molecule) 1:200 (AbCys, Paris, France, code AbC0019); rabbit anti-human S100-A4 1:200 (Dakocytomation, Glostrup, Denmark, code A5114); rabbit anti-human synaptophysin 1:100 (Dakocytomation, code A0010) (a presynaptic marker); mouse anti-syntaxin 1:200 (Sigma-Aldrich, code S0664); rabbit anti-rat spinophilin 1:200 (Chemicon, code AB5669) (marking dendritic spines); rabbit anti-human S100-A4 1:200 (Dakocytomation, code A5114); mouse anti-PSA-NCAM 1:200 (AbCys, France, code AbC0019).

Secondary Antibodies and Detection Systems. Sections were incubated for 30 min at room temperature with the following secondary antibodies: biotin-conjugated anti-rabbit IgG 1:400 (Sigma-Aldrich, code B3275) or biotin-conjugated anti-mouse IgG 1:200 (Sigma-Aldrich, code B8774) or biotin-conjugated anti-goat IgG 1:20 (Amersham Biosciences, Buckinghamshire, UK, code RPN 1025) or biotin-conjugated anti-rat IgG 1:1500 (Amersham Biosciences, code 1005); or biotin-conjugated anti-mouse IgM 1:20 (Jackson ImmunoResearch, West Grove, PA, code 115-065-020). All the secondary antibodies were detected by StreptABComplex/HRP followed by biotinylated tyramide and streptavidin-peroxidase complex (tyramide signal amplification, TSA indirect) (NEN, Life Science Products, Boston, MA, code NEL700A) prepared following manufacturer's recommendations. The immunoreaction was visualized by using DAB as a chromogen.

Controls. To evaluate the extent of non-specific binding of the antisera in the immunohistochemical experiments, 1:100–1:1000 of normal goat or donkey serum was substituted for the primary antibody step described above. Other control sections were incubated with isotypic IgG instead of the primary antibody. A further control was to pre-absorb the primary antibodies with their corresponding antigenic proteins. We used CD-14 (Santa Cruz Biotechnology, code sc-6999-P); MDA-ovalbumin (Alpha Diagnostics, code MDA-11-CO); p53 (Santa Cruz Biotechnology, code sc-4246); and cytochrome-c (Santa Cruz Biotechnology, code sc-4270). Results were considered only if these controls were negative.

In Situ Detection of DNA Fragmentation

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) was carried out using the Fragment End Labeling (FragEL) Detection Kit (Calbiochem, code QIA33). The FragEL kit contains all the materials used below and each step was carried out according to the manufacturer's recommendations. The tissue was processed and rehydrated as mentioned above, and sections were incubated with 20 μg/ml proteinase K for 20 min to strip off nuclear proteins. After immersion in equilibration buffer for 20 min, sections were incubated with TdT and biotin-labeled deoxynucleotide (dNTP-biotin) in a humidified chamber at 37°C for 1.5 hr. This was followed by wash buffer and the stop solution for 5 min at room temperature to stop the reaction. After washing in TBS and incubation in blocking buffer for 10 min, the sections were incubated with peroxidase-streptavidin for 30 min and afterward, DAB was used as chromogen. The sections were counterstained with methyl-green. Negative control sections were treated similarly but incubated in the absence of TdT enzyme or dNTP-biotin or peroxidase-streptavidin. We also compared our sections with positive control slides provided in the FragEL Detection Kit. TUNEL was compared to stainings for apoptotic markers (ssDNA, p53, cytochrome-c, and activated caspase-3). In addition, the morphologic criteria for apoptosis (cell shrinkage, formation of apoptotic bodies, membrane blebbing, no loss of cellular integrity, compactation of chromatin into uniformly dense masses) were evaluated.

Cell Counts and Statistical Analysis

In addition to morphologic evaluation, quantitation (cellular counts) of some of the variables analyzed were carried out from 0.5 mm^2 matched areas of 3 μm brain sections for statistical analysis of the results. For each parameter analyzed, brain sections from the five mice of each group were used

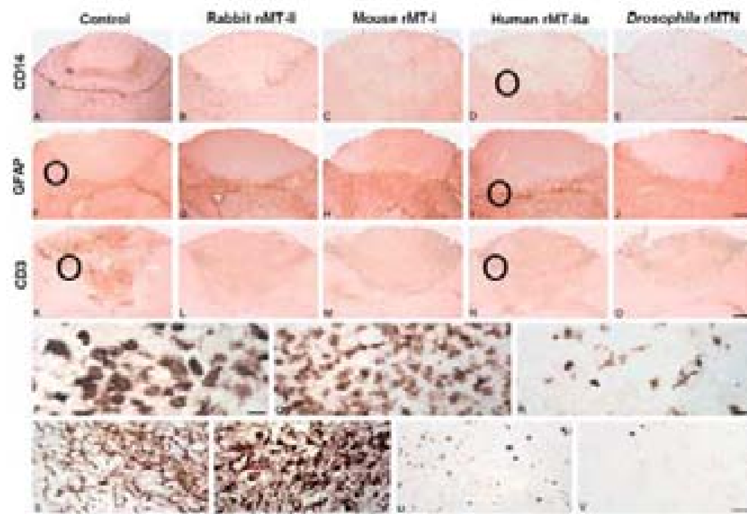

Fig. 1. Inflammatory responses to cryolesion of *Mt1&2* KO mice treated with saline (control), rabbit nMT II, mouse rMT I, human rMT II, and *Drosophila* rMTN. **A:** Numerous CD14+ macrophages are seen around and inside the lesion of control mice. At the border zone between lesioned and unlesioned tissue a population of large stout macrophages are seen (marked by *P* and also shown in **P**). Inside the lesion another population of smaller round monocytic macrophages is detected (marked by Q and also shown in **Q**). **B–E:** In the mice receiving MT medical treatment, the numbers of CD14+ macrophages recruited to the lesion are reduced relative to control mice. **F–J:** The lesion induces GFAP+ reactive astrogliosis in all the mice. The different MTs increased astrocytosis relative to controls. **K–O:** CD3+ T lymphocytes were recruited to the lesion site in all mice, although the T cell numbers were decreased in the MT treated mice relative to the controls. P: Higher magnification of the CD14+ stout macrophages of control mice. The cells shown are from the area marked by *P* in (A). Q: Higher magnification of the small round monocytic cells marked by Q in (A). **R:** Higher magnification of the framed area in (D) showing CD14+ cells of lesioned mice treated with human rMT II, which mainly display some ramified microglia and a very few round macrophages. **S:** Higher magnification of the framed area in (F) showing the GFAP+ astrocytes of control mice. **T:** Higher magnification of the perilesional field framed in (I) showing enhanced reactive astrogliosis after treatment with human rMT II treated mice. **U:** Higher magnification of the framed area of (K) showing recruited **T** cells of control mice. **V:** Higher magnification of the **T** cells of the framed area of (N), which shows reduced **T** cell recruitment of human rMT IIa treated mice after the lesion. Scale bars = 200 µm (A–O); 22 µm (P–R); 44 µm (S–V). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

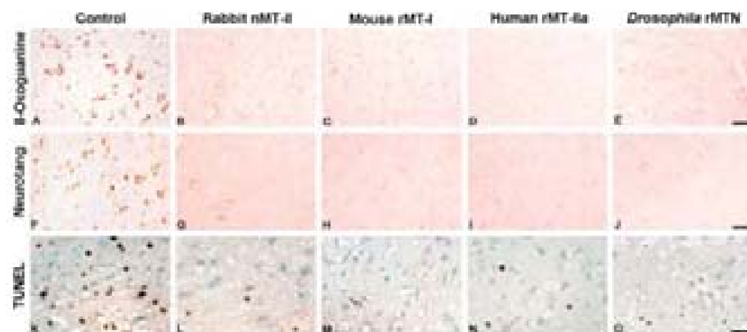

Fig. 2. Oxidative stress, neurodegeneration, and apoptosis in the tissue surrounding the necrotic area caused by cryolesion of *Mt1&2* KO mice treated with saline (control), rabbit nMT II, mouse rMT I, human rMT II, and *Drosophila* rMTN. **A:** Brain injury induced oxidative stress was evident in the perilesional tissue of control mice as judged by 8 oxoguanine levels. **B–E:** The 8 oxoguanine levels were decreased by all MT treatments. **F:** Immunoreactivity for neurofibrillary tangles was increased in the perilesional brain tissue of control mice. **G–J:** The MT treatments prevented significantly the neurodegenerative changes induced by the cryolesion. **K–O:** TUNEL+ apoptotic cell death in the brain tissue adjacent to the lesion cavity of saline injected mice (K) and mice receiving the different MTs (L–O). As shown the control treated mice showed more apoptosis than the mice treated with MT I&II and MTN. Scale bars = 50 µm (A–J); 30 µm (K–O). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

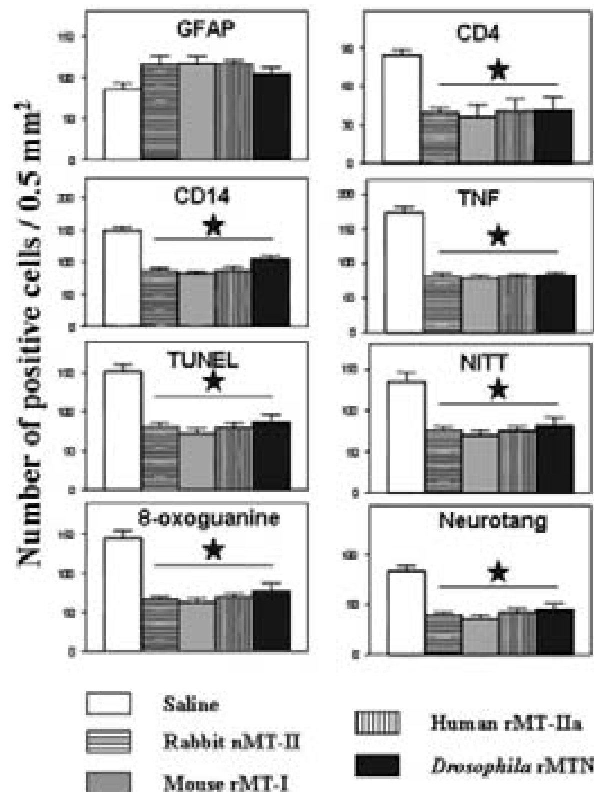

Fig. 3. Cellular counts of some of the variables analyzed (representative results of some of them are shown in Figs. 1,2) were carried out from 0.5 mm^2 matched areas at the border of the cortical lesion in five mice per group for statistical analysis (mean ± SE). Results were analyzed with one way ANOVA followed by SNK post hoc comparisons. *$P < 0.01$ vs. saline injected mice.

and a mean value was calculated. Positively stained cells were defined as cells with staining of the soma. In the case of the apoptotic markers (like TUNEL) positively stained cells were defined as cells with nuclear staining. The cell counts were carried out by the same investigator, who was blinded to animal identity and the different medical treatments. Cells were counted at the border of the cortical lesion (the rim between lesioned and unlesioned brain tissue), where inflammation is prominent. Results were analyzed with one-way ANOVA followed by SNK post-hoc comparisons.

RESULTS

Mammalian MT-I&II and Drosophila MTN Modulate Inflammation After Brain Injury

In saline-treated mice the cryolesion induced a prominent recruitment and activation of microglia/macrophages (lectin/CD14 positive) and of CD3/CD4 positive T lymphocytes, which in high numbers infiltrated the lesion site and increased the expression of proinflammatory cytokines like TNF-α and IL-1. All these responses were dramatically decreased by rabbit nMT-II (Figs. 1,3), that significantly reduced the inflammatory reaction in the lesioned area in accordance with the data reported previously (Giralt et al., 2002b). Results with human native MT-IIa were in accordance with these results. The administration of recombinant mammalian MTs (mouse rMT-I and human rMT-IIa) also decreased the inflammatory response, demonstrating total identity between recombinant and native mammalian MTs not only structurally but also functionally. Most important, the administration of the non-vertebrate *Drosophila* MTN also reduced the inflammatory response to a similar degree than mammalian MTs, although its effect tended to be somewhat smaller for macrophage recruitment (Figs. 1,3). The recruited macrophages were derived mainly from blood monocytes as determined by using MOMA immunoreactivity (not shown).

