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REGULAR PAPER

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# Treatment of experimental autoimmune encephalomyelitis in rat by 1,25-dihydroxyvitamin $D_3$ leads to early effects within the central nervous system

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**Abstract** We report here that curative treatment of the multiple sclerosis paradigm, chronic relapsing experimental autoimmune encephalomyelitis (EAE) of the Lewis rat, by 1,25-dihydroxyvitamin $D_3$ (1,25-D3) leads to a rapid clinical improvement accompanied by an inhibition of CD4, MHC class II and type II nitric oxide synthase (NOS II) expression in the posterior areas of the central nervous system (CNS). In contrast, the hormone has no effect on transforming growth factor-β1 transcripts. Computer analysis of the NOS II promoter, expressed by microglia and astrocytes, reveals consensus sequence for vitamin D receptor binding, emphasizing the idea that 1,25-D3 may regulate some aspects of EAE by acting directly on CNS constituent cells. We also demonstrate that vitamin D deprivation leads to minimal effects on the kinetic profile of EAE accompanied by a moderate exacerbation of the clinical symptoms. Interestingly, curative treatment of vitamin D-deprived rats with a non-toxic-1,25-D3 analogue (MC1288) strongly inhibited EAE symptoms, thus promulgating the potential interest of such compounds in the management of multiple sclerosis.

**Keywords** Vitamin D · Nitric oxide synthase · TGF-β · Microglia · Macrophages

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## Introduction

Multiple sclerosis (MS) is a human autoimmune and inflammatory demyelinating disease of unknown etiology that affects the central nervous system (CNS). During the 70s, the concept that vitamin D might represent an important factor in MS emerged from Goldberg's epidemiological and genetic studies. Based on the fact that the supply of vitamin D is exclusively due to UV irradiation of the skin and food supplementation, he described a very impressive negative correlation between sunlight, as well as dietary factors that affect vitamin D concentration, and MS prevalence [19, 20]. More recently, it has been demonstrated that 1,25-dihydroxyvitamin-$D_3$ (1,25-D3), the biologically active form of vitamin D, when administered at pharmacological doses during the immunization phase (prophylactic method) protects rodents against experimental autoimmune encephalomyelitis (EAE), an animal model of MS [4, 29]. Moreover, 1,25-D3 prevents the appearance of other autoimmune diseases, such as experimental autoimmune thyroiditis, lupus erythematosus, type I diabetes and arthritis [3]. Thus, the potential mechanisms for the preventive functions of 1,25-D3 on EAE may be a consequence of its wide range of immunomodulatory properties [3].

There is now growing evidence that 1,25-D3 is also directly operative within the CNS [15]. 1,25-D3 can cross the blood-brain barrier, and can also be produced by CNS constituent cells [15]. This secosteroid hormone displays extensive molecular and behavioral effects on various CNS neuronal and non-neuronal cell subpopulations expressing its receptor, the vitamin D receptor (VDR) [15]. These observations raise the possibility that during ongoing EAE or MS, when the pathology is already declared, 1,25-D3 might display within the CNS itself both immunomodulatory and neurological effects limiting the disease.

Two further studies in rats and in mice recently indicated that 1,25-D3 ameliorates the symptoms of EAE when administered after the onset of clinical signs (cura-

tive method) [6, 35]. In the rat model, the beneficial effect of the hormone treatment was accompanied, within the CNS, by a region-specific down-regulation of CD4 antigen [35], and by an inhibition of type II nitric oxide synthase (NOS II) expression [13]. NOS II (the inducible enzymatic isoform able to generate NO) and NO itself are tightly associated with MS and EAE physiopathology [18, 30, 39]. In the mouse archetype, 1,25-D3 curative treatment leads to a decrease of IL–12-dependent Th1 cell development [31] and to an increase of IL–4 and TGF-β1 transcripts in situ [7]. TGF-β1 is itself a potent inhibitor of NOS II synthesis [49] and TGF-β1 null mice spontaneously overexpress NOS II [51]. Thus, the idea that 1,25-D3 curative treatment might partially prevent the severity of EAE and reduce NOS II expression during this disease, by positively regulating the anti-encephalitogenic cytokine TGF-β1, appears attractive. However, a direct effect of the hormone on the NOS II gene remains a possibility since CNS parenchymal cells in which NOS II expression has been identified (e.g. astrocytes, microglia, oligodendrocytes, neurons) are VDR expressing cells [15].

To further clarify the mechanisms by which vitamin D ameliorates symptoms of EAE, thereby representing a potential treatment for MS, we used a curative approach for the chronic relapsing EAE of the Lewis rat. A clinical follow-up of the treatment of EAE-control rats with 1,25-D3 confirmed the beneficial effect of the hormone. Likewise, treatment of EAE-vitamin D-deprived rats, which display an EAE clinical course comparable to the one observed for EAE-control rats, by the vitamin D non-toxic 20-epi analogue MC1288 [26] strongly inhibited EAE symptoms. Moreover, we demonstrate that EAE curative treatment by 1,25-D3 leads to a CNS region-specific inhibition of NOS II gene expression and immunoreactivity, not only during the second paralytic attack as previously reported [13], but already during the first paralytic attack, closely after the onset of the treatment. We also show that 1,25-D3 treatment has no consequence on the expression of TGF-β1 transcripts in our model, indicating no direct correlation between TGF-β1 and NOS II synthesis. As sequence analysis suggests that the NOS II-gene promoter contains a consensus motif for VDR binding, our data support the concept that 1,25-D3 is able to directly regulate some molecular aspects of the ongoing EAE process on parenchymal CNS cells, in parallel to its formerly described immunomodulatory properties.

