Trabajo 5

Interleukin-6 and tumor necrosis factor-α type 1 receptor deficient mice reveal a role of IL-6 and TNF-α on brain metallothionein-I and –III regulation

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Research report

Interleukin-6 and tumor necrosis factor-α type 1 receptor deficient mice reveal a role of IL-6 and TNF-α on brain metallothionein-I and -III regulation

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Abstract

Metallothioneins (MTs) are a family of low molecular weight proteins which in rodents is comprised of several isoforms (MT-I to MT-IV). MT-I and MT-II are widely expressed isoforms, whereas MT-III is mainly expressed in the central nervous system and is the only isoform that inhibits survival and neurite formation of rat cortical neurons in vitro. However, the physiological roles and regulation of these proteins in the brain are poorly characterized. In this report we have studied the putative role of IL-6 and TNF-α on the regulation of brain MT-I and MT-III, by using mice carrying a null mutation in the IL-6 or the TNF-α type 1 receptor genes or both. In situ hybridization analysis revealed that brain MT-I induction by bacterial lipopolysaccharide (LPS) was significantly lower in IL-6- and TNFR1-deficient mice, and to a greater extent in the double mutant mice, in most brain areas studied. These results suggest that the MT-I isoform could be considered an acute-phase protein in the brain, which is consistent with previous studies in transgenic mice overexpressing IL-6 in astrocytes. In contrast to LPS, brain MT-I induction by restraint stress was not affected significantly by IL-6 or TNFR1 deficiencies, suggesting that these cytokines are not important during the stress response in the brain. In basal conditions, it was also observed that the double mutant mice had diminished MT-I mRNA levels in several brain areas. In contrast to MT-I, MT-III mRNA levels were minimally affected by either LPS or stress. Yet, significant decreasing effects of IL-6 and TNFR1 deficiencies were observed in the Purkinje neuronal layer of the cerebellum (after LPS) and ependymal cells (after LPS and stress). In contrast, significant increasing effects, especially of TNFR1 deficiency, were observed in CA1 hippocampal area, retrosplenial and parietal cortex, and in thalamic nuclei (after LPS). These results demonstrate that IL-6 and TNF-α are involved in brain MTs regulation during LPS-elicited inflammatory response but not during the stress response.

Keywords: IL-6; TNF; Metallothionein-I, -II and -III; Stress; Endotoxin; Turpentine

1. Introduction

Metallothioneins (MTs) are a family of small, cysteine-rich, metal-binding proteins [28]. There are several isoforms in mammals; MT-I and MT-II are expressed virtually in all tissues, whereas MT-III (or growth inhibitory factor) and MT-IV are two tissue specific isoforms which are mainly localized in the brain [37,49] and stratified squamous epithelia [40], respectively. The biochemical and molecular properties of the MT-I and MT-II isoforms are well documented, but their physiological role and regulation in the brain in physiological conditions are poorly characterized; the scarcity of information in this regard for MT-III is even more dramatic [25]. Uchida et al. [49] discovered MT-III unexpectedly as a protein markedly decreased in human brains with Alzheimer disease (AD). In vitro, the protein had the capability to inhibit survival and neurite formation of cortical neurons. Yuguchi et al. [55] later suggested that such an inhibiting role of MT-III could be relevant after neuronal injury in vivo. Further studies, however, have challenged not only the involve-

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ment of MT-III in AD [16] but also the view of this protein as an inhibitory factor for neurons in vivo [15,47]. Several studies have in addition demonstrated that MT-III is far less responsive to metals and other agents than the MT-I and MT-II isoforms [11,37,57]. Thus, more studies are needed to clarify the physiological regulation of brain MT isoforms.

It is known that the exogenous administration of a number of cytokines including interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) increases MT-I + II expression in different tissues [5,10,12,34,44] and cell lines [4,17,29,45,51]. Furthermore, transgenic mice overexpressing IL-6 in astrocytes also showed a marked upregulation of these MT isoforms [22]. However, whether or not IL-6 and TNF-α would be involved in brain MT regulation in physiological conditions is unknown.

IL-6 is a major mediator of the immune and inflammatory responses in the organism. Originally, IL-6 was named 'hepatocyte-stimulating factor' because of its induction of the acute-phase response in the liver [1,20]. IL-6 is also involved in the B-cell differentiation, the activation of T-cells and the promotion of hematopoiesis [50]. Furthermore, IL-6 has specific effects in the nervous system, stimulating the activity of the hypothalamus–adrenocortical axis, inducing fever or causing neuronal differentiation [35,39,42]. It has also been demonstrated that transgenic mice expressing IL-6 under the control of the glial fibrillary acidic protein gene promoter develop a chronic progressive neurodegenerative disease characterized by neurodegeneration, astrocytosis, microgliosis, angiogenesis, and induction of acute-phase protein synthesis [9]. It is therefore clear that IL-6 has major functions in both the liver and the brain. The cytokine tumor necrosis factor α (TNF-α) is also a functionally pleiotropic hormone [7,8] that shares a number of physiological functions with IL-6, including the liver acute-phase response. IL-6 and TNF-α are released not only after inflammatory stimuli but also after stressful stimuli [3,32,52,54,58]. Therefore, taken together the previous studies suggest that both IL-6 and TNF-α could have a role on brain MT regulation. In this report we have undertaken studies employing knock-out mice carrying a null mutation in the IL-6 gene [30] and/or the TNF-α type 1 receptor gene [41] which demonstrate that both cytokines are major physiological regulators of the expression of brain MT isoforms during the LPS-elicited inflammatory response but not during the stress response.

2. Materials and methods

2.1. Production of IL-6 and TNFR1 deficient mice

Generation and development of the IL-6 deficient mice (IL-$6^{-/-}$) and TNF-α type 1 receptor was as described previously [30,41]. We used as controls the wild-type strains, C57BL/6 (Jackson, Germany) and the F_1 mice C57BL/6 × 129/Sv (provided by Biological Research Lab., Basel, Switzerland).

2.2. Maintenance of the animals

The animals were maintained under standard laboratory conditions (light cycle from 07:30 to 19:30, temperature 22°C, food and water provided ad libitum) for at least one week before starting the experiments.

2.3. Experiment 1. Effect of restraint stress on brain MT-I and MT-III expression of IL-$6^{-/-}$ mice

In this first experiment we studied the widely expressed MT-I isoform and also the brain-specific isoform, MT-III, in several brain areas by using in situ hybridization experiments. To this end, some animals were immobilized for 4–5 h by wrapping them in a metallic net and then killed along *with* unstressed mice and brain MT-I and MT-III mRNAs were assayed as described below. We used C57BL/6 mice as IL-$6^{+/+}$ controls.

2.4. Experiment 2. Effect of restraint stress and lipopolysaccharide on brain MT-I and MT-III expression in IL-$6^{-/-}$ and/or TNFR1$^{-/-}$ mice

The previous experiment revealed only minor effects of IL-6 deficiency on brain MTs expression. However, since IL-6 has overlapping functions with other cytokines but especially TNF-α [8], and both are known to induce MT-I + II synthesis (see above), it was possible that brain MTs response to stress was not significantly affected by IL-6 deficiency because of a compensatory effect of TNF-α. Therefore, we carried out this experiment where brain MT response to stress was evaluated in IL-$6^{-/-}$, TNFR1$^{-/-}$ and IL-$6^{-/-}$ × TNFR1$^{-/-}$ mice. The animals were again stressed for 4–5 h and then killed along basal animals. In this experiment, we also evaluated brain MTs response to lipopolysaccharide (0.1 mg/kg, s.c.) in the three mutant mice strains, which were killed 4–5 h after the injection. As controls, we used C57BL/6 and C57BL/6 × 129/Sv mice.