Brain lesioned mice also showed the expected reactive astrogliosis around the injury by 3 dpl, and the enhancement of this response by rabbit nMT-II treatment using GFAP immunoreactivity. In line with the previous results, the recombinant mammalian proteins showed similar effects (Figs. 1,3). *Drosophila* MTN showed an intermediate effect, and, because of that, the one-way ANOVA was not significant ($P < 0.055$ with the MTN included in the analysis; <0.05 if excluded).

Mammalian MT-I&II and Drosophila MTN Modulate Oxidative Stress, Neurodegeneration, and Apoptosis After Brain Injury

Lesioned mice receiving saline showed pronounced levels of brain damage such as formation of ROS and oxidative stress as well as neurodegeneration and apoptosis by 3 dpl. Immunostainings for NITT, MDA, and 8-oxoguanine (oxidative stress markers) were increased significantly in neuronal and glial cells surrounding the lesion. Increased immunoreactivity for neurodegenerative markers APP-FM and intraneuronal neurofibrillary tangles and stainings for apoptosis markers caspase-3, cytochrome-c, p53, and TUNEL were increased significantly in saline-injected mice (Figs. 2,3). All mammalian MT-I and MT-II, both native and recombinant, decreased these effects, as did *Drosophila* MTN.

It is worth noting that in unlesioned healthy controls we did not observe effects of the injected MTs regarding the variables analyzed, which likely is due to intact BBB properties.

MT-III Does Not Modulate Inflammation, Oxidative Stress, Neurodegeneration, or Apoptosis After Brain Injury

In the second part of this study, human rMT-III was compared to human nMT-IIa, as positive control and with saline as negative control. In global, the human nMT-IIa form behaved as the other mammalian native or recombinant MTs described above, and particularly as

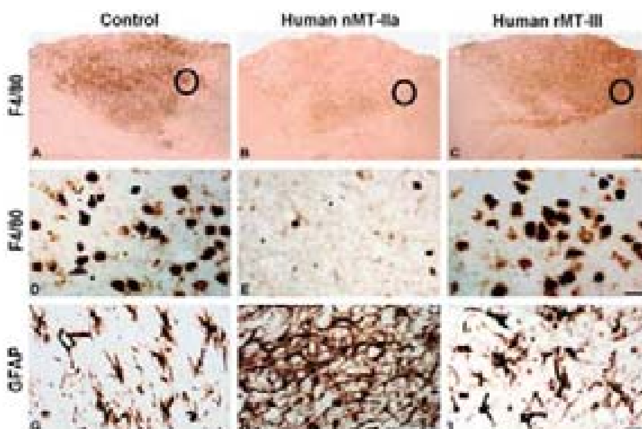

Fig. 4. Inflammatory responses of F4/80+ microglia/macrophages and GFAP+ astrocytes in brain injured *Mt1&2* KO mice treated with saline (control), human nMT IIa, or human rMT III. **A–C:** F4/80+ microglia/macrophages seen at the cortical lesion site in saline (A), nMT IIa (B), and rMT III (C) treated mice. **D–F:** Higher magnification of (A–C) showing F4/80+ microglia/macrophages located at the lesion border line (framed areas in A–C). **G–I:** GFAP+ reactive astrogliosis as seen at the lesion border line of saline (G), nMT IIa (H), and rMT III (I) treated mice. Scale bars = 200 μm (A–C); 28 μm (D–F); 33 μm (G–I). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the corresponding human rMT-IIa form. This highlights the expected striking similarities of these proteins. In contrast, the i.p. injection of rMT-III showed remarkable differences.

Although nMT-IIa administration decreased microglia/macrophages and T cells recruitment and activation and IL-1β and TNFα expression, and increased GFAP reactive astrocytes, rMT-III had no significant effects (Figs. 4,7). By 3 dpl, all groups showed cortical damage consisting of oxidative stress, neurodegenerative changes, and apoptotic cell death, which were observed mainly in the perilesional tissue surrounding the necrotic cavity. Again, human nMT-IIa, but not human rMT-III treatment, could reduce these responses significantly, as judged by detecting oxidative protein and DNA damage, intraneuronal accumulation of neurofibrillary tangles and apoptotic cell death (Figs. 5,7).

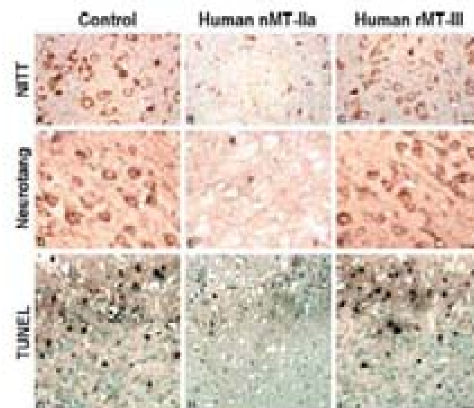

Fig. 5. Oxidative stress, neurodegeneration and apoptosis as seen in the parenchyma surrounding the lesion cavity of *Mt1&2* KO mice treated with saline (control), human nMT IIa or rMT III. **A–C:** NITT immunoreactivity in saline (A), nMT IIa (B), and rMT III (C) treated mice showing that mainly perilesional neurons are suffering oxidative stress. **D–F:** Immunoreactivity for neurofibrillary tangles indicating neurodegeneration in saline (D), nMT IIa (E), and rMT III (F) treated mice. **G–I:** TUNEL in saline (G), nMT IIa (H), and rMT III (I) treated mice showing apoptotic cell death inside the lesion (*top*) and perilesional (*bottom*) in the uninjured parenchyma. Brain injury induced oxidative stress, neurodegeneration and apoptosis were reduced by nMT IIa relative to saline treatment, whereas rMT III had no significant effects. Scale bars = 52 μm (A–C); 60 μm (D–F); 70 μm (G–I). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Growth/Trophic/Repair Factors and Synaptic Plasticity: Opposing Roles of Human nMT-IIa and Human rMT-III

After the lesion, all groups showed increased expression of repair-promoting agents such as growth factors, neurotrophins, and their receptors (FGF, FGF-R, NT-3, NT-4, BDNF, and TrkB), and the neuroprotectants involved in synaptic plasticity PSA-NCAM and S100-A4. nMT-IIa-treated mice showed increased expression of these factors when compared to the expression observed in saline treated mice, whereas the opposite was observed in rMT-III-treated mice (Figs. 6,7).

To evaluate the degree of post-lesional synaptic growth and plasticity, we immunostained for synaptophysin and syntaxin (presynaptic markers) and for spinophilin (a marker of dendritic spines). In all lesioned mice, these synaptic markers were clearly increased around the lesion cavity after the brain lesion. In comparison to saline-treated mice, nMT-IIa treatment clearly increased synaptic plasticity judging by these three markers, whereas the opposite was observed in rMT-III-treated mice (Figs. 6,7).

DISCUSSION

It has been established clearly that MT-II administered exogenously induces neuroprotection in a number of injury models as long as the BBB is altered (Penkowa and Hidalgo, 2000, 2001; Giralt et al., 2002b; Chung et al., 2003; Xie et al., 2004). It is generally agreed that mammalian MT-I and MT-II will likely be functionally equivalent, and in fact results obtained in *Mt1&2* KO mice are consistent with those obtained in

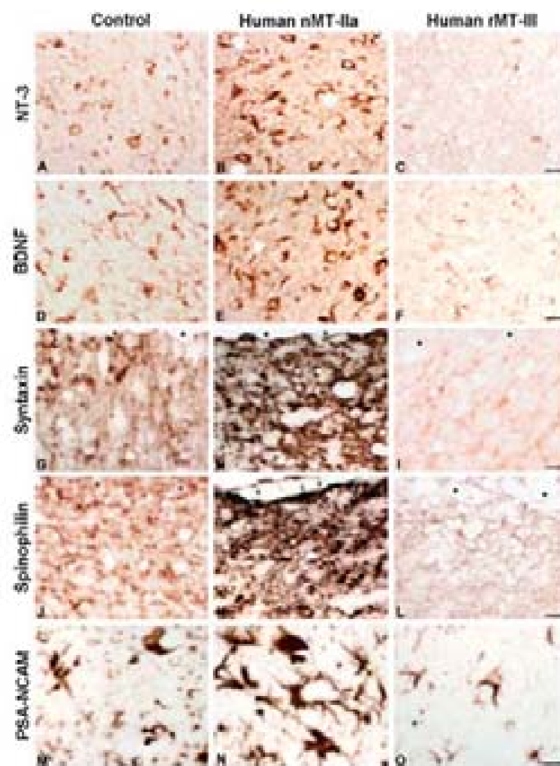

Fig. 6. Neurotrophins, synaptic plasticity, and repair factors (NT 3, BDNF, syntaxin, spinophilin, and PSA NCAM) in the tissue surrounding the lesion of *Mt1&2* KO mice treated with saline (control), human nMT II or human rMT III. **A–C:** Expression of NT 3 in saline (A), nMT II (B), and rMT III (C) treated mice. **D–F:** Expression of BDNF in saline (D), nMT II (E), and rMT III (F) treated mice. As shown, nMT IIa increased NT 3 and BDNF in neuronal and astroglial cells, whereas MT III reduced neurotrophins relative to saline. **G–I:** Syntaxin immunostainings around the lesion (marked by asterisks) in saline (G), nMT IIa (H), and rMT III (I) treated mice. **J–L:** Spinophilin expressing dendritic spines around the lesion (marked by asterisks) in saline (J), nMT IIa (K), and rMT III (L) treated mice. **M–O:** PSA NCAM expression in saline (M), nMT IIa (N), and rMT III (O) treated mice. Neurotrophins, synaptic plasticity markers and PSA NCAM were enhanced by nMT IIa relative to saline treatment, whereas rMT III tended to reduce these. Scale bars = 60 μm (A–F); 58 μm (G–L); 17 μm (M–O). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mice overexpressing MT-I regarding brain neurobiology (Hidalgo et al., 2001; Hidalgo, 2004). Nevertheless, direct comparative studies validating this have not been carried out. We compared the neuroprotective effect of native or recombinant MT-I&II from different mammalian species, including humans, to validate their functional equivalence in brain neurobiology. We also included in this study the *Drosophila* MTN, a MT nonhomologous to the mammalian forms and closer to yeast Cup1 (Peterson et al., 1996; Valls et al., 2000), because if putative protein–protein interactions exist underlying the therapeutical effects of MTs, the comparison between distantly related MTs might provide some insight.

As expected, native and recombinant mammalian MT-I&II behaved similarly, decreasing the inflammatory response, oxidative stress, neurodegeneration, and apoptotic death induced by the cryolesion. This confirms the suitability of recombinant synthesis for physiological studies, and strongly suggests that the two MT isoforms are functionally equivalent, at least in this injury model. This study shows, for the first time, that treatment with *Drosophila* MTN, although being somewhat less efficient, results in comparable and significant immunomodulation and neuroprotection relative to mammalian MT-I&II. Considering the low sequence similarity between the mammalian and *Drosophila* proteins (Table I), these results do not support protein–protein interactions (including a putative receptor) as the mechanism underlying the therapeutical effects of Zn-MTs, but rather suggest that either the zinc counterpart or other chemical properties of these proteins such as the highly reactive thiol groups could be the ones involved. The general structural properties that characterize all MTs are the formation of two metal-thiolate clusters, localized in two mutually interacting β- and α-domains (Romero-Isart and Vašák, 2002). Although the three-dimensional structure of *Drosophila* MTN is currently unknown, the available spectroscopic data suggests that also in this MT the binding of three of four zinc ions to 10 cysteine thiolates of the protein generates a metal-thiolate cluster (Valls et al., 2000). Even though the thiol groups in MTs are masked through their interaction with metal ions, they retain a substantial degree of the nucleophilicity seen with the metal-free protein. This is reflected by their high reactivity with oxidizing agents and ability to efficiently scavenge free radicals such as hydroxyl (OH·), superoxide (O_2 ·) or nitric oxide (NO). In all cases, the free radicals attack occurs at the metal-bound thiolates, leading to the protein oxidation or modification (Romero-Isart and Vašák, 2002). A consequence of this is that MT will release its zinc content, which locally might reach significant concentrations and exert potent effects (Sensi and Jeng, 2004). These structural properties of mammalian MT-I&II (presumably also of *Drosophila* MTN) are in agreement with their mediated antiinflammatory, antioxidative, and antiapoptotic actions in the brain (Hidalgo et al., 2001; this study).