## Materials and methods

### EAE induction and treatment by 1,25-D3

Age-matched 8- to 9-week-old Lewis female rats ($n$=68; Charles River, Cléon, France) were used. Control animals ($n$=3) were not immunized and EAE was induced, as described previously [35], under the same conditions in three independent experiments: rats immunized for EAE and treated with 1,25-D3 [EAE-1,25-D3 (+), $n$=12, 12 and 15 animals, respectively], and rats immunized for EAE and treated with vehicle alone [EAE-1,25-D3 (–), $n$=6, 6 and 14 animals, respectively]. Briefly, 1 g spinal cord obtained from female Dunkin-Hartley guinea pigs (Charles River) was homogenized with 1 ml saline. The homogenate was then emulsified with 2 ml Difco's bacto complete Freund's adjuvant (CFA) H37RA supplemented with 40 mg *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, Mich.); 0.1 ml of this emulsion were injected intradermally under ether anesthesia into each footpad of the rats. 1,25-D3 (a kind gift of L. Binderup, Leo Pharmaceutical Products, Ballerup, Denmark) dissolved in 80% propylene glycol/20% 0.05 M disodium phosphate pH 7.4 was injected intraperitoneally into animals in a two-step therapeutic procedure starting after the onset of EAE clinical signs and interrupted during the remission phase. Two injections of 1,25-D3 of 5 µg/kg on days 11 and 13 were followed by three injections of 1 µg/kg on days 19, 21 and 23 for EAE 1,25-3 (+) rats. Vehicle alone was administered instead of the hormone to EAE 1,25-D3 (–) rats.

### EAE in vitamin D-deprived rats and treatment by MC1288

After weaning, from 3 weeks after birth until the end of the study, 27 Lewis female rats (Charles River) were submitted to vitamin D deprivation. The vitamin D-free diet consisted of 2% Ca and 1.25% P instead of 0.64% Ca, 0.64% P and 2,000 IU/kg vitamin D in normal control diet (UAR, Epinay sur Orge, France). This vitamin D-free diet has been reported to prevent ensuing hypocalcemia and hyperparathyroidism [27]. To avoid vitamin D synthesis, vitamin D-deficient rats were housed in a dark room and had free access to food and water. EAE was induced in all animals as previously described and treatment with MC1288 (a vitamin D non-hypercalcemic 20 epi-analogue, which acts through VDR in a similar fashion as 1,25-D3 [26]; a kind gift of L. Binderup) at a dose of 0.05 µg/kg per day ($n$=9) and 0.2 µg/kg per day ($n$=9) or with vehicle alone ($n$=9) was given from day 10 to day 23.

### Clinical assessment and statistical analysis

Rats were weighed and examined daily; clinical signs were graded as: 1, loss of tail tonicity; 2, weakness of one or both hind legs or mild ataxia; 3, severe ataxia or paralysis; 4, severe hind leg paralysis accompanied by urinary incontinence. Comparisons of percentages for the incidence of score 4 at the first attack and the incidence of a second attack, were determined by the Chi-Square test. Due to the small number of animals in some groups, the Yates's correction was applied when required. Comparisons of mean clinical scores were performed by using the Student's $t$-test and confirmed by the Mann-Whitney U-test. Statistical significance ($P$ value) was determined by reporting the calculated value of $\chi^2$, $\chi^2$c, t or U in the corresponding Fisher and Yates's statistical table.

### Immunohistochemistry and in situ hybridization

A minimum of six animals were randomly chosen among each group and killed on days 13, 19 or 23 post immunization. Brains and spinal cords were surgically removed, snap frozen in isopentane cooled with liquid nitrogen and processed for immunohistochemistry (IHC) or in situ hybridization (ISH). Cryostat sections (10 µm) were made from the brain regions located at 0.7 mm, –3.5 mm and –11.0 mm from Bregma (anteroposterior stereotactic coordinates, [40]) and in the lumbothoracic spinal cord. For IHC, the previously described protocol [13, 35] and the following primary antibodies were used: a mouse monoclonal antibody directed against the complement receptor type 3 (CR3) (OX42, Serotec, Kidlington, UK), a mouse monoclonal antibody directed against the rat MHC class II molecules (MHC-II) (OX6, Cedarlane, Ontario, Canada), a mouse monoclonal antibody directed against the CD4 antigen (W3/25, Serotec), and a rabbit polyclonal antibody directed against NOS II (Transduction Laboratories, Lexington, Ky.). For ISH, detection of NOS II mRNA and TGF-β1 mRNA was carried out as previously described [13, 14]. For NOS II mRNA (GenBank accession no. U03699) the following oligodeoxynucleotide probes were used: 5'-TCCCCTCTGATGGTGCCATCGGG-

CATCTGGTAGCCAGCG-3', 5'-AGCGCATACCACTTCAGC-CCGAGCTCCTGGAACCACTCGT-3', 5'-CCTCCTGCCCAC-TTCCTCCAGGATGTTGTAGCGCTGTG-3' and 5'-CTCCTGC-ATTTCTTCCTGATAGAGGTGGTCCTCCTCTGGGTGCCTG-CA-3' ; and for TGF-β1 mRNA (GenBank accession no. X52498) the following ones: 5'-GAGCAGGAAGGGTCGGTTCATGTCA-TGGATGGTGCCCAGG-3', 5'-GTGGAGCTGAAGCAGTAGT-TGGTATCCAGGGCTCTCCGC-3', 5'-GGGCTCGTGGATCC-ACTTCCAACCCAGGTCCTTCCTA-3', 5'-GTAGAGGGCAA-GGACCTTGCTGTACTGTGTGTCCAGGCTC-3'. Those probes were 3' end-labeled using [α-$^{35}$S]deoxyadenosine triphosphate (Amersham) and terminal deoxynucleotidyl-transferase (Boehringer Mannheim, Germany). βmax Hyperfilm (Amersham) and K5 nuclear emulsion (Ilford, Cheshire, UK) were used for signal detection.

### Semiquantitative analysis of immunolabeling

MHC-II, CR-3, CD4 and NOS II semiquantitative counts were performed under medium magnification (×20) by two independent investigators on sections from EAE-control rats and EAE animals responding to 1,25-D3. Results, given as the mean cell number obtained from whole spinal cord and whole brain stem, were recorded using the following arbitrary scale: +, 2–20 cells; ++, 20–100 cells; +++, >100 cells; ++++, intense and widespread staining.

### NOS II mRNA ISH analysis

Radioautographs obtained from sections of spinal cord were quantified using the image computer analysis program ImageJ developed by the National Institutes of Health (Bethesda, Md.). For each section, the percentage of total cerebral area corresponding to foci expressing NOS II mRNA was determined. Results are given as the mean value ± standard deviation obtained from the analysis of brain sections from EAE-control rats (three animals) and EAE animals responding to 1,25-D3 (three animals).

### NOS II promoter sequence analysis

Rat NOS II promoter sequence (GenBank accession no. AF042085) was used for multiple sequence alignment by using the ClustalW computer program from the EMBL European Bioinformatics Institute (Heidelberg, Germany).