2.5. Experiment 3. Effect of turpentine

Turpentine is a toxic agent that elicits an inflammatory response where the cellular mediators released differ from those released after lipopolysaccharide, and, more specifically, IL-6 is not released by turpentine [46]. Therefore, we carried out an experiment where IL-6 deficient mice were injected with turpentine (50 μl, s.c.) and killed 4–5 h later along uninjected mice. Controls were C57BL/6 mice.

2.6. In situ hybridization

Immediately after the animals were killed, their brains were removed, frozen in liquid nitrogen and stored at −80°C. Serial coronal sections (20 μm in thickness) were obtained from the frozen brains with a cryostat and mounted on slides coated with poly-L-lysine, which were then maintained at −80°C until the day of analysis. We have analyzed the MT-I and MT-III isoforms. Since MT-I and MT-II are coordinately regulated [53], we assume that the results described for MT-I are representative of the MT-I + II isoforms.

In experiment 1, sections were prepared from several representative brain areas. For MT-I mRNA studies: (i) sections containing the hypothalamic arcuate nucleus were used for determining the MT-I expression in the hippocampal layers, habenula, cortex and hypothalamus, and (ii) in cerebellar sections MT-I expression was determined in the Purkinje cell layer, raphe nuclei and white matter. For MT-III mRNA studies the following sections were prepared: (i) sections containing the arcuate nucleus, where MT-III expression was determined in pyramidal neurons of CA1 and CA2 and in lacunosum moleculare of hippocampus, and in retrosplenial cortex; (ii) in sections obtained at the end of the pineal recess of the third ventricle, MT-III expression was studied in CA1, CA3, dentate gyrus and lacunosum moleculare of hippocampus, and in retrosplenial cortex; (iii) in sections starting at the beginning of the supraquiasmatic nuclei, MT-III mRNA was determined in globus pallidus and piriform and parietal cortex; and (iv) we finally determined MT-III expression in sections which included the cerebellar cortex and white matter, and the raphe nuclei. In Experiments 2 and 3, we studied MT-I and MT-III mRNA levels in sections containing the hypothalamic arcuate nucleus and in cerebellar sections.

For MT-I mRNA studies, we used the mouse cDNA generously provided by Dr. R.D. Palmiter (University of Washington, Seattle, WA). For MT-III mRNA studies, and in order to avoid cross-hybridization with MT-I and MT-II mRNAs, we have used a specific DNA fragment of 153 bp that contains the coding region for the terminal 15 amino acids and the 3′ untranslated region until the poly G stretch of MT-III mRNA (generously provided by Dr. G.K. Andrews, Dept. Biochemistry, Kansas City, KS, USA). Both the MT-I and MT-III cDNAs were labelled with [^{35}S] α-UTP using a SP6/T7 transcription kit (Boehringer

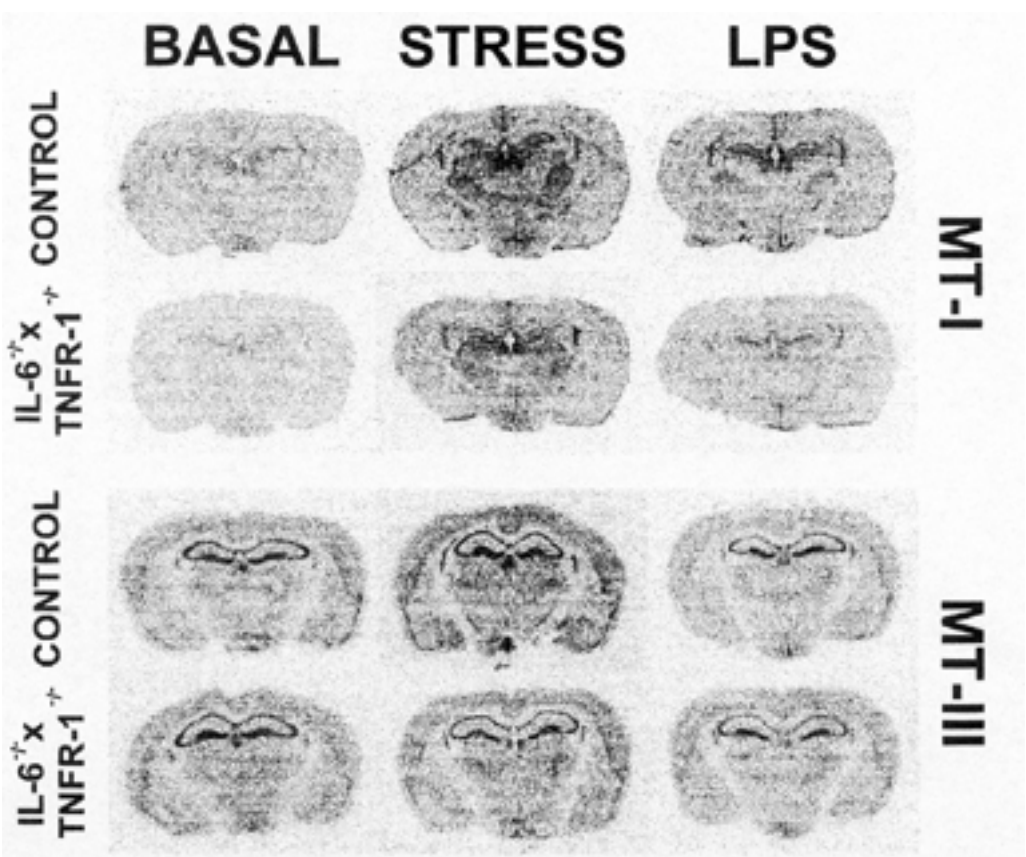

Fig. 1. Representative in situ hybridization analysis of the effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-I and MT-III expression in coronal sections of the cerebrum. Significant effects on MT-I expression was observed in stressed and endotoxin-injected mice. IL-6 and/or TNF-α functional deficiencies affected significantly MT-I response to LPS, and to a much lower extent, to stress. In contrast, MT-III expression was only modestly affected. Several animals per group were studied and the mRNA levels quantitated in specific brain areas, which are shown in Figs. 3 and 4.