When human rMT-III was administered exogenously, a very different pattern of neurobiologic effects emerged, as it was totally inefficient in controlling inflammatory response, cytokine production, oxidative stress, neurodegeneration, and apoptosis. This functional divergence was further highlighted when post-lesional synaptic plasticity was evaluated, because rMT-III showed an opposite pattern of effects compared to human nMT-IIa (detrimental vs. positive, respectively). The detrimental effects of rMT-III could be significantly mediated by the concomitant decrease of a number of growth factors, neurotrophins, and neuroprotective fac-

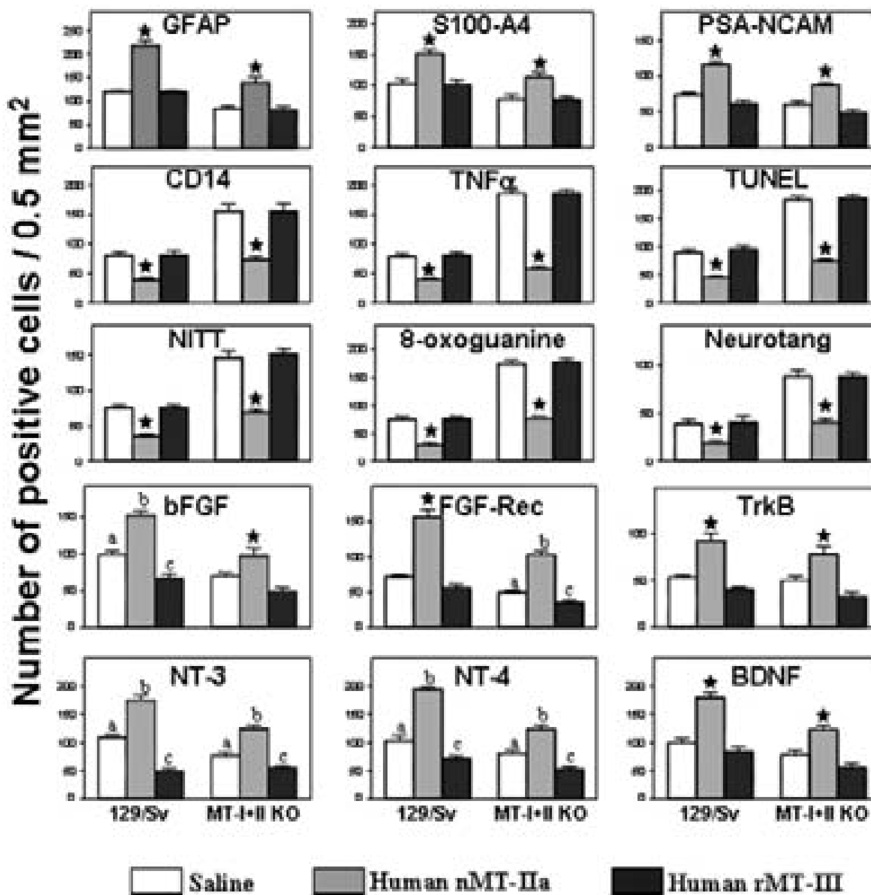

Fig. 7. Cellular counts of some of the variables analyzed in nMT IIa vs. rMT III experiment were carried out from 0.5 mm^2 matched areas at the border of the cortical lesion of five mice per group for statistical analysis (mean ± SE). Results were analyzed with two way ANOVA with strain and treatment as main factors. The *Mt1&2* KO mice were significantly different ($P < 0.001$) from WT mice for all variables except BDNF. One way ANOVA was done separately for each strain followed by SNK post hoc comparisons. *$P < 0.05$ vs. saline injected mice; groups labeled with different letters are significantly different from each other ($P < 0.05$).

tors, mechanism(s) that could also be underlying the neuroprotective effects of nMT-IIa. To our knowledge, this is the first study reporting that exogenously administered MT-III causes such prominent neurobiologic effects in vivo, although confirmation seems already at hand because Chung and West (2004) and Chung et al. (2003) mentioned a similar detrimental role after cortical brain injury. It is essential to realize that the effects with exogenous rMT-III reported in this study are consistent with those observed in *Mt-3* KO mice (Carrasco et al., 2003), fully supporting their physiologic relevance.

The present results clearly demonstrate specific and divergent roles of MT-III vs. the normal counterparts MT-I&II in vivo despite the evident structural similarities between these MT isoforms regarding metal-thiolate clusters (Table I). It has long been suspected this would be the case (Uchida et al., 1991), but clear evidences were scarce. Both MT-I&II and MT-III effects in the brain seem to emerge after injury because there is basically no brain alterations in the corresponding KO mice in normal conditions. Beyond this coincidence, diverging roles of MT-I&II vs. MT-III in emergency situations are demonstrated in the present study. Because nMT-IIa and rMT-III showed opposite effects on neuronal plasticity, whereas only the former affected oxidative stress, it is unlikely that the different effects they cause are related simply to modulation of oxidative stress in the injured brain. This points to specific mechanisms that might be the consequence of the structural differences known to exist between MT-III and MT-I&II, and shows the involvement of the MT-III $C_{(6)}$PCP and $T_{(5)}$CPCP sequence motifs in its inhibitory neuronal survival properties (Sewell et al., 1995; Romero-Isart et al., 2002). On the contrary, MT-I&II may promote neurite growth (Uchida et al., 1991; Erickson et al., 1994; Chung et al., 2002; Kohler et al., 2003). Structural divergence may also be the basis of the specific protein–protein interactions that recent studies have shown. Hence, molecular interaction between the G protein-Rab3A vesicular transporter and metal-loaded MT-III has been identified by the yeast two-hybrid method (Kang et al., 2001) and confirmed by affinity precipitation and surface plasmon resonance (Knipp et al., 2005). Several additional MT-III interacting partners, such as heat-shock proteins 84, hsp70, dihydropyrimidinase-like protein 2, creatine kinase, and β-actin, have been detected by immunoaffinity chromatography (Lahti et al., 2005). This opens new perspectives in the field.

Based on the present results, we can not rule out effects of the injected MTs outside the CNS as part of their neurobiologic effects. Although the outcome of the present experiments do show a clear consistency with that obtained in experiments with transgenic mice, further studies are needed for establishing more precisely the specific contribution of peripheral vs. central effects. We have shown that a cryolesion to the cortex does affect the number of lectin positive macrophages and CD3-positive T cells in the spleen in addition to the CNS. ZnMT-II injection reduces the former effect but not the latter, and the same thing occurs in MT-I overexpressing mice (Giralt et al., 2002b). It can be speculated that the peripheral effects of the injected (or endogenously expressed) MT-I&II could contribute to some extent to the reduction of macrophages observed in the brain, whereas it does not regarding T cells (although once again this might be indirectly influenced by the concomitant reduction in the number of macrophages). Regarding MT-III, there is no evidence to our knowledge of peripheral effects on the immune system or other physiologic compartments relevant to the brain. Obviously we can not rule out this completely and further work is clearly needed. Nevertheless, the effects we observe in the injured brain are more consistent with neuronal functions than with immune-affected physiological variables. Whereas we have observed previously a clear presence of injected rabbit MT-II in brains with compromised BBB quite rapidly (Penkowa and Hidalgo, 2000; Giralt et al., 2002b), this has not been tested here. The results suggest strongly that the different MTs used in this study behave similarly to the rabbit nMT-II. We have never analyzed how fast the MT-III protein reaches the brain and how similar this process is compared to the MT-I&II counterparts. This is a logical aim for future studies.

In conclusion, our results demonstrate specific and divergent roles for MT-III vs. MT-I&II in vivo in the cryolesioned mouse brain, which are consistent with those of the endogenously expressed protein. These results, together with the confirmation of the effectiveness of recombinant proteins, indicates that both types of proteins can be considered for use as potential therapeutic factors for the brain.

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IV. DISCUSIÓN

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1. Dinámica de la expresión génica en la criolesión

La respuesta inflamatoria desencadenada por la criolesión ya había sido, tal y como se comenta en la introducción, caracterizada por nuestro grupo desde un punto de vista primordialmente histopatológico (Giralt *et al.*, 2002b, Penkowa *et al.*, 1999a). Nos interasaba a continuación determinar la dinámica de activación de los diferentes factores implicados en la respuesta a este tipo de lesión. En este sentido, la tecnología de los *chips* de ADN (*microarrays*), se ha mostrado como una herramienta de elevado potencial para analizar la implicación de miles de genes al mismo tiempo en una situación concreta. Esta técnica, usada en otros tipos de lesión traumática (Knoblach *et al.*, 1999, Kury *et al.*, 2004, Natale *et al.*, 2004), permite analizar, dependiendo del experimento y del *chip* usado, la respuesta de entre 10.000 y 20.000 genes expresados en la corteza cerebral ipsilateral a diferentes tiempos después de la lesión, concretamente y tras la realización de tres experimentos, con dos cepas de ratones diferentes, hemos podido cubrir desde 1 día post lesión (dpl) hasta 16dpl.

1.1. Selección de los genes

El análisis de los resultados de los microarrays lleva asociado el empleo de herramientas informáticas, en este caso el programa dChip (Li y Wong, 2001), que facilitan el filtrado de los genes afectados según sus cambios en la expresión (*fold change*). Estos genes posteriormente son analizados mediante un test estadístico convencional (ANOVA de dos factores) para obtener así una lista manejable y estadísticamente significativa. Esta aproximación ha sido propuesta como la más apropiada para el análisis de los datos generados por microarrays (Guo et al., 2006) y fue usada en dos de los tres experimentos. En el último, sin embargo, y debido a que una nueva versión del programa dChip permitía realizar directamente un test ANOVA de dos factores, se optó por usar este sistema. El número de genes cuya expresión se vio afectada por la criolesión se situó alrededor de 320 en los dos primeros experimentos y de 459 en el último. Las diferencias en los valores entre experimentos, aunque mínimas si pensamos en el número total de genes analizados, pueden explicarse por diferentes motivos como el própiamente metodológico así como por el hecho

de que en el último experimento se han tenido en cuenta sondas redundantes, lo que conlleva que el número de genes afectados real sea algo menor. Otro factor a tener en cuenta es que, pese a que en este apartado nos centramos en el papel *per se* de la criolesión, no debemos olvidar que los experimentos fueron diseñados desde la perspectiva de evaluar el efecto de la deficiencia bien de TNFR1/ TNFR2, IL-6 o MT1+2 para elucidar su implicación, por lo que, dependiendo de los genotipos estudiados pueden existir diferencias puntuales en el test ANOVA de dos factores. Finalmente, el último experimento fue realizado en ratones de la cepa 129Sv/J, mientras que los dos anteriores se realizaron en animales C57Bl6/J, por lo que, dadas sus marcadas diferencias anatómicas y funcionales (Kalueff y Tuohimaa, 2004, van Bogaert *et al.*, 2006), pueden existir diferencias en la respuesta entre las dos cepas.