## Results

### EAE curative treatment by 1,25-D3 and MC1288: clinical course analysis

To determine whether biologically active vitamin D-related compounds represent valuable tools for curative treatment of rat EAE, 1,25-D3 (or vehicle alone) and MC1288 (or vehicle alone) were administered, respectively, to EAE-control animals and EAE-vitamin D-deprived animals after clinical signs had begun (Fig. 1A, day 11, and Fig. 1B, day 10).

The observations for 1,25-D3-treated EAE rats show that this treatment influences both the amplitude and the kinetics of the clinical course of EAE (Fig. 1A). An inhibition of the severity of the first paralytic attack (day 12/13) was observed (Fig. 1A), with a decrease (almost 50%) in the incidence of maximal score 4 at that stage

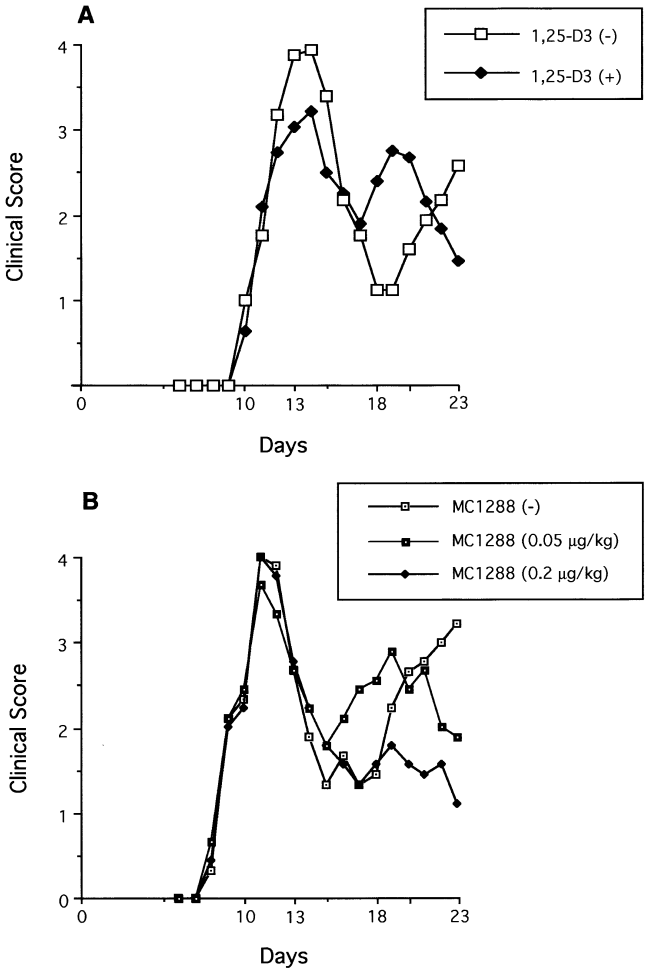



**Fig. 1** **A** 1,25-D3 treatment of rats immunized for EAE modifies the clinical course of the disease both in amplitude and kinetics. Vehicle alone [1,25-D3 (–)] or 1,25-D3 [1,25-D3 (+)] were administered on days 11, 13, 19, 21 and 23. Treated animals present a reduced clinical score during the first attack, a less marked remission phase, and an earlier and less intense second attack. **B** Rats deprived of vitamin D and immunized for EAE tend to exacerbation of the clinical signs; MC1288 treatment of these animals results in an improvement of the clinical scores. Vehicle alone [MC1288 (–)] or MC1288 (0.05 µg/kg or 0.2 µg/kg) were administered everyday from day 10 to day 23 (*1,25-D3* 1,25-dihydroxyvitamin $D_3$, *EAE* experimental autoimmune encephalomyelitis)

(Table 1). Although not statistically significant, the occurrence of a second paralytic attack was reduced in 1,25-D3-treated EAE animals (52.9% compared to 72.7%, Table 1). When observed, this second paralytic attack occurred earlier in these rats (Fig. 1A), leading only to a partial remission phase (score >1 in most animals, Fig. 1A), and also to a significant reduction of the mean clinical scores observed on day 23 (Fig. 1A and Table 1).

To address the hypothesis that endogenous vitamin D may be involved in EAE physiopathology, rats submitted to a diet excluding vitamin D were immunized for EAE. Clinically, these animals presented a similar kinetic profile of EAE with a moderate exacerbation of the symptoms. This included an earlier appearance of the clinical

**Table 1** Clinical effects of 1,25-D3 curative treatment on EAE-control rats and of MC1288 curative treatment on EAE-vitamin D-deprived rats. Vehicle alone [1,25-D3(–)] or 1,25-D3 [1,25-D3 (+)] were administered on days 11, 13, 19, 21 and 23 in EAE-control rats. Vehicle alone [MC1288 (–)] or MC1288 (0.05 µg/kg or 0.2 µg/kg) were administered everyday from day 10 to day 23 in EAE-vitamin D-deprived rats (*1,25-D3* 1,25-dihydroxyvitamin $D_3$, *EAE* experimental autoimmune encephalomyelitis)

| Experimental group | Incidence of score 4 at the first paralytic attack | Incidence of a second paralytic attack | Mean clinical score on day 23 |
|---|---|---|---|
| 1,25–D3 (–) | 100.0% (n=26) | 72.7% (n=11) | 2.45±1.13 (n=11) |
| 1,25–D3 (+) | 51.3%*** (*n*=39) | 52.9% (*n*=17) | 1.53±1.12* (*n*=17) |
| MC1288 (–) | 100.0% (*n*=9) | 100.0% (*n*=9) | 3.22±0.97 (*n*=9) |
| MC1288 (0.05 µg/kg) | 66.6% (*n*=9) | 44.4%$^{\#}$ (*n*=9) | 1.88±0.60$^{\#\#}$ (*n*=9) |
| MC1288 (0.2 µg/kg) | 100% (*n*=9) | 22.2%$^{\#\#}$ (*n*=9) | 1.11±0.33$^{\#\#\#}$ (*n*=9) |

*** $p<0.001$, * $p<0.05$: comparisons between EAE-control rats and 1,25-D3-treated EAE rats.
$^{\#\#\#}$ $p<0.001$, $^{\#\#}$ $p<0.01$, $^{\#}$ $p<0.05$: comparisons between vitamin D-deprived EAE rats and MC1288-treated vitamin D-deprived EAE rats

signs (after 8.70±0.47 days post induction compared with 10.85±0.81 days for rats on normal diet, Student's *t*-test: $P<0.001$), a non-classical remission phase (clinical score >1 in 44.4% of the animals instead of 0% with normal diet, $\chi^2$ test: $P<0.05$) and a second paralytic attack, reaching 100% of incidence, characterized by a marked ataxia (Fig. 1B and Table 1). Treatment of vitamin D-deprived EAE rats by the non-toxic MC1288 vitamin D analogue at low doses (0.05 and 0.2 µg/kg per day) led to nonsignificant effects on the first paralytic attack, but displayed dramatic inhibitory effects on both the incidence and the amplitude of the second paralytic attack with a maximal effect at 0.2 µg/kg per day (Fig. 1B and Table 1).