Mannheim, Mannheim, Germany). In brief, the DNA was linearized by digestion with restriction endonucleases (*Hin*dIII to antisense probes and *Xho*I to sense probes). Six μl of the linearized DNA (0.5 μg/μl) was incubated with 2 μl of transcription buffer, 2 μl of 100 mM dithiotreitol, 0.8 μl of RNase inhibitor, 1 μl of 10 mM ATP, CTP and GTP, 2.4 μl of 0.1 mM cold UTP, 2 μl of [^{35}S] α-UTP (20 μCi/μl) (Amersham), 0.8 μl of RNase free H_2O and 1 μl of RNA polymerase (T7 RNA polymerase for antisense probes, SP6 RNA polymerase for sense probes), at 37°C for 30 min. After the transcription process, the DNA was digested by adding 1 μl of DNase and incubating for 30 min at 37°C. After DNAse treatment, the RNA was extracted with phenol and phenol:chloroform:isoamyl alcohol (25:24:1). The upper phase (200 μl) was recovered and incubated overnight with 1 μl of tRNA (10 mg/ml), 10 μl of 3M sodium acetate and 500 μl of 100% ethanol to precipitate the RNA. After centrifugation the precipitated probe was dissolved in 50 μl of RNAse free H_2O. In situ hybridization was performed using procedures described by Yuguchi et al. [55] with some modifications: the sections were incubated with 0.1 N HCl instead of proteinase K, and we used RNAse at 10 μg/ml instead of 1 μg/ml to digest the free probe. The concentration of probe used was approximately 1×10^6 dpm/90 μl/slide. Autoradiography was performed exposing the film (hyperfilm-MP, Amersham) to the slides for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were semiquantitatively determined in four sections per brain area and animal, by measuring the optical densities and the

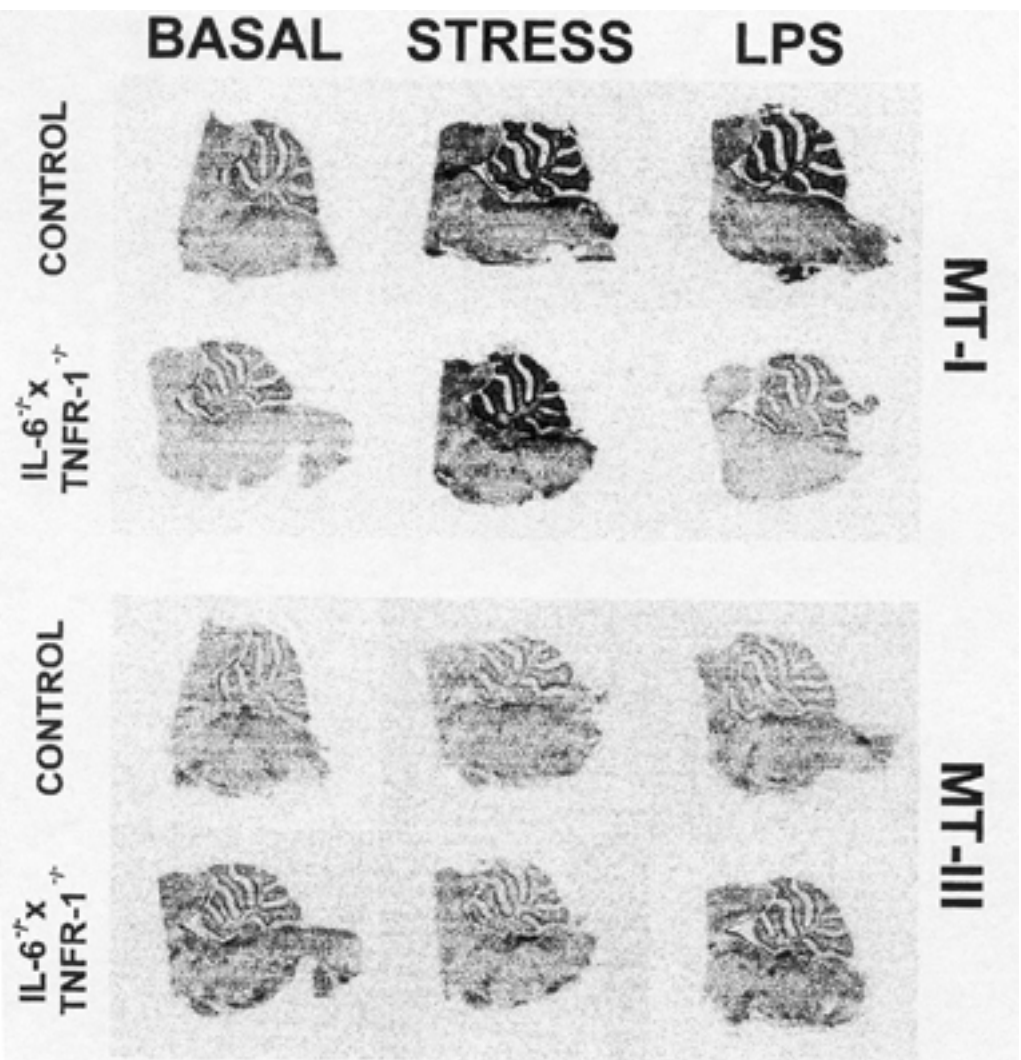

Fig. 2. Representative in situ hybridization analysis of the effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-I and MT-III expression in sagittal sections of the cerebellum and brain stem. Significant effects on MT-I expression was observed in stressed and endotoxin-injected mice. IL-6 and/or TNF-α functional deficiencies affected significantly MT-I response to LPS, but not to stress. MT-III expression was also modestly affected. Several animals per group were studied and the mRNA levels quantitated in specific brain areas, which are shown in Figs. 5 and 6.

number of pixels in defined areas with a Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels × optic density).

2.7. Statistical assays

Results were analyzed with Two-way and/or One-way ANOVA followed by SNK multiple comparisons of the means.

3. Results

3.1. Experiment 1

Figs. 1 and 2 show representative in situ hybridization results in sections from the cerebrum (coronal sections containing the hypothalamic arcuate nucleus) and the cerebellum, respectively. It is clear that the sense probes produced a very weak signal compared with the antisense MT-I and MT-III probes. Also, the pattern of hybridization

Table 1
In situ hybridization of MT-I mRNA in brain of control and IL-6$^{-/-}$ mice in basal and stress situations

Areas	Film	MT-I			
		C57BL/6		IL-6$^{-/-}$	
		Basal	Stress	Basal	Stress
CA1 (Pyramidal neurons)	1	11.7 ± 1.2	61.3 ± 11.5*	43.6 ± 14.7▲	120.4 ± 15.4*▲
CA2 (Pyramidal neurons)	1	120.8 ± 23.4	187.8 ± 38.0	145.6 ± 37.9	197.4 ± 41.6
CA1 (Lacunosum moleculare)	1	83.0 ± 27.0	379.8 ± 61.6*	125.0 ± 27.3	352.0 ± 39.1*
Dentate gyrus	1	96.6 ± 22.7	553.8 ± 70.9*	145.8 ± 16.6	510.6 ± 39.3*
Habenula	1	213.2 ± 16.9	688.4 ± 80.3*	172.0 ± 49.6▲	570.6 ± 17.8*▲
Choroid plexus	1	421 ± 48.4	893.4 ± 50.1*	455.2 ± 13.7	839.8 ± 41.1*
Retrosplenial cortex	1	45 ± 19.5	274.2 ± 70.1*	94.4 ± 26.6	310.8 ± 52.6*
Parietal cortex	1	83.6 ± 13.3	282.8 ± 20.7*	143.4 ± 36.1	257.4 ± 36.8*
Hypothalamus	1	138.0 ± 50.6	337.0 ± 91.6*	122.2 ± 7.6	335.8 ± 62.6*
Cerebellar cortex	2	254.7 ± 41.4	1531.6 ± 101.0*	158.3 ± 48.6	1579.4 ± 338.0*
Raphe nucleus	2	24.7 ± 4.0	44.4 ± 9.5*	29.9 ± 6.0	68.5 ± 19.1*

C57BL/6 and IL-6$^{-/-}$ mice were killed in unstressed (basal) or stressed (stress, 4 h of immobilization) conditions. Coronal sections (4 per area and animal) were prepared containing the hypothalamic arcuate nucleus, processed simultaneously and exposed to the same autoradiographic film (film 1). Other sections were prepared for the cerebellum which were processed separately and exposed to a different film (film 2). MT-I signal was semiquantitatively determined by measuring the optical densities and the number of pixels in defined areas of the stated brain regions. Results are mean ± S.E. ($n = 3$–5); they were analyzed with Two-way MANOVA with strain and stress as main factors. * $p < 0.05$ vs. basal mice, ▲ $p < 0.05$ vs. C57BL/6 strain.