1.2. Caracterización del patrón temporal de expresión en la criolesión

Si bien es importante identificar los genes alterados en nuestro modelo de lesión, no lo es menos el poder clasificarlos según su patrón de expresión, agrupando todos aquéllos que respondan de manera similar a la criolesión y, de esta forma, poder definir qué funciones y/o mecanismos celulares están implicados en cada momento.

El programa dChip permite la agrupación jerárquica de los genes obtenidos, creando diferentes *clusters* donde se muestra el nivel de expresión de un gen en comparación con los niveles basales en cada tiempo estudiado. Gracias a este tipo de análisis hemos podido identificar grupos de genes que muestran cambios en la expresión, ya sea incrementándola (aproximadamente un 70% de los genes) o disminuyéndola (30% de los genes), en algunos de los tiempos estudiados. Después de un análisis funcional mediante el programa EASE (Hosack *et al.*, 2003) pudimos identificar las categorías sobre-representadas en cada uno de los *clusters* seleccionados.

Nuestros resultados permiten afirmar que, si bien la criolesión induce en gran medida la expresión de genes encaminados a favorecer la respuesta inflamatoria, la entrada leucocitaria y la resolución del daño, iniciando procesos de supervivencia o apoptóticos en las diferentes células afectadas, también provoca la disminución de la función neuronal y la plasticidad sináptica, aunque probablemente exista una interrelación entre estas dos acciones, siendo aquí donde cobra vital importancia el estudio del papel de las citoquinas, que como ya se ha descrito anteriormente, participan en las primeras fases de la respuesta.

En las primeras 24 horas después de la lesión encontramos un marcado incremento en la expresión de genes implicados en procesos de señalización tales como factores de transcripción, en muchas ocasiones relacionados con la señalización de citoquinas y factores de crecimiento, destacando a *nfkb*, *c-fos*, *c-jun* y *stat3.* La expresión de estos factores ha sido ampliamente descrita en respuesta a lesiones traumáticas (Hang *et al.*, 2006, Michael *et al.*, 2005, Suzuki *et al.*, 2005). Estos factores son a su vez los responsables de la activación de multitud de genes, sugiriendo una activación de múltiples mecanismos de supervivencia y/o apoptosis después de la lesión. Algunos ejemplos serían g*add45b* y *gadd45g*, implicados tanto en mecanismos neuroprotectores como neurotóxicos (Yoo *et al.*, 2003) o genes implicados en la respuesta inflamatoria como las quimioquinas (*ccl2, ccl3, ccl12*) (Rebenko-Moll *et al.*, 2006) o las proteínas de fase aguda metalotioneína 1 y 2, que son inducidas tras lesión en parte por IL-6 (Lee *et al.*, 1999), marcando así una posible primera respuesta rápida al daño.

En el periodo comprendido entre 1 día y 4 días post lesión los resultados sugieren que están siendo activados mecanismos de respuesta intracelular al daño, aumenta la expresión de moléculas implicadas en el control del ciclo celular y apoptosis, como *p21* o *CLIC4* relacionados con procesos apoptóticos inducidos por p53 (Kassouf *et al.*, 2006, Siever y Erickson, 1997). Otros mecanismos claramente activados sugieren la remodelación del tejido, observándose un incremento en la expresión de genes que codifican proteínas del citoesqueleto, como es el caso de genes de astrocitos como GFAP o vimentina, dando lugar a una mayor reactividad glial, hecho que se ha demostrado tanto en la criolesión (Penkowa *et al.*, 2000b) como en otros tipos de lesiones (Dutcher *et al.*, 1999). Otros genes que codifican factores del citoesqueleto de diferentes tipos celulares como la calponina 3 o la transgelina también se ven incrementados, así como genes asociados a la matriz extracelular, tales como las anexinas, implicadas en procesos de adhesión celular y fibrinolisis (Siever y Erickson, 1997), que facilitarán la entrada de leucocitos así como la exacerbación del proceso inflamatorio.

Entre los días 4 y 8 post lesión se confirma una mayor entrada de leucocitos, observándose un marcado incremento en el número de genes implicados en los procesos de respuesta inmune, con especial énfasis en genes pertenecientes al complejo mayor de histocompatibilidad tipo II, presente en células presentadoras de antígeno profesionales (Abbas *et al.*, 2000) y factores del complemento. También hemos descrito un incremento en la expresión de genes relacionados con la reconstrucción celular y con la respuesta a daño. Se ha podido observar el incremento de la expresión de diversas catepsinas, implicadas tanto en procesos de proteolisis intracelular como de la matriz extracelular (Felbor *et al.*, 2002). A los

8dpl, la única categoria de genes con respuesta significativa implica la remodelación de la matriz extracelular. A los 16 días post-lesión la expresión génica, en general, vuelve a los niveles basales.

No debemos olvidar, sin embargo, que un 30% de los genes seleccionados mostraron una disminución en su expresión después de la criolesión. La gran mayoría de estos genes están relacionados con la diferenciación neuronal, el desarrollo del sistema nervioso y mecanismos de transporte intracelular. Nuestros resultados confirman el descenso de la función neuronal tras la lesión, pudiendo ser sus causas primarias (debidas a la lesión) y/o secundarias (por un descenso de factores tróficos en la denominada zona de penumbra) (Wieloch y Nikolich, 2006). Así, podemos destacar el gen que codifica la subunidad beta de la proteína quinasa II dependiente de calcio/calmodulina – *Camk2b*-, implicada en procesos de memoria y plasticidad (Colbran y Brown, 2004) y el gen para la proteína asociada a microtúbulo 2 (*mtap2*), implicado en la estabilidad de los microtubulos y en procesos de plasticidad sináptica (Harada et al., 2002).

2. El TNF-α en el sistema nervioso central

Nos hemos centrado en la participación de cada uno de los receptores del TNF-α por separado gracias al empleo de animales knock-out para el receptor 1 (TNFR1KO) o para el receptor 2 (TNFR2KO) en un modelo de lesión traumática, en concreto la criolesión, aunque cabe destacar que no hemos olvidado posibles acciones de este factor en el organismo sano.

2.1. Implicación del TNF-α en la actividad motora/exploratoria

De los resultados que hemos obtenido con animales deficientes bien para el receptor 1 o para el receptor tipo 2 del TNF-α se desprende que este factor está implicado en el desarrollo y/o en la modulación de algunos de los patrones conductuales del ratón.

En este sentido tanto los animales deficientes para el receptor 1 como para el receptor 2 del TNF-α muestran una mayor actividad locomotora, cuantificada por un incremento en el número de ambulaciones exteriores en el test *hole board* (tabla de agujeros), confirmando así resultados de otros grupos que muestran como una sobre-expresión de TNF-α afecta de manera global la conducta de los ratones (Fiore *et al.*, 1996), observándose una menor

actividad locomotora (Fiore *et al.*, 1998). El test *hole board* es también un indicador de la actividad exploratoria de los animales, habiéndose descrito como el incremento de TNF-α hipocampal tras lesión en ratón reduce la capacidad exploratoria medida por el número de *rearings* (Pan *et al.*, 2003). Nuestro estudio confirma estos resultados, caracterizando además el papel específico de TNFR2 en este patrón de conducta, al observar un incremento tanto en el número de *rearings* como de *head dippings* –también indicador de conducta exploratoria- únicamente en los animales TNFR2KO. Una posible explicación de las diferencias conductuales de los animales deficientes en TNFR2 puede encontrarse en la alteración en la expresión de diversos genes, identificados gracias a los microarrays, implicados tanto en la formación como en la actividad de las sinapsis. El TNF-α, mediante su unión al receptor 2, podría estar modulando el patrón de expresión génica dando como resultado final las diferencias conductuales que describimos en adultos. En este sentido se ha descrito una acción del TNF-α en procesos de refuerzo sináptico (Stellwagen y Malenka, 2006). El estudio de la contribución específica de cada uno de los genes en el fenotipo observado se presenta como un interesante proyecto para elucidar las acciones centrales del TNF-α en condiciones fisiológicas.

El test *hole board*, permite además evaluar la ansiedad/emotividad de los animales, ya que las ambulaciones por cuadrantes internos, desprotegidos, serán más frecuentes en animales menos ansiosos. Nuestros resultados no muestran ningún efecto del TNF-α en este parámetro, ni tampoco se observan diferencias entre cepas en un test específico para evaluar la ansiedad de los animales, como es el *elevated plus maze* (laberinto en cruz elevado). En este último el tiempo que los animales pasan en los brazos abiertos (no protegidos) del laberinto correlaciona inversamente con la ansiedad del animal. Los resultados sugieren que el TNF-α, a nivel fisiológico no estaría implicado en la ansiedad o emotividad de los animales, aunque otros laboratorios, usando animales TNFKO, han sugerido un papel de esta citoquina en el establecimiento de conductas ansiogénicas (Yamada *et al.*, 2000) o tras infección vírica (Silverman *et al.*, 2007). Las diferencias podrían explicarse por un lado por los efectos que puede causar una deficiencia total de TNF-α durante el desarrollo, y por otro por que las acciones del TNF-α a las concentraciones en las que se encuentra en respuesta a infecciones pueden diferir de las observadas a nivel fisiológico, mostrando nuevamente la complejidad de la señalización de este factor. Otras citoquinas pro-inflamatorias como la IL-1 o la IL-6, sin embargo, sí que han mostrado efectos sobre la ansiedad, (Armario *et al.*, 1998, Swiergiel y Dunn, 2007), debe tenerse en cuenta, sin embargo, que niveles elevados de estos factores

pueden inducir la respuesta de enfermedad (*sickness behaviour*), la cual a su vez puede alterar los resultados de los tests conductuales usados normalmente.

2.2. Papel de los receptores del TNF-α en un modelo de lesión traumática

Múltiples mecanismos se activan en respuesta a daño en el sistema nervioso central, por lo que no es de extrañar que, tal y como se ha descrito en otros modelos experimentales (Li *et al.*, 2004, von Gertten *et al.*, 2005), en nuestro modelo de lesión se observe un claro patrón de activación de grupos de genes. De entre los grupos obtenidos se puede identificar la inducción de genes implicados en procesos inflamatorios, sobre todo en tiempos cortos después de la lesión, así como genes relacionados con la respuesta a daño. Por el contrario, entre los genes cuya expresión disminuye después de la lesión se pueden encontrar factores implicados en la diferenciación neuronal y, posteriormente, en la estabilidad de los microtubulos, y, por extensión, de la célula.

Una vez visto el mecanismo general iniciado frente a una lesión nuestro objetivo ha sido caracterizar el papel individual de cada uno de los receptores del TNF-α en esta respuesta, habiéndose analizado a diferentes niveles. En primer lugar, sé usó un test para evaluar la función motora tras lesión, el *horizontal ladder beam* (escalera horizontal elevada – HLB-). En este test, se enseña a los ratones a pasar sobre una escalera horizontal para llegar a una caja oscura en un extremo. Una vez los ratones han aprendido a ejecutar la prueba sin errores, son lesionados y se anota el número de fallos que cometen al pasar sobre la escalera horizontal. Nuestros resultados muestran como tanto los animales TNFR1KO como los animales TNFR2KO presentan una mejor respuesta, comparados con animales de la cepa control, lo que sugiere una implicación neuropatológica para ambos receptores. En este sentido, la ausencia de TNF-α se ha mostrado positiva tanto en procesos isquémicos como tras administración de ácido caínico (Bruce *et al.*, 1996, Gary *et al.*, 1998). En otros casos, sin embargo, se ha descrito un papel diferencial de los dos receptores, por ejemplo en la EAE el TNFR1 desempeña un papel negativo mientras que TNFR2 participa en funciones neuroprotectoras (Suvannavejh *et al.*, 2000), aunque lo contrario se ha descrito en procesos isquémicos (Akassoglou *et al.*, 2003) y por último, después de axotomía se ha descrito un papel perjudicial mediado por los dos receptores al mismo tiempo (Raivich *et al.*, 2002), estando más en línea con nuestros resultados en la criolesión.