## Histo-molecular characterization of EAE curative treatment by 1,25-D3

To gain a better understanding of the mechanisms by which 1,25-D3 improves clinical symptoms of EAE early after the onset of treatment, we primarily focused on days 13 (first paralytic attack) and 19 (recovery) to study various aspects of the inflammatory reaction. We investigated the expression of markers specific for cell types or for activation stages (e.g., CR3, MHC-II and CD4) and analyzed gene expression and immunoreactivity of NOS II, a well-characterized molecule involved in the progression of EAE.

On day 13, CR3 and MHC-II labeling were observed in discrete areas of the anterior brain, cerebellum/brain stem and spinal cord on small inflammatory infiltrates and on activated and reactive microglial cells surrounding the infiltrates (Fig. 2A). On day 19, inflammatory infiltrates were much larger and more widely distributed throughout these CNS regions (Fig. 2A). CD4-positive cells (e.g., lymphocytes, activated and reactive microglia and perivascular cells) were observed in the ventricles and the meninges, on blood vessels scattered throughout the CNS parenchyma and around inflammatory infiltrates. On days 13 and 19, CD4 immunostaining was reduced as compared to the CR3 and the MHC-II immunostaining, but displayed a very similar topographic distribution (Fig. 2A). After 1,25-D3 treatment, a strong decrease of CR3, MHC-II and CD4 immunolabeling in inflammatory infiltrates and surrounding areas was predominantly observed on day 13 in spinal cord and brain stem/cerebellum of animals, presenting an improved clinical score (below 4) (Table 2, Figs. 2B, C; 3A–F). These data illustrate the rapid effect of 1,25-D3 on cellular reaction during EAE particularly in posterior CNS areas.

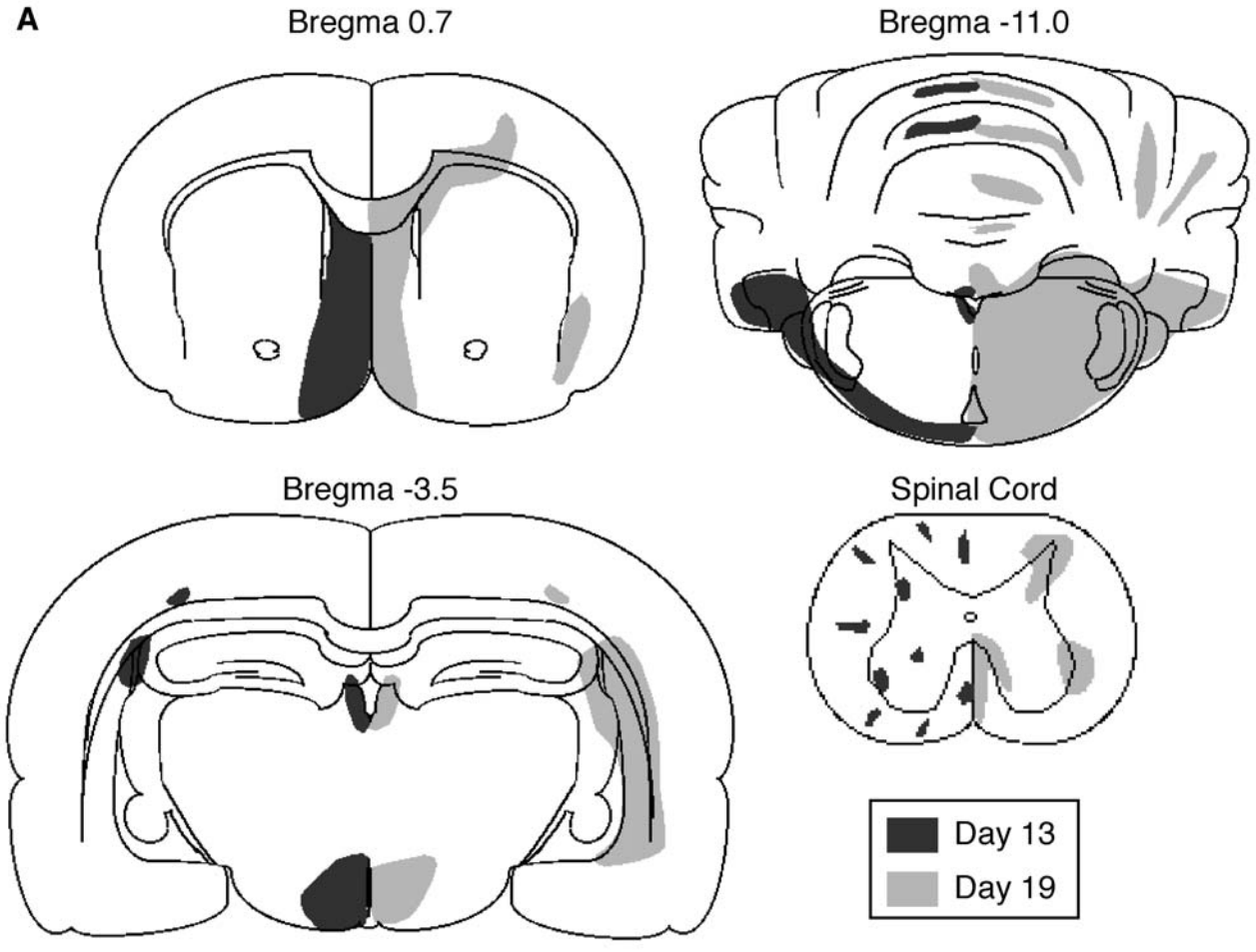
On day 13, NOS II immunostaining was observed on microvessels throughout the brain parenchyma, and on some inflammatory infiltrates, mainly in spinal cord and posterior brain sections (Fig. 3G). NOS II mRNA was expressed by almost all the inflammatory infiltrates in all CNS regions examined and was particularly abundant in the spinal cord (Fig. 4A, D). On day 19, NOS II mRNA was less frequently expressed than on day 13, especially in the spinal cord. In contrast, a progression in the number of NOS II-immunolabeled cells was observed primarily in the spinal cord and brain stem/cerebellum. 1,25-D3 treatment exerted a drastic inhibitory effect on NOS II mRNA expression and NOS II immunolabeling on days 13 and 19, which correlated with the decrease in the clinical scores on day 13 but not on day 19. Interestingly, this inhibition mainly concerned the spinal cord and brain stem/cerebellum (Table 2, Figs. 3H, 4B, C, E).