Table 2
In situ hybridization of MT-III mRNA in brain of control and IL-6$^{-/-}$ mice in basal and stress situations

Areas	Film	MT-III			
		C57BL/6		IL-6$^{-/-}$	
		Basal	Stress	Basal	Stress
CA1	1	483.0 ± 68.8	557.2 ± 62.2	670.2 ± 36.6▲	683.3 ± 45.7▲
CA2	1	800.1 ± 101.7	862.3 ± 102.4	1064.0 ± 31.1	923.0 ± 29.5
Lacunosum moleculare	1	410.9 ± 36.8	403.8 ± 37.2	440.9 ± 22.8	469.1 ± 24.5
Retrosplenial cortex	1	434.1 ± 69.2	571.5 ± 75.4	599.1 ± 84.9	650.2 ± 21.6
CA3	2	325.6 ± 31.6	372.2 ± 138.7	369.9 ± 30.6	356.1 ± 16.8
Dentate gyrus	2	298.1 ± 30.1▼	230.3 ± 23.4▼	213.0 ± 13.3▼	315.7 ± 27.9▼
Lacunosum moleculare	2	117.5 ± 8.7▼	83.7 ± 10.4▼	71.1 ± 5.6▼	128.6 ± 13.8▼
Parietal cortex	3	553.4 ± 170.1	470.0 ± 90.8	408.8 ± 27.8	228.7 ± 52.2
Piriform cortex	3	436.4 ± 163.3	421.3 ± 76.5	478.4 ± 45.2	314.3 ± 42.9
Globus pallidus	3	381.9 ± 147.6	425.0 ± 103.4	516.8 ± 46.5	354.3 ± 71.7
Cerebellar cortex	4	313.8 ± 46.8	440.2 ± 44.9*	271.1 ± 21.5	385.4 ± 48.5*
Raphe nucleus	4	153.9 ± 16.8	170.3 ± 11.5	140.0 ± 20.9	164.8 ± 38.5

C57BL/6 and IL-6$^{-/-}$ mice were killed in unstressed (basal) or stressed (stress, 4 h of immobilization) conditions. Coronal sections (4 per area and animal) were prepared containing the hypothalamic arcuate nucleus (autoradiographic film 1), the end of pineal recess of the third ventricle (film 2), the beginning of the supraquiasmatic nuclei (film 3), and cerebellar tissue (film 4). All sections to be compared were processed simultaneously and exposed to the same film. MT-III signal was measured similarly to MT-I. Results are mean ± S.E. $n = 3$–5. * $p < 0.05$ vs. basal, ▲ $p < 0.05$ vs. C57BL/6 strain. ▼ $p < 0.05$ indicates interaction between strains and stress, presumably because IL-6 deficiency dramatically changed both the basal MT-III expression and its response to stress compared to the C57BL/6 mice. If analyzed separately from control mice, stress significantly decreased MT-III expression in pyramidal CA2 neurons and in piriform and parietal cortex in IL-6 deficient mice.

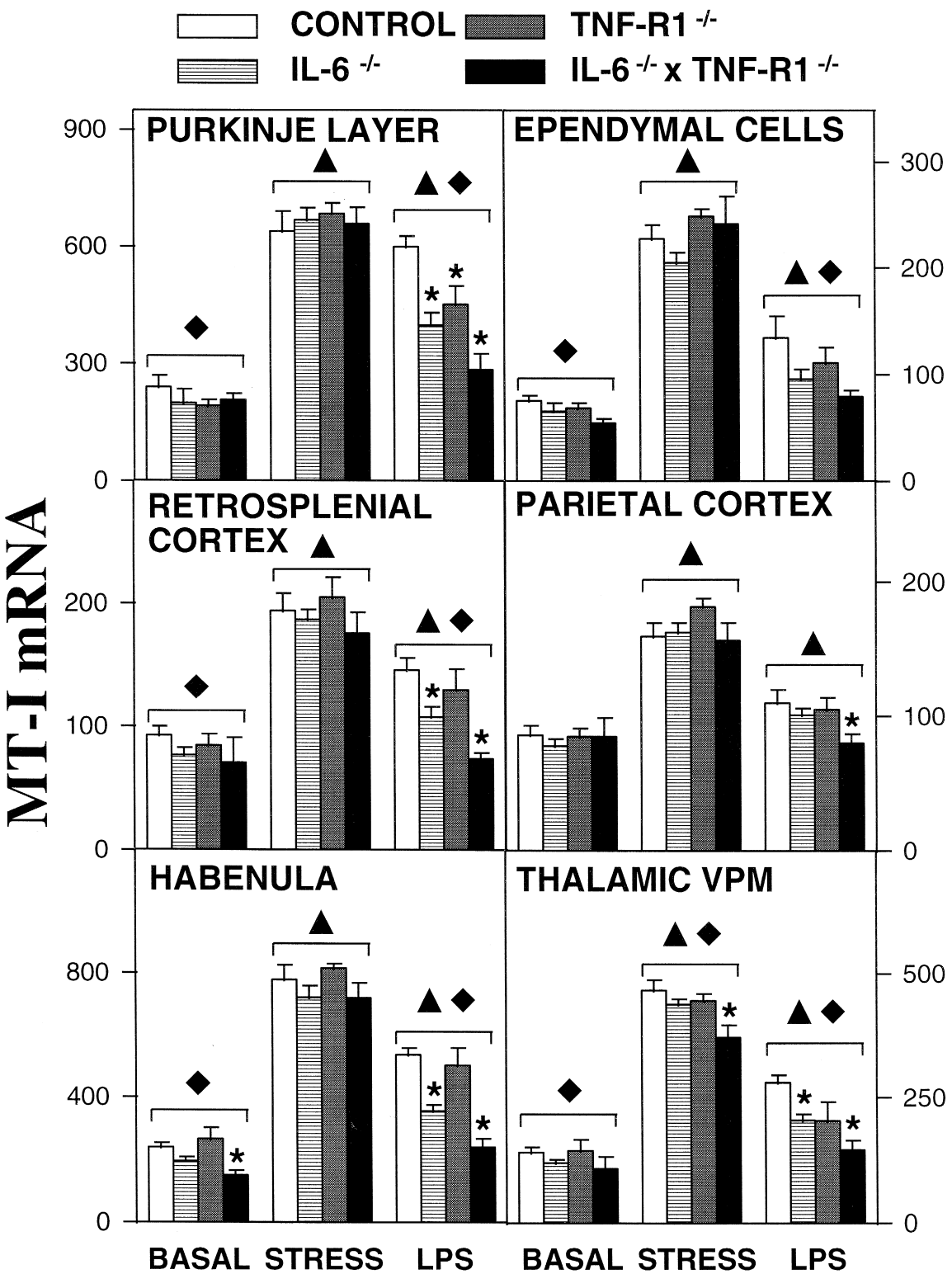

Fig. 3. Effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-I mRNA levels in specific brain areas as determined by in situ hybridization analysis (see Figs. 1 and 2). All animals to be statistically compared were processed simultaneously and exposed to the same autoradiographic film. Results are mean ± S.E. ($n = 5–12$). Data were evaluated with Two-way ANOVA with strain and stress, or strain and LPS, as main factors. When the Two-way ANOVA was significant, One-way ANOVA followed by post-hoc comparisons of the means (SNK) for the basal, stress, or LPS conditions were carried out. Two-way ANOVA revealed the following: ▲ $p < 0.05$ vs. basal mice, and (♦) overall significant ($p < 0.05$) effect of the null mutations. * $p > 0.05$ vs. respective control mice.