Por este motivo, nuestro esfuerzo se ha encaminado en encontrar los mecanismos de la señalización de cada uno de los receptores en nuestro modelo, para en un futuro poder estudiar los mecanismos concretos que puedan explicar la diversidad de acciones.

2.2.1. TNFR1 media la respuesta inflamatoria desencadenada por la criolesión

Una de las acciones mejor descrita para TNF-α es su capacidad pro-inflamatoria por lo que en este trabajo hemos caracterizado la respuesta inflamatoria subsiguiente a la criolesión en animales TNFR1KO y TNFR2KO.

Nuestros resultados sugieren un papel de TNFR1 pero no de TNFR2 en el establecimiento de la respuesta inflamatoria, habiéndose observado que sólo los animales TNFR1KO presentan un menor número de astrocitos reactivos, menor microglia activada/macrófagos (células positivas para F4/80 así como células positivas para IL-1β), linfocitos T (CD3 positivos) infiltrados y células apoptóticas (positivas para caspasa-3 y TUNEL) tanto 3 como 7 días después de la lesión, pero sin estar asociada a una reducción en el estrés oxidativo (células positivas para proteínas nitrosiladas y para 8-oxoguanina, un marcador de daño en el ADN).

Un estudio más exhaustivo a nivel de expresión génica mediante diferentes técnicas nos ha permitido describir que esta menor respuesta inflamatoria y apoptosis observada en los animales TNFR1KO podría ser consecuencia de la menor activación génica en las primeras 24 horas de la respuesta a la lesión. Una primera aproximación mediante RPA (*ribonuclease protection assay*) nos permitió confirmar esta hipótesis, presentando los animales TNFR1KO una menor activación de genes implicados en la vía de señalización de TNFR1 (*rip*), una disminución en la expresión de citoquinas pro-inflamatorias, acompañada de una menor expresión de *gfap* y de *mac1*, lo que sugeria una menor activación astroglial e infiltración de macrófagos respectivamente, y de genes implicados en la respuesta inflamatoria tales como la proteína de fase aguda *eb22/5*, el factor de adhesión celular *icam*, las metaloproteinasas de matriz (*mmps*) y su inhibidor (*timp1*) y finalmente en genes implicados en vías apoptóticas (*fas*). Estos resultados permitirían explicar los resultados obtenidos por otros grupos, en tanto en cuanto el TNF-α estaría implicada en la respuesta inflamatoria, regulando el reclutamiento de leucocitos y la gliosis (Munoz-Fernandez y Fresno, 1998, Raivich *et al.*, 1999).

La reducción observada en la expresión de estos genes sugería la implicación del factor de transcripción más importante en la señalización de TNF-α, el factor nuclear (NF)kB, habiéndose descrito una expresión reducida de este factor previamente en animales con diferentes déficits en la vía del TNF-α (Kim *et al.*, 2001, Sullivan *et al.*, 1999). Mediante el uso de una técnica de screening de amplio espectro (*microarrays*) con sondas para aproximadamente 20.000 genes pudimos identificar efectivamente una menor expresión de *nfkB* así como de otros factores de transcripción c/ebp, c-jun, c-fos en los animales TNFR1KO a las 24 horas post lesión. NFkB puede activar mecanismos tanto antiinflamatorios como pro-inflamatorios entre los que se encuentran tanto c-fos como c-jun, que una vez dimerizados constituyen el factor de transcripción AP-1, que puede iniciar procesos apoptóticos (Hallenbeck, 2002).

La disminución en la expresión de factores de transcripción está acompañada de una disminución en la expresión de genes implicados en la respuesta inflamatoria en los animales TNFR1KO. Aunque los microarrays no detectaron la expresión de citoquinas pro-inflamatorias, probablemente por problemas de sensibilidad, a 1 dia post-lesión encontramos menor expresión de genes relacionados con la señalización de citoquinas tales como el receptor 1 de la IL-1 o *stat3*, factor de señalización de IL-6 o *socs3*, implicado en la regulación de las acciones de citoquinas (Emery *et al.*, 2006), lo que se corresponde con las disminuciones en la expresión de IL-1α, IL-1β, IL-6 y TNF-α observadas con el RPA. Las citoquinas inducen a su vez la expresión de múltiples factores, entre ellos quimioquinas (Borish y Steinke, 2003), que a su vez, dada la implicación de estos factores en la migración leucocitaria (Rebenko-Moll *et al.*, 2006), explicarían la menor presencia de macrófagos y leucocitos observados tanto a 3 como a 7dpl.

Los genes de las metalotioneínas 1 y 2 son inducidos por la criolesión y muestran una disminución a 1dpl asociada a la deficiencia de TNFR1. Las metalotioneínas poseen una clara acción anti-inflamatoria y anti-oxidante (Carrasco, 2000, Hidalgo *et al.*, 2001) y son inducidas por IL-6 a través de STAT3 (Lee *et al.*, 1999). Pese a que a nivel histoquímico no observamos diferencias en cuanto a células positivas para marcadores de estrés oxidativo la disminución en los niveles de metalotioneína puede condicionar en estadios posteriores la capacidad antioxidante en los animales TNFR1KO. En este sentido, el estrés oxidativo desempeña un papel muy importante en el mantenimiento de las vías apoptóticas inducidas por el TNF-α (Kamata *et al.*, 2005). Hemos descrito que la criolesión induce la expresión de las metaloproteinasas MMP3, MMP9 y MMP12 y de su inhibidor TIMP1. Este dato es

consistente con los resultados descritos en un modelo de impacto cortical (Wang *et al.*, 2000), en el que se observa una menor inducción en la expresión de estos factores en los animales TNFR1KO a un día post lesión. Los resultados sugerirían una menor degradación de la matriz extracelular, que podría ser causa y consecuencia al mismo tiempo de una menor infiltración de células circulantes (Justicia *et al.*, 2003). Finalmente también hemos podido observar que la criolesión induce la expresión de genes implicados en procesos apoptóticos tales como c-fos, c-jun, fas y a20, siendo este último un gen con acciones antiapóptoticas inducido por NFκB (Bubici *et al.*, 2006), los animales TNFR1KO presentan nuevamente una inducción menor de todos estos factores, siendo posiblemente éste el motivo de la menor muerte celular apoptótica detectada en el borde de la lesión tanto a 3 como a 7dpl.

En vista de nuestros resultados parecería claro que TNFR1 está mediando la activación de los mecanismos pro-inflamatorios en respuesta a criolesión a tiempos cortos, tal y como se ha descrito en otros modelos experimentales (Suvannavejh *et al.*, 2000). En fases posteriores (4dpl) no encontramos diferencias en cuanto a la expresión de *Gfap* o del gen de fase aguda *eb22/5* (Campbell *et al.*, 1994) o de *Timp1*, observándose incluso un incremento de los valores de este último a los 3dpl, sugiriendo probablemente un retraso en su dinámica de activación. Los resultados no hacen más que demostrar que, si bien los cambios iniciales a los 7dpl causados por la deficiencia de TNFR1 se muestran beneficiosos tanto histopatológica como funcionalmente (medido por el test HLB), no podemos descartar que los mecanismos de signo contrario se inicien en estadios posteriores, tal y como ha sido postulado por otros autores (Munoz-Fernandez y Fresno, 1998, Shohami *et al.*, 1999).

2.2.2. TNFR2 es el principal mediador de las acciones del TNF-α independientes de la inflamación

El hecho de que una menor presencia de mediadores inflamatorios, como pasa en los animales TNFR1KO, motivase una disminución en el daño celular y una mejora en la respuesta funcional después de daño encajaba totalmente con la descripción de las acciones descritas para este receptor. Los animales TNFR2KO, como ya hemos comentado, presentan también una mejor respuesta en el test HLB, sugiriendo que el TNF-α puede tener acciones

independientes de la inflamación mediadas por TNFR2 que desembocan en una peor respuesta funcional tras criolesión.

El uso de los microarrays nos ha permitido detectar una gran variedad de genes afectados constitutivamente por la deficiencia de TNFR2, por lo que es probable que combinaciones de éstos sean los causantes de los efectos observados. Una vez agrupados los genes según sus funciones descritas, pudimos observar como la mayoría de ellos estan implicados en desarrollo, memoria, plasticidad sináptica y estabilización de los microtúbulos, por lo que nuestros resultados abren múltiples posibilidades futuras para la clarificación de las acciones del TNF-α tanto en situaciones fisiológicas como frente a daño.

En este sentido, y a modo de ejemplo de las posibilidades que ofrecerá en el futuro el estudio exhaustivo de cada uno de los genes seleccionados, se puede resaltar un posible mecanismo implicado en la muerte celular por vías alternativas a las descritas actualmente para TNFR1. Se ha descrito una vía de muerte celular inducida por TNF-α causada por la disfunción de los microtúbulos inducida por la fosforilación de la proteína stathmin (Vancompernolle *et al.*, 2000). En nuestro estudio hemos observado como stathmin se encuentra disminuído en los animales TNFR2KO de manera constitutiva, de la misma manera que lo están los genes de factores asociados a los microtúbulos map1b y map2. El mismo grupo (Van Herreweghe *et al.*, 2002) ha descrito que este proceso viene mediado por la fosforilación de la glioxilasa-1 por acción de la PKA, induciendo muerte celular por una vía dependiente de productos finales de glicosilación avanzada (*advanced glycation end-product* –AGE-). En este sentido hemos identificado que el gen de la subunidad catalítica de la PKA (*Prkacb*) también muestra una expresión reducida en los animales TNFR2KO. En conclusión, la deficiencia de TNFR2 nos muestra una implicación de este receptor en mecanismos de mantenimiento celular diferentes a las descritas para el TNFR1, tanto a nivel fisiológico como después de lesión.

En nuestro modelo la ausencia y, por lo tanto el bloqueo de las vías de señalización de cualquiera de los dos receptores se ha mostrado beneficioso en las primeras fases de la respuesta, aunque, dado el papel dual descrito para el TNF-α no podemos afirmar que sea así durante la progresión de la respuesta al daño (Shohami *et al.*, 1999).

3. Implicación de la IL-6 en el sistema nervioso central

El objetivo principal del estudio ha sido la determinación de los genes implicados en la diferente respuesta a la criolesión que observamos en los animales con deficiencia en IL-6 (IL6KO) (Penkowa *et al.*, 1999b, Penkowa *et al.*, 2000b) o que sobre-expresan IL6 en el sistema nervioso central (GFAP-IL6) (Penkowa *et al.*, 2003c). Estos resultados, principalmente obtenidos mediante técnicas histopatológicas, sugieren un papel dual de la IL-6, estando primeramente implicada en el establecimiento de la respuesta inflamatoria, pero al mismo tiempo participando en la disminución del estrés oxidativo y de los procesos apoptóticos, pudiendo estar estos efectos a su vez implicados en la facilitación de la recuperación después de lesiones traumáticas (Swartz *et al.*, 2001), así como en modelos de lesión axonal *in vitro* (Hakkoum *et al.*, 2007). En vista de estos resultados, es de vital importancia, por tanto, elucidar los mecanismos desencadenantes.

Nuestros resultados, mediante el empleo de *microarrays*, nos han permitido determinar la implicación de la IL-6 tanto en situaciones fisiólogicas como después de la lesión, habiendo observado cambios en la expresión de diferentes genes, algunos no descritos previamente, que pueden mediar los importantes efectos establecidos para la IL-6 bien en la supervivencia celular bien en la respuesta inflamatoria tras criolesión.