TGF-β1 is known to be up-regulated by 1,25-D3 curative treatment in mice during EAE [7] and to down-regulate NOS II in macrophages [49]. In contrast, TGF-β1 deficiency leads to ectopic expression of NOS II [51]. To investigate whether the observed effects of 1,25-D3 on NOS II gene were related to an increase of TGF-β1 synthesis, we studied the gene expression of TGF-β1 in our rat model by ISH. On day 13, no labeling was observed in the anterior and median sections, whereas some small infiltrates were weakly labeled in the brain stem/cerebellum (Fig. 4F) and spinal cord. On day 19, TGF-β1 mRNA was more strongly expressed. Some infiltrates were positive in the hippocampus, and the majority of them were highly labeled in the posterior brain section (Fig. 4G) and the spinal cord. Although inflammatory infiltrates were still present on day 23 [13, 35], TGF-β1 mRNA expression was no longer detected at this stage (Fig. 4H). 1,25-D3 cura-

tive treatment of EAE did not exert any effect on the distribution and the levels of TGF-β1 transcripts at any time point examined. On the basis of these observations, no direct correlation can be made between TGF-β1 and NOS II gene expression during EAE in rat.

## Computer identification of a putative vitamin D-responsive elements in the rat NOS II promoter

The large majority of the presently known vitamin D-responsive elements (VDREs) constitute a direct repeat of two hexameric core binding motifs, spaced by three nucleotides (DR3-type VDREs), which mediate selective recog-



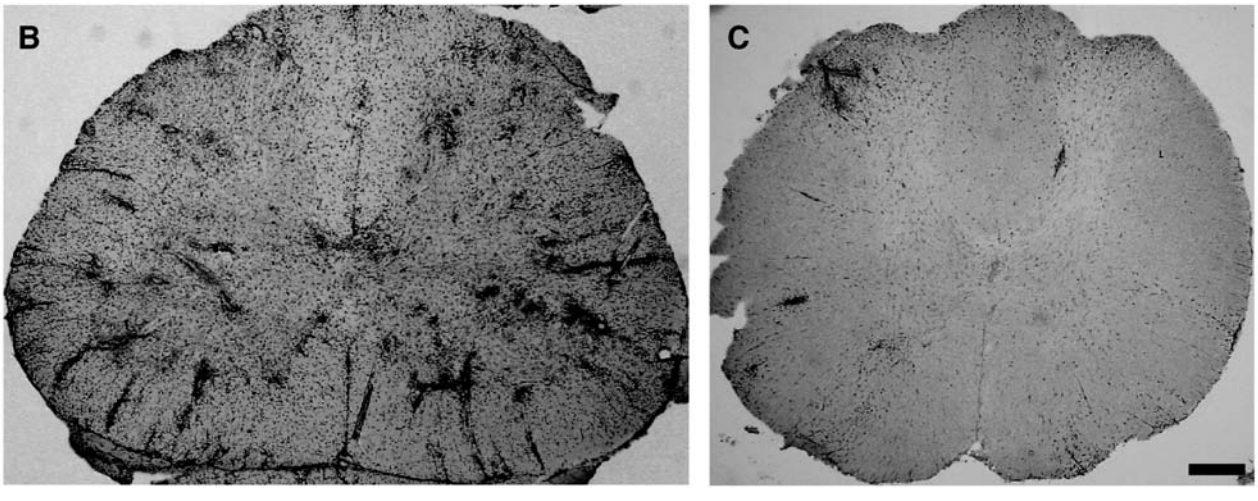



**Table 2** Immunohistochemical analysis of EAE-control rats compared to 1,25-D3-responsive animals

| Immunolabeling | Experimental group | Spinal cord | Brain stem |
|---|---|---|---|
| OX42 | 1,25–D3 (–) | ++++ | ++ |
| | 1,25–D3 (+) | ++ | + |
| MHC class II | 1,25–D3 (–) | ++++ | ++ |
| | 1,25–D3 (+) | + | + |
| CD4 | 1,25–D3 (–) | +++ | ++ |
| | 1,25–D3 (+) | + | + |
| NOS II | 1,25–D3 (–) | +++ | ++ |
| | 1,25–D3 (+) | + | + |

nition and VDR-protein binding. To determine such sequences in the rat NOS II-gene promoter, we performed computer multiple sequence alignments with a consensus motif RRKNSA (R=A or G, K=G or T, S=C or G), which has already been used by others for successful identification of VDREs [50]. This allowed us to identify different pairs of matches from which the best sequence for a VDRE was AGGACAGGAAGGTCA located between the positions 2,947 and 2,933. Interestingly, this sequence possesses 86.67% homology with one of the most potent DR3-type VDRE found for instance in the rat atrial natriuretic factor (ANF) promoter. The hexameric core binding motifs present 100% homology with others core binding motifs able to bind VDR as a heterodimer with RXR in 5'-RXR-VDR-3' polarity [50].