of the two MT probes were in clear agreement with previously published results in mice [36]. Thus, we observed the known major differences in the expression of these two MT isoforms in unstressed mice, namely the intense hybridization of the MT-III probe to pyramidal neurons of the hippocampus in the CA1–CA3 regions as well as granule cells of the dentate gyrus, whereas MT-I expression was conspicuously absent in all but the CA3

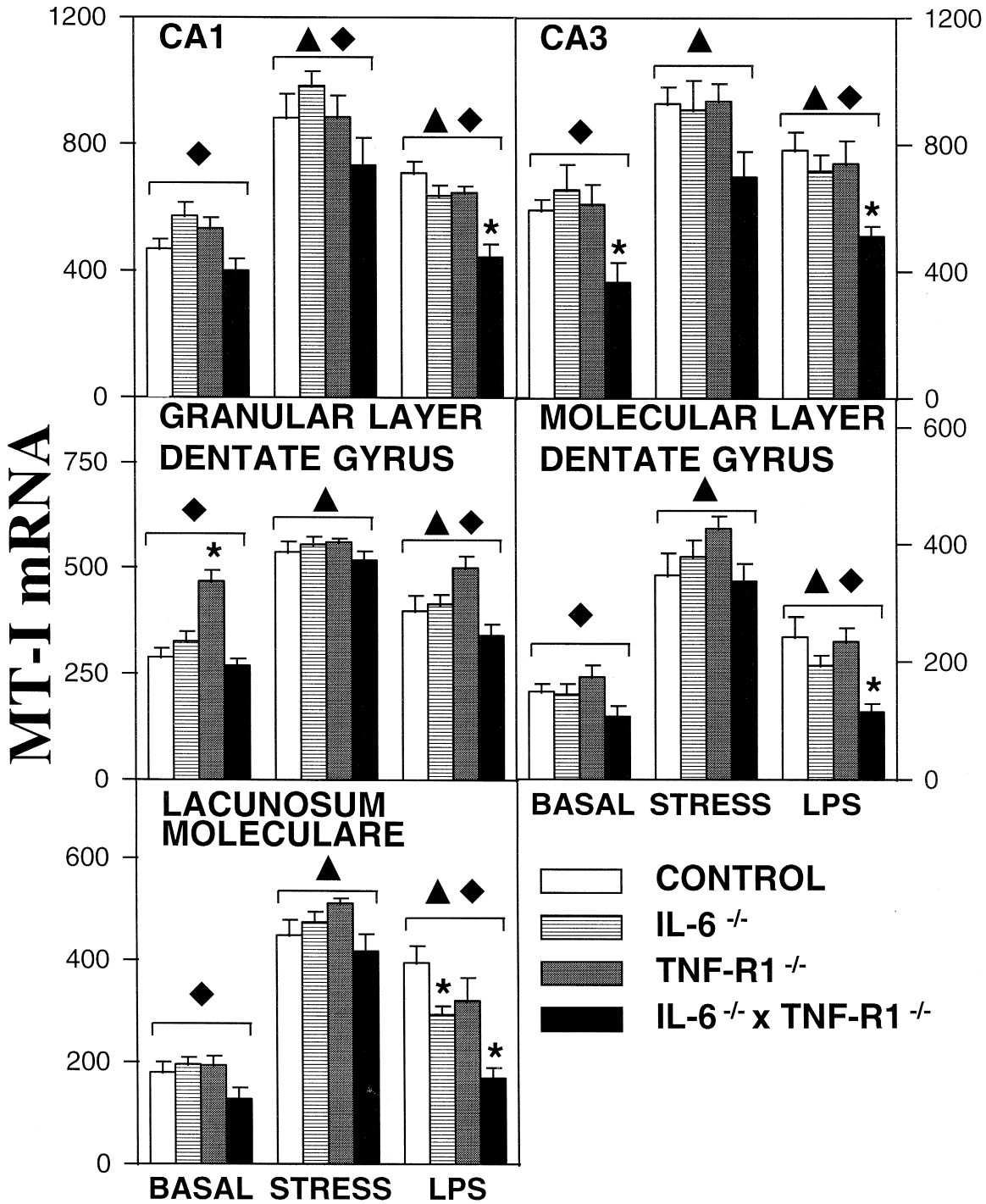

Fig. 4. Effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-I mRNA levels in specific hippocampal areas as determined by in situ hybridization analysis (see Fig. 1). All animals to be statistically compared were processed simultaneously and exposed to the same autoradiographic film. Results are mean ± S.E. ($n = 5–12$). Data were evaluated with Two-way ANOVA with strain and stress, or strain and LPS, as main factors. When the Two-way ANOVA was significant, One-way ANOVA followed by post-hoc comparisons of the means (SNK) for the basal, stress, or LPS conditions were carried out. Two-way ANOVA revealed the following: ▲ $p < 0.05$ vs. basal mice, and (◆) overall significant ($p < 0.05$) effect of the null mutations. * $p > 0.05$ vs. respective control mice.