3.1. La señalización de la IL-6 en condiciones fisiológicas

La expresión de diversos genes se ha visto alterada de manera constitutiva por la deficiencia o sobre-expresión de IL-6 en el SNC, sirviendo de base para explicar las múltiples observaciones sobre la implicación de la IL-6 en diferentes funciones en el SNC. Los genes han podido ser agrupados en diferentes categorias. Entre ellas, encontramos genes implicados en procesos de plasticidad sináptica, en este sentido factores como la alfa-sinucleina o la sinapsina II, que modulan la función de las vesículas sinápticas (Austin *et al.*, 2006, Samigullin *et al.*, 2004), muestran una expresión incrementada en los animales IL6KO, mientras que por el contrario *vamp2* (sinaptobrevina 2), gen involucrado en la transmisión sináptica (Blagoveshchenskaya et al., 2002), está disminuído en los animales que sobreexpresan IL-6, por lo que podrían estar implicados en las alteraciones en los niveles de

diferentes proteínas sinápticas inducidas por IL-6 y descritas recientemente (Vereyken *et al.*, 2007) que pueden explicar, a su vez, las diferencias en la conducta emocional observadas en los animales IL6KO (Armario *et al.*, 1998)

También hemos podido observar como la IL-6 modula la expresión de genes implicados en el desarrollo del SNC. Se ha sugerido una acción de la IL-6 en la diferenciación preferencial de las células precursoras neurales en astrocitos (Taga y Fukuda, 2005), habiéndose observado una disminución de la neurogénesis en adultos (Vallieres *et al.*, 2002). Nuestros resultados confirmarían la implicación de IL-6 en estos procesos; así la deficiencia de IL-6 está asociada a un incremento en la expresión de los factores de transcripción *Sox2,* expresados en células precursoras embrionarias (Miyagi *et al.*, 2004), o el factor de transcripción 12, que participa en el desarrollo de los oligodendrocitos (Riemenschneider *et al.*, 2004).

Por último, otras funciones en las que hemos descrito genes alterados por la deficiencia o sobre-expresión de IL-6 son la síntesis y metabolismo protéico y de ácidos nucléicos así como genes implicados en la citoarquitectura celular, que probablemente estén involucrados en todos los procesos descritos anteriormente, y que, sin lugar a dudas, condicionan la posterior respuesta a situaciones lesivas descrita para los animales IL6KO (Penkowa *et al.*, 2000b) o GFAP-IL6 (Penkowa *et al.*, 2003c) tanto en la criolesión como en otros modelos (Penkowa *et al.*, 2003b, Swartz *et al.*, 2001).

Nuestros resultados, en conjunto, confirman la naturaleza pleyotrópica de la IL-6 así como sirven de base para un estudio pormenorizado gen a gen que puede resultar clave en la identificación de nuevos mecanismos de acción de la IL-6 en la fisiología del SNC.

3.2. Implicación de la IL-6 en el sistema nervioso central en un modelo de lesión traumática

3.2.1. La IL-6 modula la función neuronal

Nuestros resultados muestran una disminución de la expresión tras lesión de diversos genes implicados en la función neuronal, en la transducción de señales y en el control de la transcripción génica, encontrándose una implicación de la IL-6 en todas estas categorias, lo que sugiere importantes y variadas acciones de esta citoquina. De entre todas las categorias,

quizás la más destacada y en la que más genes se han visto afectados por la deficiencia o sobre-expresión de IL-6 es la de la señalización del calcio. Este dato no es soprendente si se tiene en consideración que la alteración de la homeostasis del calcio es una de las características de las lesiones traumáticas, al activar mecanismos de muerte celular por excitotoxicidad y alterar los mecanismos de aprendizaje y memoria (Yun *et al.*, 2002).

Se ha descrito una disminución en los niveles de CAMKII en respuesta a lesiones isquémicas (Uemura et al., 2002), epilepsia (Liang y Jones, 1997) y daño excitotóxico *in vitro* (Churn et al., 1993). Nosotros observamos que la deficiencia de IL-6 mantiene la expresión de la proteina quinasa dependiente de calcio/calmodulina II (*Camk2*), mientras que la sobre-expresión de IL-6 provoca una reducción mucho más marcada. De la misma manera, el gen de la quinasa 2 de la CaMKII (*Camkk2*) se encuentra disminuído en los animales GFAP-IL6. Dado que la vía de la CaMKII es uno de los mecanismos más importantes en el control de la plasticidad sináptica y la memoria (Colbran y Brown, 2004) podemos sugerir que la IL-6 presenta un papel inhibidor de estas funciones y en este sentido, se podrían explicar los deficits en actividad hipocampal (Steffensen et al., 1994) y aprendizaje (Heyser et al., 1997) descritos en los animals GFAP-IL6 y la inhibición de los procesos de memoria y plasticidad sináptica descritos para la IL-6 (Balschun et al., 2004).

Después de la lesión, la IL-6 puede estar actuando de una manera más compleja en la homeostasis del calcio. En este sentido hemos podido identificar una menor expresión de genes como Atp2b2 (*Pmca2*) o *Achyl1*, implicados en la salida de calcio de las neuronas tras lesión (Cooper *et al.*, 2006, Pottorf *et al.*, 2006) en los animales GFAP-IL6. De la misma manera, la administración crónica de IL-6 en neuronas cerebelares también se ha descrito que induce la liberación de calcio desde compartimentos intracelulares (Nelson et al., 2004).

El objetivo principal de la respuesta a una lesión es la limitación del daño y uno de los mecanismos, como ya se ha comentado, es la disminución de la función sináptica. En este sentido, ha sido ampliamente descrita la capacidad de la microglia activada para eliminar las sinapsis excitadoras en neuronas dañadas, en un proceso conocido como *synaptic stripping* (Cullheim y Thams, 2007), impidiendo así una activación neuronal y favoreciendo la iniciación de mecanismos de supervivencia.

3.2.2. La IL-6 es pro-inflamatoria en la criolesión

La IL-6 ha sido extensamente descrita como factor pro-inflamatorio (Munoz-Fernandez y Fresno, 1998, Van Wagoner y Benveniste, 1999) y, en este sentido, nuestros resultados claramente muestran la contribución de la IL-6 en la expresión de genes implicados en la respuesta inflamatoria. En primer lugar, el trabajo con animales deficientes en IL-6 nos ha permitido observar una menor activación en dos de las principales vías de señalización mediadas por citoquinas pro-inflamatorias, como son la via JAK/STAT observando una menor activación de *Socs3*, gen diana de STAT3, mediador de la respuesta inflamatoria de IL-6 e indicativo del *status* inflamatorio de la célula (Emery *et al.*, 2006). Asimismo, la señalización por NFκB, descrito anteriormente, también se encuentra alterada en los animales sin IL-6, donde observamos una menor indución de *Ikba*, que codifica para el inhibidor de NFκB, IκBα, lo que sugiere bien una respuesta defectuosa en estos animales al daño o bien una alteración en el control de los genes diana de NFκB, que a su vez es el mediador principal de TNF-α, lo dará lugar a una menor presencia de citoquinas pro-inflamatorias, como había sido previamente observado en un modelo de isquemia (Clark *et al.*, 2000).

Una de las acciones mejor establecidas para la IL-6 es su participación en la inducción de la activación glial y en la infiltración de células circulantes (Kopf *et al.*, 1994, Penkowa *et al.*, 1999b). En este sentido, no es de extrañar que observemos una menor expresión de diferentes genes implicados en estos procesos, como es el caso de *Cd14*, que codifica para el co-receptor de LPS y que se expresa en monocitos y macrófagos (Beschorner *et al.*, 2002) en los animales IL6KO. De la misma manera, se observa una disminución en diferentes genes del complejo de histocompatibilidad tipo II, también expresados por monocitos y otras células presentadoras de antígenos en situaciones de inflamación (Cresswell, 1996). Se ha señalado una menor respuesta inflamatoria en los animales IL6KO frente a diferentes situaciones patológicas (Eugster *et al.*, 1998, Raivich *et al.*, 1999).

La sobre-expresión de IL-6, produce, como era de esperar, efectos totalmente opuestos, observándose incrementos en la expresión de los genes del complejo mayor de histocompatibilidad II, o de genes como *Iftm1* e *Ifitm2* que se expresan en macrófagos y son inducidos en procesos inflamatorios (Johnson et al., 2006), genes para diferentes subunidades del receptor para la fracción constante de las IgG, implicados en la atracción leucocitaria (Hamaguchi et al., 2006), o genes del complemento como *C4b*, sugiriendo una mayor respuesta inflamatoria y un incremento en la infiltración de leucocitos, que ha sido descrita

en animales GFAP-IL6 tras criolesión (Penkowa *et al.*, 2003c). De la misma manera, la sobreexpresión de IL-6 induce la expresión de *Gfap*, principal filamento astrocitario. La expresión de GFAP es uno de los mejores marcadores de inflamación y es una clara indicación de una mayor reactividad astroglial (Zeng *et al.*, 2000). En este sentido aunque los animales GFAP-IL6 presentan claros signos de neuroinflamación y astrogliosis en algunas áreas cerebrales (Campbell et al., 1993), en condiciones basales no muestran diferencias en cuanto a los niveles de GFAP en el córtex cerebral (Chiang et al., 1994), pero sí que los presentan cuando son sometidos a algun modelo de lesión tal como la criolesión, (Penkowa *et al.*, 2003c), por lo que nuestros resultados apoyarían y extenderían los de estos trabajos, al identificar algunos de los genes y mecanismos que pueden estar orquestando las respuestas observadas.

3.2.3. La expresión de IL-6 promueve la supervivencia neuronal

La IL-6, además de tener un marcado papel pro-inflamatorio, pertenece a la familia de las neuropoyetinas (Bauer et al., 2007), por lo que puede presentar acciones neurotróficas, habiéndose establecido que favorece la supervivencia neuronal en diferentes situaciones patológicas (Murphy *et al.*, 2000, Swartz *et al.*, 2001, Westberg *et al.*, 2007).

En el modelo de la criolesión, nuestro grupo ha determinado un incremento en el número de células apoptóticas (positivas para marcaje por la técnica de TUNEL) en los animales IL6KO así como un incremento en los niveles de estrés oxidativo celular (Penkowa *et al.*, 2000b) , mientras que lo contrario se ha descrito en los animales GFAP-IL6 (Penkowa *et al.*, 2003c).

Nuestros resultados nos han permitido demostrar la implicación, a nivel transcripcional, de la IL-6 en los procesos de supervivencia y de muerte celular contribuyendo a la identificación de vías apoptóticas implicadas en la respuesta. Hemos identificado como la IL-6 promueve la expresión de genes de factores antioxidantes que pueden ser los involucrados en la reducción del estrés oxidativo observado en los animales GFAP-IL6 a la vez que, su expresión defectuosa en los animales IL6KO puede explicar el incremento de los niveles del mismo.

Respecto al papel neuroprotector de la IL-6, se ha descrito su acción en la producción de factores neurotróficos como GM-CSF (Penkowa *et al.*, 1999b) o BDNF (Murphy *et al.*,

2000) así como en la limitación del estrés oxidativo y de la función mitocondrial en diferentes situaciones patológicas (Jin *et al.*, 2007, Penkowa *et al.*, 2000b, Penkowa y Hidalgo, 2000a).

Tanto la deficiencia de IL-6 como su sobre-expresión comportan una alteración en la expresión de los factores de transcripción c-fos y c-jun, que, como ya se ha comentado, conforman AP-1. En este aspecto, c-fos ha sido señalado como un factor esencial en la supervivencia neuronal regulando la expresión de diferentes factores neurotróficos, entre ellos BDNF (Cui *et al.*, 1999, Zhang *et al.*, 2002). Hemos podido determinar como la expresión del gen de respuesta inmediata c-fos se encuentra reducida, acompañándose de una disminución en los niveles de BDNF, en los animales IL6KO, mientras que su expresión está aumentada en los animales GFAP-IL6, por lo que este podría ser uno de los primeros partícipes de la mayor supervivencia mediada por IL-6.