◀ **Fig. 2** **A** Topographic distribution of MHC-II immunolabeling in four cross-sections of EAE-rat CNS. The *left half* of the figures represents the labeling observed 13 days after EAE induction (*dark gray*) and the *right half* these observed on day 19 (*light gray*). The labeled regions illustrate areas where labeled infiltrates and surrounding immunoreactive cells are observed. The areas devoid of infiltrates are not indicated, even though positive cells may be found. *Bregma 0.7 mm*: On day 13, MHC-II expression is restricted to the septum and diagonal band of Broca. On day 19, the immunolabeling is more widespread and also concerns the corpus callosum, the subjacent area of caudate putamen and the frontoparietal cortex. A basal region around the clostrum and endopiriform nuclei is also labeled. *Bregma –3.5 mm*: On day 13, MHC-II is expressed in different regions: all along the third ventricle around the hypothalamus nuclei and at the level of median habenula, and sometimes in the frontoparietal cortex. Immunoreactivity is also observed in the fimbria and the hippocampus. On day 19, the same areas are labeled and, in addition, MHC-II is widely expressed in the vicinity of the lateral ventricles, as well as in the amygdala and endopiriform nuclei. *Bregma 11.0 mm*: On day 13, MHC-II immunolabeling is observed at the basal region of the brain stem periphery and in the cerebellum, both in a restricted area around the cochlear nuclei and the flocculus and in the 4th and 5th lobules. On day 19, the labeling has extended to cover all the brainstem and the cerebellum lobules. *Spinal cord*: In contrast to the other CNS sections, MHC-II expression is observed on day 13 throughout the whole parenchyma, with small to medium-sized infiltrates, especially in the white matter. On day 19, immunoreactive inflammatory infiltrates are larger and more concentrated in the gray matter and the adjacent areas of the white matter. **B** MHC-II immunostaining on day 13 within the spinal cord of EAE-control rats. **C** MHC-II immunostaining on day 13 within the spinal cord of EAE rats responding to 1,25-D3 treatment. Note the almost complete disappearance of the immunostaining (*MHC-II* MHC class II). *Bar* **B**, **C** 350 μm

## Discussion

The present study demonstrates that the curative treatment of chronic relapsing EAE in the Lewis rat, with 1,25-D3, leads to a rapid amelioration of symptoms, which correlates to a region-specific inhibition of the inflammatory process within the CNS. The histo-molecular effects of the hormone include a reduction in the size of the infiltrates, and a down-regulation of MHC-II and CD4 expression and of NOS II synthesis. They are observed as early as day 13 and mainly concern CNS posterior areas and more potently the spinal cord. This site may represent a privileged CNS target for 1,25-D3 [34, 35, 43, 48]. Alternatively, it is possible that inflammatory cells present a region-dependent functional heterogeneity, so that their response to 1,25-D3 varies from one CNS structure to another. Such observations are in accordance with the caudorostral evolution of the disease [9, 35], and suggest that EAE severity, especially at the first attack, could be linked to inflammatory lesions of the spinal cord. During the recovery phase, the clinicopathological correlation is less apparent with a progression of the lesions in rostral CNS areas. This discrepancy could be explained by several immunological changes in the local brain environment, such as an increased production of anti-inflammatory cytokines [24], leading to a decrease in CNS damage and to an extensive destruction of T lymphocytes [41]. Our results showing that TGF-β1 mRNA are weakly expressed on day 13 and highly expressed on day 19 support this notion.

The recruitment of inflammatory cells in the CNS is possibly the earliest mechanism during EAE after the initial infiltration of T cells [42]. Likewise, it has been demonstrated that blood-borne macrophages, which are effector cells involved in EAE pathology, represent a very large population of the reactive cells observed in the perivascular lesions [22, 23, 33]. However, microglial cells, which are CNS resident cells able to modify their phenotypic properties in graded responses from quiescent to activated and reactive stages, are also involved in T cell-mediated injury and represent the major intrinsic immune and inflammatory effector cell population of the brain [16, 17, 21, 33]. Therefore, our results demonstrating that, in some CNS areas, there is a complete disappearance of CR3-positive cells (which express MHC-II and display the morphology of both macrophages and activated/reactive microglia) after 1,25-D3 treatment of EAE suggest an early action of the hormone on both cell pools. Our data support the hypothesis that the early decrease of CD4 immunolabeling on day 13 after 1,25-D3 treatment is a down-regulation of CD4 antigen expression by inflammatory cells as previously demonstrated on day 23 [35]. This inhibitory effect of the hormone is reminiscent of those observed on isolated immune cells, on molecules involved in the antigen presentation to lymphocytes comprising