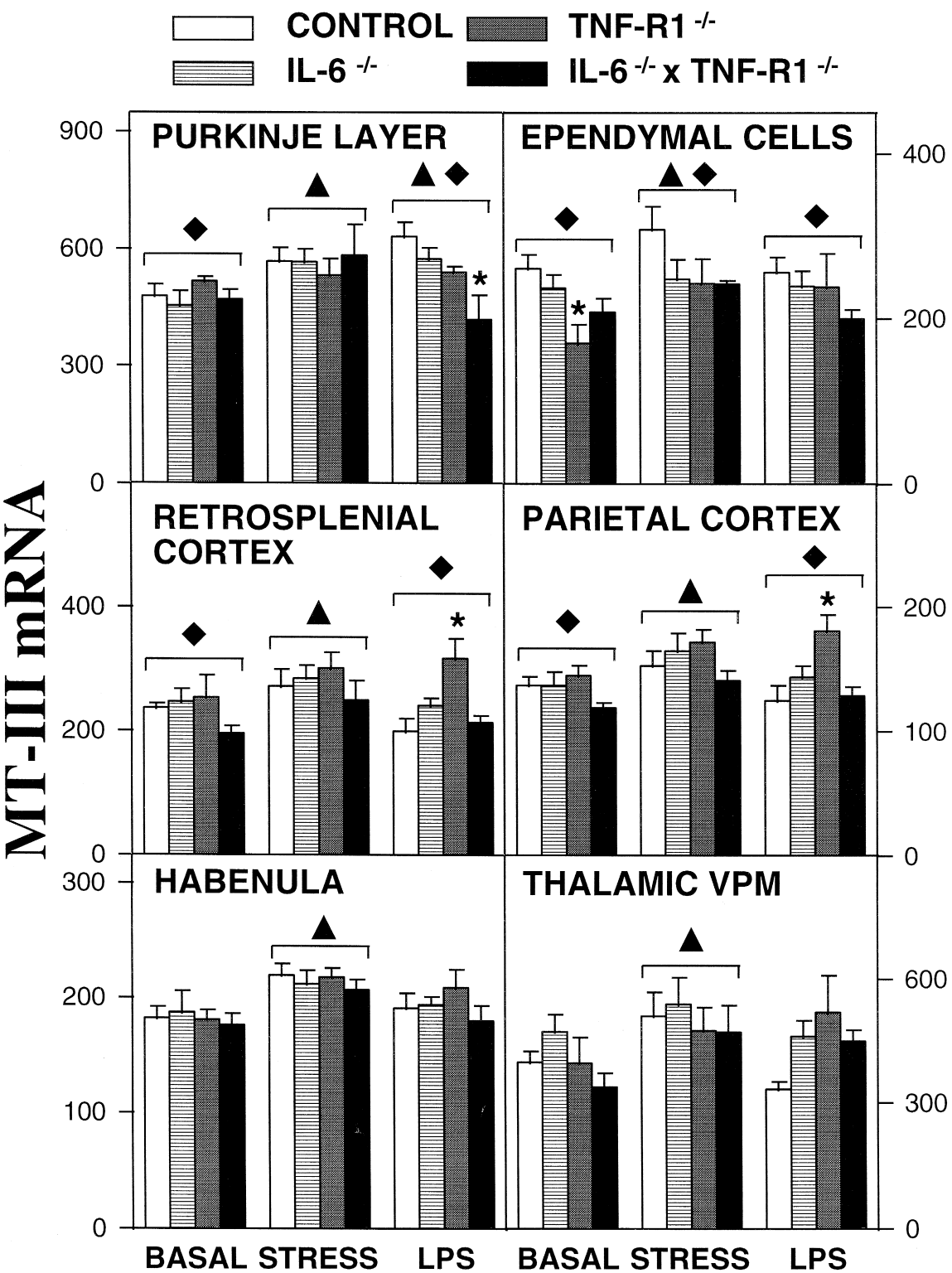

Fig. 5. Effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-III mRNA levels in specific brain areas as determined by in situ hybridization analysis (see Figs. 1 and 2). All animals to be statistically compared were processed simultaneously and exposed to the same autoradiographic film. Results are mean ± S.E. ($n = 5–12$). Data were evaluated with Two-way ANOVA with strain and stress, or strain and LPS, as main factors. When the Two-way ANOVA was significant, One-way ANOVA followed by post-hoc comparisons of the means (SNK) for the basal, stress, or LPS conditions were carried out. Two-way ANOVA revealed the following: ▲ $p < 0.05$ vs. basal mice, and (♦) overall significant ($p < 0.05$) effect of the null mutations. * $p > 0.05$ vs. respective control mice.

neurons but was prominent in the lacunosum moleculare, a structure abundantly populated of astrocytes. In the remaining brain areas, the MT-I and MT-III antisense probes produced comparable signals in unstressed mice, with clear hybridization throughout the neocortex (with significantly lower levels in layer IV than in the remaining layers), thalamus, hypothalamus, brainstem and cerebellar cortex (especially the Purkinje cell layer). A prominent MT-III signal was also observed in the choroid plexus and ependymal cells, and of MT-I in the later.

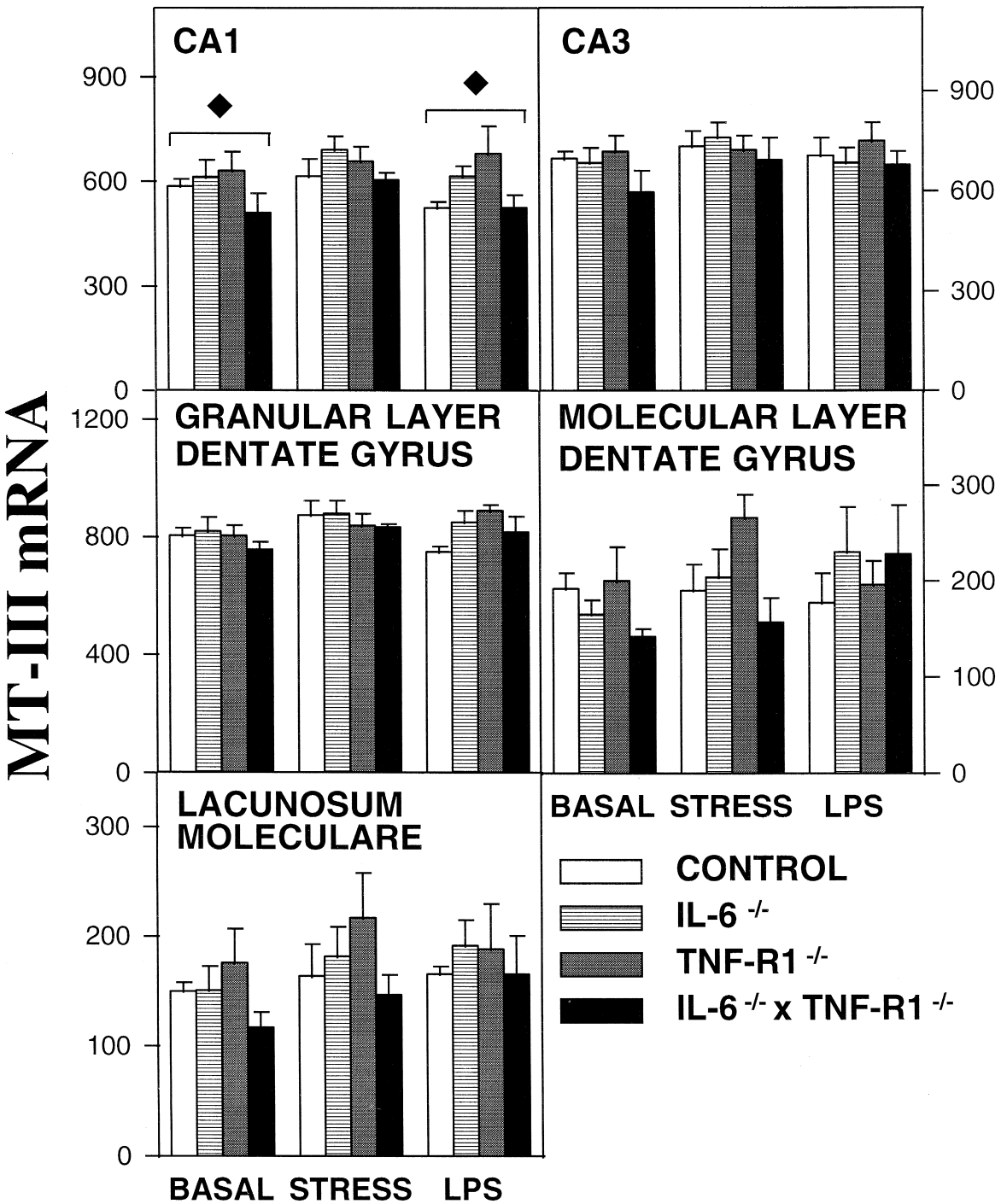

Fig. 6. Effect of immobilization stress (STRESS) and lipopolysaccharide (LPS) on MT-III levels in specific hippocampal areas as determined by in situ hybridization analysis (see Fig. 1). All animals to be statistically compared were processed simultaneously and exposed to the same autoradiographic film. Results are mean ± S.E. ($n = 5–12$). Data were evaluated with Two-way ANOVA with strain and stress, or strain and LPS, as main factors. When the Two-way ANOVA was significant, One-way ANOVA followed by post-hoc comparisons of the means (SNK) for the basal, stress, or LPS conditions were carried out. Two-way ANOVA revealed the following: (♦) overall significant ($p < 0.05$) effect of the null mutations.