De entre los diferentes genes implicados en procesos apoptóticos identificados en nuestros resultados es de especial importancia, al haber sido descrita por primera vez su modulación por IL-6, el canal de cloro intracelular 4 (chloride intracellular channel 4,*Clic4*). Este gen codifica para un canal de cloro presente tanto en el citoplasma como en la mitocondria y puede ser regulado por p53 y TNF-α, estando implicado en la pérdida de potencial de membrana mitocondrial (Fernandez-Salas *et al.*, 2002). Por otro lado, se ha descrito recientemente que puede translocar al núcleo y acelerar la apoptosis inducida por p53 en respuesta al estrés (Suh *et al.*, 2004). La expresión de *Clic4* se encuentra incrementada y se mantiene durante más tiempo en los animales IL6KO mientras que en los animales GFAP-IL6 se encuentra disminuída, por lo que es un claro candidato a mediar las acciones anti-apoptóticas de IL-6 y por tanto confirmando el papel de la IL-6 en el control de procesos apoptóticos.

Por otra parte, de acuerdo con la bibliografía existente, la IL-6 afecta la expresión de genes relacionados con la generación o con el control del estrés oxidativo. El incremento de la expresión observado para factores como peroxiredoxin 5 o *Sh3bgrl*, ambos inducidos en respuesta a daño oxidativo (Mazzocco *et al.*, 2002, Wang *et al.*, 2003), en los animales IL6KO sugiere también un mayor daño, mientras que la reducida presencia de *Atf4*, factor implicado en la respuesta a estrés reticular (Harding et al., 2003) y el incremento del coactivador 1 de PPARγ (*Pgc1a*), implicado en la inducción de diferentes enzimas anti-oxidantes (St-Pierre et al., 2006) en los animales GFAP-IL6, refuerza la visión del papel regulador del estrés oxidativo por parte de la IL-6.

En cuanto a genes antioxidantes relacionados con la IL-6 debemos destacar los de las Metalotioneínas 1 y 2, que muestran reducción de su expresión en los animales IL6KO e

indución en los animales GFAP-IL6. Estas observaciones concuerdan con estudios anteriores en animales deficientes para IL-6 en los que la inducción de MTs está notablemente disminuida, mostrando el importante papel de esta citoquina en la inducción de las MTs tras lesión traumática (Penkowa *et al.*, 1999b, Penkowa *et al.*, 2000b), administración de LPS (Carrasco *et al.*, 1998) o administración de la gliotoxina 6-AN (Penkowa y Hidalgo, 2000a). Por el contrario, la sobreexpresión de MTs en los animales GFAP-IL6 se ha propuesto como un mecanismo neuroprotector en la criolesión (Penkowa *et al.*, 2003c) así como frente a la administración de 6-AN (Penkowa *et al.*, 2003b), donde se ha observado que la sobreexpresión MT produce un menor daño y muerte neuronal (Penkowa *et al.*, 2004). Los resultados sugieren a las metalotioneínas como uno de los importantes mecanismos de protección frente al estrés oxidativo promovidos por IL-6.

4. Implicación de las MTs en el la respuesta a lesión traumática en el sistema nervioso central

Múltiples laboratorios han caracterizado extensamente diversas acciones beneficiosas de las metalotioneínas en diferentes modelos de lesión (Chung y West, 2004, Hidalgo *et al.*, 2001, Hozumi *et al.*, 2004). Existen, sin embargo, múltiples incógnitas tanto en sus acciones como en los mecanismos mediante los cuales las llevan a cabo. En este sentido nuestros resultados con animales *knock-out* para las metalotioneínas 1 y 2 (MTKO) nos han permitido caracterizar de una manera global sus implicaciones, a nivel transcripcional, identificando nuevos mecanismos de acción en condiciones fisiológicas, así como describiendo los genes implicados en sus funciones en el control del estrés oxidativo y en la reducción de la apoptosis tras criolesión, con lo que se refuerzan así las evidencias previas que sugerían a las MTs como potenciales factores terapéuticos frente a diferentes patologías.

4.1. Los animales MTKO presentan una mayor predisposición a estrés oxidativo y muerte neuronal

Los ratones MTKO, generados hace algo más de 15 años por el laboratorio del Dr. Richard Palmiter (Masters *et al.*, 1994a), han permitido caracterizar extensamente las implicaciones de las metalotioneínas en el contexto de un organismo vivo. Una de las primeras observaciones realizadas con estos animales fue su aparente normalidad fenotípica, que descartaba una implicación esencial de estos factores durante el desarrollo o en la fisiología de los animales. Estas primeras conclusiones se han ido matizando a medida que se han ido caracterizando más exhaustivamente, habiéndose descrito alteraciones conductuales (Yoshida *et al.*, 2006), entre ellas déficits de aprendizaje (Levin *et al.*, 2006) en los ratones MTKO. En nuestros resultados hemos identificado, entre otros factores, una menor expresión de genes tales como la mono-oxidasa A o el receptor 4 de la serotonina, implicados ambos en un amplio rango de funciones en el SNC, incluyendo la modulación de los patrones conductuales (Cases *et al.*, 1995), por lo que podrían ser las primeras bases para explicar las diferencias conductuales observadas en los animales MTKO. Hemos identificado también genes implicados en procesos de desarrollo y plasticidad sináptica que se ven afectados por la deficiencia de MT-I y MT-II, tales como la serpina-12 (*4632419J12Rik*) (Molinari *et al.*, 2003). Cabe destacar, además, que hemos podido identificar numerosos genes implicados en la síntesis y metabolismo proteico cuya expresión se ve alterada por la deficiencia de MTs, si bien son demasiado numerosos y heterogéneos para discutirlos individualmente, sí que sugieren un papel complejo y amplio de las metalotioneínas en el control de la función celular normal.

Pese a que, como se ha comentado anteriormente, los animales MTKO se desarrollan en una aparente normalidad, muestran un claro fenotipo diferenciado al ser sometidos a situaciones de lesión o daño. Las características más destacables observadas en estos ratones además de una mayor sensibilidad al envenenamiento por cadmio (Masters *et al.*, 1994a), son: una respuesta inflamatoria alterada, un incremento del estrés oxidativo y un descenso de la supervivencia neuronal, habiendo sido descritas por nuestro grupo tanto tras la administración del epileptógeno ácido caínico (Carrasco *et al.*, 2000), como tras criolesión (Penkowa *et al.*, 1999a) o en la EAE (Penkowa *et al.*, 2001). Por otra parte, la deficiencia de MT I y II exacerba la neuroinflamación observada en los animales GFAP-IL6 (Giralt *et al.*, 2002a).

Otros laboratorios han llegado a las mismas conclusiones respecto al papel de las metalotioneínas en la respuesta frente a diversos tipos de daño (Chung *et al.*, 2004, Fitzgerald *et al.*, 2007, Potter *et al.*, 2007).

La sobre-expresión de MT-I en los ratones TgMT, generados también por el Dr. Richard Palmiter (1993), ha confirmado estas hipótesis, mostrándose protectora en la criolesión, tras administración de la gliotoxina 6-AN o de ácido caínico, así como disminuyendo la neuroinflamación y muerte neuronal presente en los animales GFAP-IL6 (Giralt *et al.*, 2002b, Penkowa *et al.*, 2003a, Penkowa *et al.*, 2004, Penkowa *et al.*, 2005).

En vista de la bibliografía existente, era de esperar que la deficiencia de MT-I y II alterara el patrón de expresión de genes relacionados con la respuesta inflamatoria. Uno de los cambios a nivel génico que más nos sorprendió fue la expresión de aquaporin 2 (*Aqp2*), que se encuentra reducida en los animales MTKO. En primer lugar, este es uno de los primeros trabajos donde se describe su expresión en SNC, habiendo confirmado además la presencia de la proteína mediante inmunohistoquímica. Las acuaporinas son una familia de canales de membrana que permiten el paso de agua (King *et al.*, 2004), con importantes funciones por lo tanto en el control del edema post-lesión (Gunnarson *et al.*, 2004). Habida cuenta de la comprometida respuesta que presentan los animales MTKO al daño, el incremento en el volumen de lesión y el retraso en la recuperación del tejido, podría ser que la menor expresión de acuaporina sea uno de los mecanismos implicados. Por otra parte, hemos descrito una menor expresión de netrina-1 tras criolesión en los animales MTKO. La netrina-1 es importante para el desarrollo del SNC, la formación de conos de crecimiento y de la regeneración tras lesión (Manitt y Kennedy, 2002), por lo que su menor expresión explicaría la menor presencia de células madres neurales en los animales MTKO, sugiriendo a su vez una implicación notable de las MTs en el proceso de neurogénesis y regeneración.

La deficiencia de MTs observamos que altera la inducción de genes en respuesta a la criolesión, entre otros diferentes miembros del complejo mayor de histocompatibilidad II, el antígeno CD34, expresado en células implicadas en la neurovascularización de tejido isquémico (Taguchi *et al.*, 2004), eotaxina (*Ccl11*), implicada en la atracción de eosinófilos, basófilos y linfocitos (Ogilvie *et al.*, 2004), lo que sugiere cuáles pueden ser los mecanismos implicados en la alteración en la respuesta inflamatoria, así como en la atracción anómala de leucocitos a la zona de la lesión en los animales MTKO.

Se ha descrito que se produce un incremento en los niveles de estrés oxidativo en los animales MTKO, en este sentido hemos detectado incrementos en los niveles de expresión del

gen de la proteína regulada por glucosa 78 (glucose-regulated protein 78, *Grp78*), factor sensible a estrés reticular (Misra *et al.*, 2002), así como una mayor expresión y producción de la catalasa 1, uno de los principales factores implicados en el control del estrés oxidativo (Ho *et al.*, 2004), así como del receptor de los productos finales de glicosilación avanzada (*receptor of advanced glycation endproducts* – RAGE-), implicado en la activación de microglia y macrófagos en respuesta a estrés oxidativo (Stuchbury y Munch, 2005), lo cual concuerda perfectamente con el fenotipo establecido para los animales MTKO tras lesión.

Por último, hemos observado que en los animales MTKO hay incrementos en la expresión de genes implicados en el control del ciclo celular y en la activación de vías apoptóticas. Entre estos genes podemos destacar *Ercc3*, que participa en la reparación del daño del DNA y que parece que está inducido por la vía apoptótica iniciada por p53 (Wang *et al.*, 1996). De esta manera los animales MTKO presentarían una predisposición hacia la activación de mecanismos apoptóticos, lo cual confirmaría y explicaría el papel de las MTs en la reducción de la muerte neuronal.

4.2. La administración exógena de MT-I y II tiene una función neuroprotectora no dependiente de receptor

El papel neuroprotector de las metalotioneínas I y II en diversas patologías ha sido ampliamente estudiado (cabe aclarar que generalmente se han observado las mismas acciones para MT-I y MT-II, por lo que se usan indistintamente en diferentes experimentos con los mismos resultados). La gran mayoría de los trabajos se han servido de animales modificados genéticamente, de tal forma que carezcan o sobre-expresen MTs, obteniendo una gran cantidad de resultados sobre las acciones de las MTs endógenas en respuesta a daño. Sin embargo, aún quedan muchas preguntas por responder, y una de las más controvertidas es la referente a la distribución celular de las metalotioneínas y si su acción es intra o extracelular, y en este sentido se han descrito mecanismos por los cuales podrían ser liberadas pese a no disponer de secuencias de secreción (Chung y West, 2004).

La administración de MTs exógenas ha permitido confirmar que poseen acciones extracelulares, al haberse observado, siempre que la barrera hematoencefálica se encuentre alterada, una mejora de la respuesta general frente a diferentes situaciones patológicas,

aunque el mecanismo de acción no ha sido completamente elucidado. Estos resultados abren la puerta a su posible utilización terapéutica como fármacos anti-inflamatorios y neuroprotectores, siendo de vital importancia establecer su mecanismo de acción.