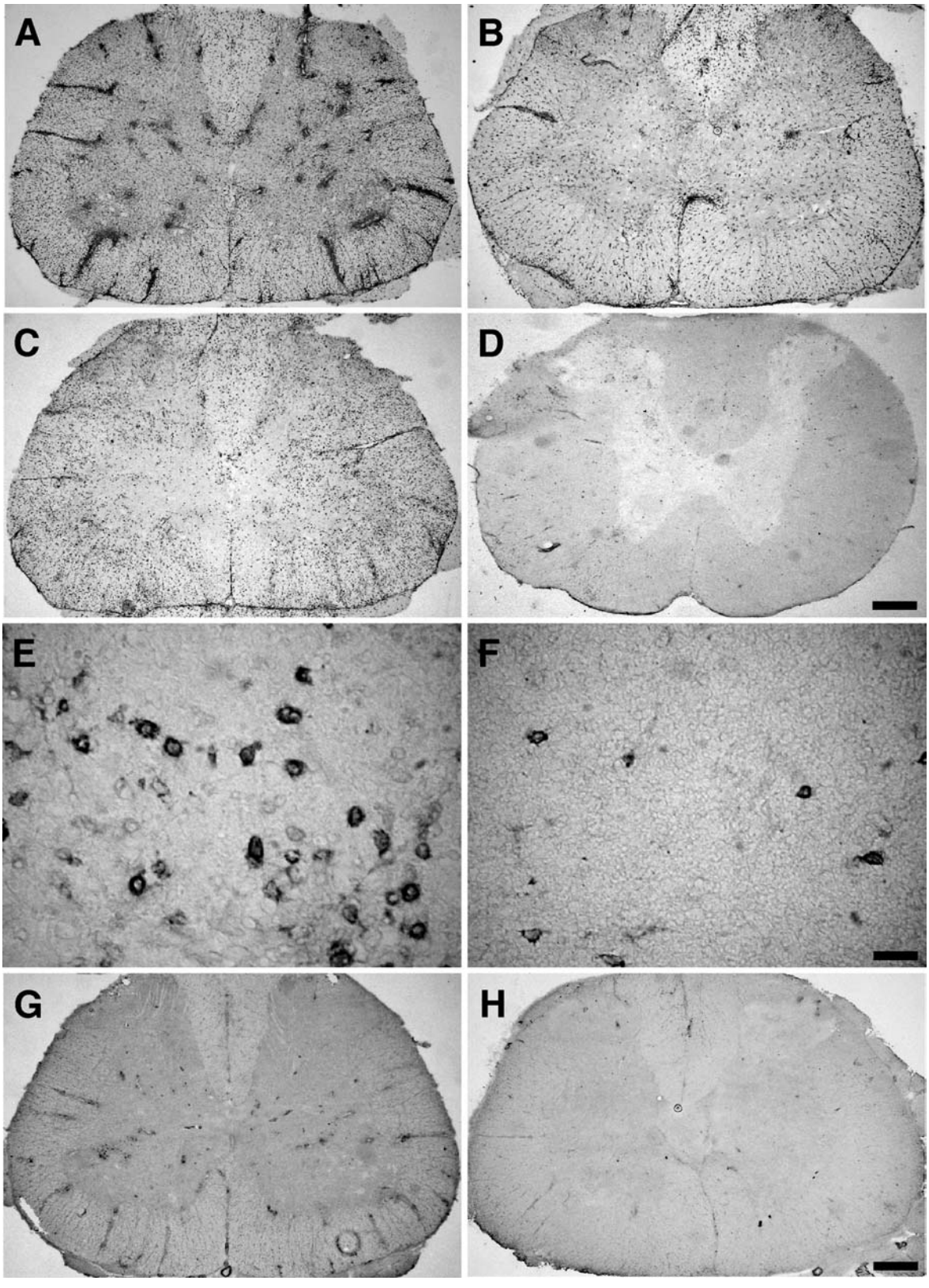



**Fig. 3** CR3, CD4 and NOS II immunolabeling on day 13 in the spinal cord of EAE-control rats (**A**, **C**, **E**, **G**) and EAE rats responding to 1,25-D3 treatment (**B**, **D**, **F**, **H**). **A**, **B** CR3; **C**, **D** CD4, **E**, **F** $CD4^+$ cells in the spinal cord gray matter; **G**, **H** NOS II (*NOS II* type II nitric oxide synthase). *Bars* **A**–**D**, **G**, **H** 350 μm; **E**, **F** 20 μm

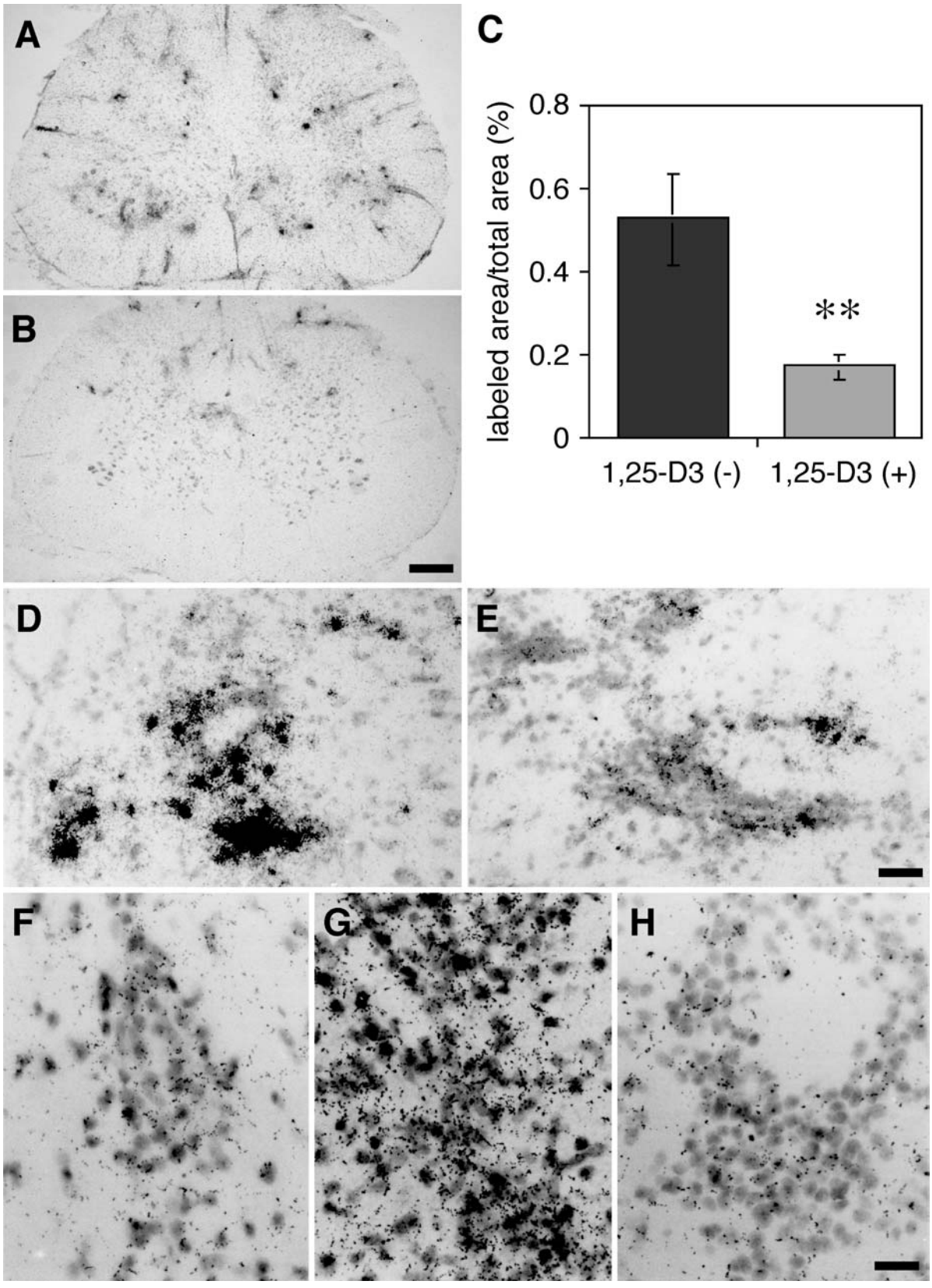



**Fig. 4** Study of NOS II and TGF-β1 mRNA expression by in situ hybridization. Comparison of NOS II gene expression on day 13 within the spinal cord of EAE-control rats (**A**) and of EAE rats responding to 1,25-D3 treatment (**B**) reveals a marked reduction of the size of the labeled area after vitamin D treatment (**C**). Whereas NOS II mRNA is intensely expressed on D13 in perivascular inflammatory infiltrates of the brain stem of non-treated rats (**D**), a reduced expression is observed at the same time point in 1,25-D3-treated animals (**E**). TGF-β1 mRNA is weakly expressed in inflammatory infiltrates of the brain stem of EAE rats on day 13 (**F**), intensely labeled at day 19 (**G**), and their expression is reduced at day 23 (**H**). Student's *t*-test: $^{**}P<0.01$. *Bars* **A**, **B** 350 μm; **D**, **E** 40 μm; **F**–**H** 20 μm