For studying the effect of IL-6 deficiency in both basal and stress situations, MT-I and MT-III mRNA levels of 3–5 mice per group were quantitated in defined working fields in representative brain areas stated in Section 2. The results demonstrate that the effects of stress and IL-6 deficiency differ for both brain MT isoforms (Tables 1 and 2). Thus, there was a clear general increasing effect of stress on MT-I mRNA levels in most brain areas studied (with the notable exception of the medial thalamic nuclei). The effect of IL-6 deficiency was not dramatic, since statistical significant effects were only observed in the CA1 (pyramidal neuron layer) area and the habenula, increasing and decreasing MT-I mRNA levels, respectively.

The results for MT-III mRNA (Table 2) demonstrated that this isoform behaves differently from MT-I. Since the hippocampus has been suggested as one of the brain areas where MT-III might be physiologically relevant [36], we evaluated the effect of stress and IL-6 deficiency on pyramidal neurons of the CA1–CA3 areas, granule cells of the dentate gyrus (DG) and lacunosum moleculare of rostral and caudal sections of this brain area. Only minor effects of both stress and IL-6 deficiency were noticed in all brain areas studied, in contrast to MT-I mRNA, with tendencies to increase or decrease MT-III mRNA depending on the brain area and physiological condition. Finally, MT-III expression was significantly increased in the cerebellum in both control and IL-6 deficient mice.

3.2. Experiment 2

Figs. 3 and 4 show brain MT-I mRNA levels of the three mutant mice studied in comparison with controls. Since the C57BL/6 mice did not differ from the F1 C57BL/6 × 129/Sv mice, they were pooled. In line with the previous experiment, stress increased MT-I mRNA levels in all areas studied. Also as expected, LPS significantly increased MT-I expression throughout the brain. In general, the brain MT-I response to stress was mostly unaltered by the IL-6 and TNFR1 deficiencies, although a general tendency was observed in the double mutants to have lower MT-I mRNA levels than the other mice. This probably mostly reflects simply what is observed in basal double mutant mice, which also tend to have lower levels, while the fold induction in all mice is comparable. Interestingly, IL-6 deficiency increased MT-I mRNA levels in the hippocampal CA1 area in basal and stress conditions, and tended to decrease them in the habenula, in agreement with the previous experiment. In contrast, the response of brain MT-I mRNA to LPS was significantly impaired in IL-$6^{-/-}$ and TNFR$1^{-/-}$ mice in most brain areas. The response to LPS in the double mutant was almost completely blunted in all brain areas studied.

Figs. 5 and 6 show brain MT-III mRNA levels. In contrast to MT-I mRNA, stress and endotoxin had only minor effects on MT-III expression, although significant increases were observed in stressed mice in brain areas such as the cerebellar Purkinje layer, the cerebrum cortex, habenula, thalamic VPM, and ependymal cells. As for MT-I, the double mutant mice appeared to have lower MT-III mRNA in basal and stress conditions in most brain areas. LPS was without effect in any of the brain areas analyzed other than the Purkinje cell layer, where the cytokine deficiencies appeared to mediate the moderate MT-III mRNA increases. Interestingly, the TNFR$1^{-/-}$ mice showed higher MT-III mRNA levels in the retrosplenial and parietal cortex in LPS-injected animals.

3.3. Experiment 3

Table 3 shows the effect of turpentine on brain MT-I and MT-III mRNA levels of control and IL-6 deficient mice. This inflammatory agent significantly increased MT-I mRNA levels throughout the brain. IL-6 deficiency had

Table 3
Effect of turpentine oil administration on brain MT-I and MT-III mRNA levels as determined by in situ hybridization

	MT-I				MT-III			
	Control		Turpentine		Control		Turpentine	
	C57BL/6	IL-$6^{-/-}$	C57BL/6	IL-$6^{-/-}$	C57BL/6	IL-$6^{-/-}$	C57BL/6	IL-$6^{-/-}$
CA1	427 ± 41	573 ± 42▲	803 ± 72*	1037 ± 66*▲	583 ± 25	613 ± 49	663 ± 23*	708 ± 45*
CA3	579 ± 50	658 ± 79	958 ± 107*	1072 ± 66*	705 ± 31	684 ± 46	763 ± 37	758 ± 24
Granular layer dentate gyrus	269 ± 27	327 ± 23	516 ± 47*	570 ± 34*	831 ± 37	820 ± 47	816 ± 29	887 ± 37
Molecular layer dentate gyrus	142 ± 18	145 ± 17	349 ± 44*	402 ± 33*	224 ± 15	165 ± 15	197 ± 21	218 ± 48
Lacunosum moleculare	158 ± 20	196 ± 13▲	407 ± 37*	509 ± 34*▲	162 ± 11	151 ± 22	184 ± 21	220 ± 39
Purkinje layer	281 ± 42	198 ± 4	693 ± 46*	648 ± 63*	468 ± 39	454 ± 37	553 ± 21*	567 ± 24*
Ependymal + choroid plexus cells	69 ± 4.5	65 ± 8	209 ± 14*	180 ± 13*	279 ± 10	237 ± 16▲	331 ± 19*	278 ± 22*▲
Retrosplenial cortex	83 ± 6.2	77 ± 6	174 ± 12*	183 ± 12*	245 ± 12	247 ± 21	298 ± 25*	270 ± 18*
Parietal cortex	86 ± 11	78 ± 5	149 ± 11*	145 ± 11*	155 ± 7	137 ± 11	153 ± 10	161 ± 9
Habenula	234 ± 12	193 ± 13	680 ± 62*	625 ± 71*	197 ± 7	187 ± 19	219 ± 14	202 ± 9
Thalamic VPM	145 ± 5	118 ± 7	425 ± 36*	397 ± 45*	460 ± 26	471 ± 42	532 ± 43*	575 ± 52*

Sagittal sections from the cerebellum and coronal sections from the cerebrum were processed simultaneously and exposed to the same autoradiographic film. MT mRNAs were measured as above. Values represent mean ± S.E. ($n = 4$–11). Results were statistically analyzed by Two-way ANOVA test with strain and turpentine oil treatment as main factors. * $p < 0.05$ vs. control, ▲ $p < 0.05$ vs. C57BL/6. Thalamic VPM, ventral posteromedial nucleus.

only modest effects, but, again interestingly, MT-I mRNA levels of IL-6$^{-/-}$ mice were increased in the hippocampal CA1 area and tended to decreased in the habenula, in agreement with the previous experiments. MT-III mRNA levels were also increased in many of the brain areas studied, but to a lower extent than MT-I isoform. IL-6 deficiency did not affect the MT-III expression.