En trabajos previos no se había caracterizado ningún posible receptor para las MTs, por lo que nos planteamos usar metalotioneínas de diferentes especies en el modelo de lesión traumática para poder evaluar posibles diferencias en su acción, dependiendo del grado de homología con la especie de estudio, ratón. Las diferencias nos hubieran permitido afirmar la existencia de una interacción proteína-proteína en el mecanismo de acción de las MTs. Por otra parte, optamos por usar proteínas recombinantes y compararlas con nuestro "patrón oro", la MT-II nativa de conejo, con la que se han publicado las acciones neuroprectoras de la MT-II (Penkowa y Hidalgo, 2000b).

El uso de MTs recombinantes nos ha permitido confirmar y extender los resultados obtenidos mediante el uso de animales modificados genéticamente, habiendo observado una disminución en la respuesta inflamatoria, el estrés oxidativo, la neurodegeneración y la muerte celular apoptótica inducida por la criolesión. Por otro lado, hemos comprobado que la MT de *Drosophila*, muy alejada estructural y filogenéticamente a la del ratón, ejerce una protección similar aunque algo menor a las propias de ratón, lo que permite descartar, a priori, una implicación proteína-proteína en la acción de las MTs.

Si se descarta la interacción proteína-proteína en la acción de las MT-I y II, la explicación más plausible es que bien el zinc coordinado a la proteína, o alguna otra propiedad química de las MTs estarían implicadas en sus acciones. La estructura de las MTs está caracterizada por dos *clusters* de grupos tiol coordinados con metales localizados en dos dominios proteícos. Estos grupos tiol son altamente reactivos y podrían ser los involucrados en la respuesta, ya que presentan una elevada reactividad frente a agentes oxidantes, contrarrestando radicales libres tales como hidroxilos, superóxido o óxido nítrico, lo cual conduce a su vez a una oxidación o modificación de las MTs (Romero-Isart y Vasak, 2002), que provoca la liberación de los átomos de zinc unidos, y éstos, a altas concentraciones locales pueden ejercer efectos neuroprotectores (Sensi y Jeng, 2004). Cabe destacar, sin embargo, que en un modelo de esclerosis múltiple en rata fue la administración de MT-II, pero no de Zinc, la que redujo los síntomas y la inflamación (Penkowa y Hidalgo, 2000b), aunque no se puede descartar que la función neuroprotectora del Zinc requiera de concentraciones locales más elevadas a las obtenidas mediante la administración sistémica.

4.3. La administración de MT-III resulta perjudicial tras lesión al inhibir la síntesis de factores neurotróficos

Si bien hemos caracterizado que el papel de las Metalotioneínas I y II en la disminución de la inflamación, del estrés oxidativo y de la muerte apoptótica las convierte en moléculas con un potencial terapéutico, nuestros resultados confirman que las MT-III desempeñan un papel totalmente distinto.

La administración de MT-III recombinante no tuvo ningún efecto en la respuesta inflamatoria pero indujo un descenso en los niveles de diferentes factores de crecimiento, neurotrofinas y factores neuroprotectores. El papel inhibidor de la MT-III sobre el crecimiento neuronal ya había sido sugerido desde su identificación, hecho por el cual se la denominó inicialmente factor inhibidor del crecimiento (*growth inhibitory factor* – GIF-) (Uchida *et al.*, 1991). De la misma manera, se ha descrito un mayor crecimiento neuronal en animales deficientes en MT-III (Carrasco *et al.*, 2003, Ceballos *et al.*, 2003) y el efecto inverso tras administración de este factor tras lesión (Chung y West, 2004).

La acción inhibidora de la MT-III debe ser consecuencia de los motivos $C_{(6)}$PCP y $T_{(5)}$CPCP existentes exclusivamente en su secuencia (Romero-Isart *et al.*, 2002), siendo esta diferencia probablemente la mediadora de la interacción de elevada afinidad observada entre la MT-III y la proteína G de transporte vesicular Rab3A (Kang *et al.*, 2001, Knipp *et al.*, 2005), aunque se han descrito otras posibles interacciones (Lahti *et al.*, 2005).

Por tanto, pese a que nuestro trabajo ha permitido demostrar las diferentes funciones de la MT-III respecto a MT-I y II en un modelo de lesión traumática, se está todavía lejos de descifrar totalmente los mecanismos implicados.

5. Conclusiones generales

En el presente trabajo nos hemos centrado en la caracterización del TNF-α, la IL-6 y las MTs en la respuesta inflamatoria en un modelo de lesión traumática, como es la criolesión. Para poder analizar la acción de cada uno de estos factores en primer lugar era esencial caracterizar el modelo de lesión en sí. Si bien la criolesión es un modelo utilizado y descrito en numerosas publicaciones, en este trabajo hemos caracterizado extensamente los cambios que induce en la dinámica de expresión génica.

En este sentido, hemos podido observar:

- El número considerable de genes que son alterados por la lesión, mayoritoriamente incrementando la expresión de genes implicados en el establecimiento de la respuesta inflamatoria y minoritariamente inhibiendo la expresión de genes implicados con la función neuronal.

En cuanto a la implicación del TNF-α en esta respuesta:

- Hemos descrito un papel negativo para este factor, aunque mediante diferentes mecanismos. La vía del receptor 1 (TNFR1) participa, primordialmente, en el establecimiento de la respuesta inflamatoria e incrementa la muerte celular.

- La unión al receptor 2 (TNFR2) modula la expresión de diferentes genes implicados en diversos mecanismos de función neuronal que resulta en un empeoramiento de la respuesta funcional de los animales sin afectar la respuesta inflamatoria.

Respecto al papel de la IL-6: .

- Hemos descrito que la IL-6 presenta un papel dual tras criolesión, habiendo identificado por un lado los genes que participan en la iniciación de la respuesta inflamatoria, la gliosis y la infiltración leucocitaria y por otra parte los implicados en la reducción del estrés oxidativo y la muerte celular, mostrándose por tanto como un

factor neuroprotector, probablemente por su acción como inductor de la expresión de diferentes factores neurotróficos y anti-oxidantes.

En este aspecto:

- Hemos descrito diferentes argumentos que relacionarían el papel protector descrito para la IL-6 con las metalotioneínas. En primer lugar, los ratones IL6KO y los ratones MTKO presentan un fenotipo parecido en respuesta a daño, con un incremento del estrés oxidativo y de la muerte neuronal, en segundo lugar, la IL-6 es uno de los principales inductores de MT-I y II y, por último, las acciones neuroprotectoras de la IL-6 se ven incrementadas con la sobre-expresión de MT-I mientras que su deficiencia exacerba el daño.

Es por tanto lógico pensar que:

- Las metalotioneínas I y II podrían ser importantes mediadores de los aspectos neuroprotectores de la IL-6 y en este sentido los resultados recogidos en este trabajo han identificado, en muchos casos por primera vez, numerosos genes que pueden participar en esta respuesta. Además, hemos podido demostrar las diferencias en la acción de MT-I y II respecto a MT-III, estando las primeras implicadas en la disminución de la respuesta inflamatoria mientras que la última tendría un papel inhibidor del crecimiento neuronal.

Por último:

- Hemos demostrado la utilidad de la administración de MTs recombinantes en la criolesión, abriendo las puertas a un posible uso terapéutico de estos factores como factores neuroprotectores en el futuro.

V. BIBLIOGRAFÍA

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VI. ANEXOS

Anexo 1.

Familias de las quimioquinas y receptores.
Extraído de **Charo y Ransohoff, 2006**.

Table 1. CC Family of Chemokines and Chemokine Receptors.*

Receptor	Chemokine Ligands	Cell Types	Disease Connection
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC1)	T cells, monocytes, eosinophils, basophils	Rheumatoid arthritis, multiple sclerosis
CCR2	CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), CCL16 (HCC4)	Monocytes, dendritic cells (immature), memory T cells	Atherosclerosis, rheumatoid arthritis, multiple sclerosis, resistance to intracellular pathogens, type 2 diabetes mellitus
CCR3	CCL11 (eotaxin), CCL13 (eotaxin-2), CCL7 (MCP-3), CCL5 (RANTES), CCL8 (MCP-2), CCL13 (MCP-4)	Eosinophils, basophils, mast cells, Th2, platelets	Allergic asthma and rhinitis
CCR4	CCL17 (TARC), CCL22 (MDC)	T cells (Th2), dendritic cells (mature), basophils, macrophages, platelets	Parasitic infection, graft rejection, T-cell homing to skin
CCR5	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CCL14 (HCC1), CCL16 (HCC4)	T cells, monocytes	HIV-1 coreceptor (T-tropic strains), transplant rejection
CCR6	CCL20 (MIP-3β, LARC)	T cells (T regulatory and memory), B cells, dendritic cells	Mucosal humoral immunity, allergic asthma, intestinal T-cell homing
CCR7	CCL19 (ELC), CCL21 (SLC)	T cells, dendritic cells (mature)	Transport of T cells and dendritic cells to lymph node, antigen presentation, and cellular immunity
CCR8	CCL1 (I309)	T cells (Th2), monocytes, dendritic cells	Dendritic-cell migration to lymph node, type 2 cellular immunity, granuloma formation
CCR9	CCL25 (TECK)	T cells, IgA+ plasma cells	Homing of T cells and IgA+ plasma cells to the intestine, inflammatory bowel disease
CCR10	CCL27 (CTACK), CCL28 (MEC)	T cells	T-cell homing to intestine and skin

* MIP denotes macrophage inflammatory protein, MCP monocyte chemoattractant protein, HCC hemofiltrate chemokine, Th2 type 2 helper T cells, TARC thymus and activation-regulated chemokine, MDC macrophage-derived chemokine, LARC liver and activation-regulated chemokine, ELC Epstein–Barr I1-ligand chemokine, SLC secondary lymphoid-tissue chemokine, TECK thymus-expressed chemokine, CTACK cutaneous T-cell–attracting chemokine, and MEC mammary-enriched chemokine.

Table 2. CXC, CX_3C, and XC Families of Chemokines and Chemokine Receptors.*

Receptor	Chemokine Ligands	Cell Types	Disease Connection
CXCR1	CXCL8 (interleukin-8), CXCL6 (GCP2)	Neutrophils, monocytes	Inflammatory lung disease, COPD
CXCR2	CXCL8, CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6	Neutrophils, monocytes, microvascular endothelial cells	Inflammatory lung disease, COPD, angiogenic for tumor growth
CXCR3-A	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	Type 1 helper cells, mast cells, mesangial cells	Inflammatory skin disease, multiple sclerosis, transplant rejection
CXCR3-B	CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	Microvascular endothelial cells, neoplastic cells	Angiostatic for tumor growth
CXCR4	CXCL12 (SDF-1)	Widely expressed	HIV-1 coreceptor (T-cell–tropic), tumor metastases, hematopoiesis
CXCR5	CXCL13 (BCA-1)	B cells, follicular helper T cells	Formation of B-cell follicles
CXCR6	CXCL16 (SR-PSOX)	CD8+ T cells, natural killer cells, and memory CD4+ T cells	Inflammatory liver disease, atherosclerosis (CXCL16)
CX_3CR1	CX3CL1 (fractalkine)	Macrophages, endothelial cells, smooth-muscle cells	Atherosclerosis
XCR1	XCL1 (lymphotactin), XCL2	T cells, natural killer cells	Rheumatoid arthritis, IgA nephropathy, tumor response

* GCP denotes granulocyte chemotactic protein, COPD chronic obstructive pulmonary disease, GRO growth-regulated oncogene, ENA epithelial-cell–derived neutrophil-activating peptide, MIG monokine induced by interferon-γ, IP-10 interferon-inducible protein 10, I-TAC interferon-inducible T-cell alpha chemoattractant, PF platelet factor, SDF stromal-cell–derived factor, HIV human immunodeficiency virus, BCA-1 B-cell chemoattractant 1, and SR-PSOX scavenger receptor for phosphatidylserine-containing oxidized lipids.