MHC-II [44], CD4 [44] and B7.2 [8]. Hence, 1,25-D3 may limit the ability of monocyte/macrophages and microglial cells to function as antigen-presenting cells during EAE. This may also be linked to the amelioration of the symptoms on day 13, since it has been demonstrated that CD4 function-blocking strategies after the onset of the clinical signs led to an improvement of EAE symptoms [25, 38].

The clinical data indicate that 1,25-D3 exerts beneficial effects by reducing both the severity and the duration of the disease. However, it appears that when the hormone exerts an important effect during the first attack, the second attack occurs earlier and there is not a complete remission phase. This could be ascribed to the inhibition of NOS II synthesis by 1,25-D3 and to the timely NO contribution to the evolution of EAE. Interestingly, it has been observed that in NOS II-knockout mice EAE developed at a very early time point and that the course of the disease was acute, progressive and more severe [12, 46]. The fact that in control-EAE rats NOS II protein expression is globally more intense on day 19 (remission phase) than on day 13 also supports the ambiguity of NO action. Thus, depending on the stage of the disease and the producing cell sub-population, NO might display beneficial effects in parallel to its detrimental ones [18]. Indeed, NO is able to inhibit leukocyte proliferation, adhesion and migration [28], and may trigger the apoptosis of both macrophages [1, 32, 36, 37, 52] and CD4-positive T lymphocytes [11, 52], events which are likely involved in the control of EAE evolution [2]. This explanation is also relevant to the data obtained in a model of focal rat brain inflammation, which suggest that the down-regulation of NOS II expression by 1,25-D3 may lead to an inhibition of macrophage/microglia apoptosis [14]. In this context, it is important to note that EAE treatment with NOS II inhibitors leads to conflicting results. A suppression of clinical signs was sometimes observed [5, 10, 47], whereas in other cases they aggravated the disease [45, 55].

Considering the present data together with our previous work, we have demonstrated that 1,25-D3 acts by reducing NOS II expression during the first and second attacks of EAE, on both CNS-infiltrating cells and CNS constituent cells represented by microglial cells and astrocytes [13]. Different lines of evidence support a direct effect of the hormone on NOS II gene in CNS constituent cells during EAE. (1) Both astrocytes and microglial cells are VDR-expressing cells (see introduction). (2) The hormone effects on NOS II gene take place very early after the beginning of the treatment. (3) Using computer analysis, a putative VDRE has been shown in the rat NOS II-gene promoter, which is very similar to the one found in the rat ANF gene promoter. Rat ANF gene expression has been shown to be inhibited in vivo by vitamin D treatment [50]. (4) 1,25-D3 effects are independent of a modulation of TGF-β1 expression. This last statement does not mean that 1,25-D3 and TGF-β1 could not be both involved in the inhibition of NOS II synthesis and in the reduction of the severity of the clinical scores during EAE, as seen in the mouse model [7], but that, if this were the case, the modalities in the rat model are distinct. Indeed, TGF-β1 is already synthesized on day 13, concomitantly to the 1,25-D3 effects. Moreover, they both share the ability to decrease the expression of NOS II (see introduction). Finally, it has recently been demonstrated that specific SMAD proteins, activated by TGF-β1, could act as a coactivator for ligand-induced transactivation of VDR by forming a nuclear complex with a member of the steroid receptor coactivator-1 protein family [53]. Further studies on synergistic/cooperative action of 1,25-D3 and TGF-β1 for the regulation of NOS II expression during EAE are needed to confirm this conjecture. Alternatively, the action of 1,25-D3 on NOS II might also lie at the level of NF-κB activation, since in other systems, a modulation of NF-κB protein levels by 1,25-D3 has been reported [54]. Other transcription factors of potential relevance for NOS II inhibition are c-Jun N-terminal kinase and activator protein-1 [49], which might also represent potential targets for 1,25-D3.

In parallel, in experiments made on rats deprived in vitamin D, we observed a tendency for an exacerbation of EAE clinical signs (earlier first attack, no true remission phase/score >1 and higher clinical scores at the second attack). This could be ascribed to the lack of constitutive immunosuppressive effects of the hormone, potentially available in the CNS in rats submitted to a classical diet. Since vitamin D-deprived EAE animals display a comparable profile of EAE clinical signs, it seems likely that the endogenous hormone is not fundamentally involved in the succession of molecular and cellular events that govern the EAE clinical course of the Lewis rat. Rats treated with MC1288, a less hypercalcemic 1,25-D3 analogue, displayed at low dose the effect already observed in standard EAE animals treated by 1,25-D3 with a precocious relapse. Moreover, it is noteworthy that, with both doses used, a significant clinical improvement was observed with respect to the incidence and mean clinical score of the relapse, which disappear totally using the highest dose.

In conclusion, this study demonstrates that, during chronic relapsing EAE of the Lewis rat, 1,25-D3 administered in a curative way exerts beneficial effects on clinical symptoms and on cellular and molecular events inside the CNS. These effects take place on CNS-infiltrating cells and CNS constituent cells very early after the commencement of treatment, modifying both the amplitude and the kinetics of the disease. NOS II, a key enzyme involved in EAE progression, constitutes a major target of the hormone, which, in contrast, does not modulate TGF-β1 gene expression. Finally, clinical results obtained in vitamin D-deprived EAE animals treated by the MC1288, an analogue of 1,25-D3, suggest a potential use of such compounds, which are devoid of the harmful calcemic effects found using the hormone, in the development of new therapeutic approaches for the treatment of MS.

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