4. Discussion

The presence of MTs in the brain has long been recognized [25]. However, the research on brain MT has gained great importance since the discovery of the brain specific isoform, MT-III (or growth inhibitory factor), and its putative relationship to Alzheimer disease [49]. However, the latter has been challenged [16], and, furthermore, MT-III has now been shown to be expressed in tissues other than the brain [33]. In addition, the isoforms MT-I + II may also be equally important for normal brain physiology since significant upregulations have been observed in human pathologies such as Alzheimer and Pick diseases [13] and amyotrophic lateral sclerosis [48], and in animals subjected to stress, LPS or brain damage [22,24,38]. Presumably, this upregulation could provide protection against free radical induced brain damage [43], or could assist in zinc and/or copper metabolism in the brain. Recent studies with transgenic and knock-out mice for MT-III also provide evidence of a protective role of this isoform against kainic acid brain damage [14]. A number of previous studies demonstrated that MT-III responds promptly to brain damage in several animal models [2,55,56]. Thus, the three brain isoforms may be relevant for brain physiology, and it is therefore of the utmost importance to characterize their regulation. In this study, brain MT regulation was examined in mice carrying a null mutation in the IL-6 gene, TNF-α type 1 receptor gene, or both, a unique approach for determining the physiological role of these cytokines.

In previous studies it has been established that IL-6 and TNF-α are significant inducers of the MT-I + II isoforms in tissues such as the liver [12,44], but little is known of their effect in the brain. We have shown that the i.c.v. injection of IL-6 in rats increases MT-I + II levels in some brain areas [21], and transgenic mice with targeted IL-6 expression to astrocytes also show increased MT-I + II levels in those brain areas with significant transgenic IL-6 expression [22], suggesting that IL-6 could be important in brain MT regulation. However, IL-6$^{-/-}$ mice did not differ dramatically from the control mice in basal conditions, and the only consistent effects noticed were in the CA1 pyramidal neurons and the habenula, where IL-6 deficiency increased and decreased MT-I mRNA levels, respectively. This general lack of effect of IL-6 deficiency is in principle not surprising since the MT-I + II isoforms are multiregulated proteins, but nevertheless significant effects, and more important, opposite trends, were observed in specific areas. TNFR1$^{-/-}$ mice did not differ dramatically either from the control mice, although in the granular layer of the dentate gyrus a significant increase of MT-I mRNA levels was observed. Interestingly, the double mutant mice showed a much more significant alteration of their brain MT-I mRNA levels, since a clear trend to have decreased levels was observed in most brain areas. IL-6 and TNF-α are pleiotropic cytokines which share some functions [8], and thus it seems feasible that in basal conditions the two of them participate in the control of MT-I expression and could compensate to some extent the functional loss of each other, so that a significant effect is observed when both cytokines are missing simultaneously. Regarding MT-III mRNA, no dramatic effects of the functional IL-6 and TNF deficiencies were noticed, although in agreement with MT-I mRNA a clear trend to show decreased MT-III signal in most brain areas was observed in the double mutant.

We next examined the putative mediating role of IL-6 and TNF-α on brain MT response to stress and LPS, two major physiological inducers of the MT-I + II isoforms. In agreement with previous reports [6,18,19], stress significantly upregulated MT-I expression, indicating that this MT isoform (and likely MT-II, which is coordinately regulated with MT-I) is important for the adaptation of the organism to stress [26]. This response was basically unaltered in the IL-6$^{-/-}$ and/or TNFR1$^{-/-}$ mice, indicating that a mediating role of IL-6 or TNF-α during stress is unlikely. Since both acute and chronic increases of IL-6 do increase brain MT-I + II levels (see above), it might be concluded that IL-6 is not increased during stress in the brain, or that necessary factors are not co-released during stress in the brain. It is important to mention that in contrast to the brain, we find a significant effect of IL-6 deficiency on liver MT response to stress (manuscript in preparation), which is consistent with the increased circulating IL-6 levels during stress and the role of the cytokine on liver MT regulation (see above). Whether this highlights a tissue-specific role of IL-6 regarding brain MT regulation during stress or reflects the inability of the cytokines to cross the blood–brain barrier remains to be established.

A different pattern emerged when brain MT-I was induced with LPS. As expected [27], the MT-I signal was significantly increased throughout the brain. Although the mechanism is unclear, it is well known that peripheral LPS administration increases cytokine production including IL-6 in the brain [31]. The present results clearly demonstrate that brain MT-I response to LPS is mediated by IL-6 and TNF-α, since the IL-6$^{-/-}$ and TNFR-1$^{-/-}$ mice showed significant diminished responses which were almost completely blunted in the double mutants. This is the first time that a physiological role of these cytokines on brain MT-I + II regulation is demonstrated.

We also evaluated the effect of another inflammatory model, turpentine oil administration. A very significant MT-I upregulation was observed throughout the brain, suggesting again a relevant role of this MT isoform. IL-6 deficiency did not affect this response in any of the brain areas analyzed, which is in contrast with the inflammatory response elicited by LPS. A likely explanation of this apparent discrepancy is that turpentine and LPS could produce a different set of pro-inflammatory cytokines. For instance, a previous study [46] has demonstrated that LPS but not turpentine caused an upregulation of IL-1, IL-6 and TNF-α in circulating monocytes, peritoneal macrophages and the liver. Despite these results, we do find a reduction of liver MT induction by turpentine in IL-$6^{-/-}$ mice (manuscript in preparation), suggesting again a tissue-specific role of IL-6. It is even feasible that brain MT-I induction by turpentine is at least in part a stress response, but in any case it is clear that IL-6 is not relevant in the inflammatory response elicited in the brain regarding MT-I regulation.

Regarding MT-III, the basically brain-specific MT isoform, the in situ hybridization results demonstrate that it remains mostly unaffected in comparison with MT-I, in agreement with previous studies [11,37,57]. Nevertheless, stress slightly but consistently increased MT-III mRNA levels in most brain areas analyzed. We have studied previously the effect of stress by Northern-blot and dot-blot in normal mice and rats [6,23]. Taken together, the results suggest that stress increases brain MT-III mRNA levels although to a lower extent than MT-I isoform, and that the apparent discrepancies between experiments may be explained by stress-induced changes in γ-actin mRNA levels. IL-6 and TNF-α functional deficiencies appeared to blunt the increase only in the ependymal + choroid plexus cells. The double mutant tended to have less MT-III mRNA levels than the other mice, but this appears to simply reflect what is observed in basal mice. In contrast to stress, LPS (at the dosage given) did not affect significantly MT-III mRNA levels in most of the brain areas studied, with the exception of the Purkinje cell layer of the cerebellum where a slight increasing effect was noticed. The IL-6 and TNF-α functional deficiencies diminished the effect of LPS in this area. Interestingly, the TNFR1$^{-/-}$ LPS-injected mice showed higher MT-III mRNA levels in the retrosplenial and parietal cortex, and the same tendency was observed in the hippocampal CA1 area, the habenula and the thalamic VPM, suggesting an inhibitory role of TNF-α in these brain areas. Finally, turpentine significantly increased MT-III mRNA in several brain areas, and IL-6 deficiency decreased the MT-III signal in the ependymal + choroid plexus cells, which also supports the assumption above stated that brain MT response in this case is at least partially a stress response.

In summary, the present results demonstrate that in vivo IL-6 and TNF-α are major regulators of the metal-binding proteins, metallothioneins, in the brain. Their role is important in one scenario, the inflammatory response elicited by LPS, but not in others, namely the stress response and the inflammatory response elicited by turpentine.

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