

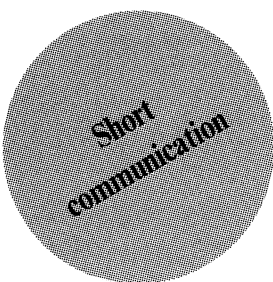
Difluoromethylornithine counteracts lesion-induced astrogliosis in rat hippocampus: enhancement of inhibitory effect by combined treatment with GM1 ganglioside

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Abstract. The effect of difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), the rate limiting enzyme of polyamine biosynthesis, and its combined action with GM1 ganglioside, was studied on the GFAP content in a model of remote astrogliosis evoked in the hippocampus by lateral fimbria transection. DFMO markedly suppressed hippocampal gliosis as measured by GFAP immunoblotting seven days postsurgery. Combined treatment with DFMO and GM1 ganglioside - a substance which alone also counteracts hippocampal gliosis, produced a stronger suppressive effect than DFMO. The results support the hypothesis of a causal link between lesion induced events: polyamine biosynthesis and astroglial reaction. Potentiation of the inhibitory effect of DFMO by GM1 ganglioside suggests that the latter does not act through the mechanism involving ODC suppression.

Key words: astrogliosis, glial fibrillary acidic protein, brain injury, ornithine decarboxylase, difluoromethylornithine, GM1 ganglioside, hippocampus



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Astrocytes respond to a variety of central nervous system (CNS) injuries by reactive gliosis that can be monitored by immunocytochemistry or immunoassay of the major intermediate filament protein of this cell type, glial fibrillary acidic protein (GFAP) (see for review Norton et al. 1992). Induction of ornithine decarboxylase (ODC), the rate limiting enzyme in the biosynthesis of polyamines has been also shown to occur in response to a variety of CNS insults (eg. Agnati et al. 1985a, Walsh et al. 1989, Skup and Grądkowska 1990). Since polyamine biosynthesis is critical for a number of cellular growth processes in the CNS (see eg. Bell et al. 1986, Slotkin and Bartolome 1986), the possible relationship between the lesion-induced activation of polyamine biosynthesis and the activation of astroglial cells has been postulated (Zini et al. 1990). For instance, irreversible inhibition of ODC elicited by difluoromethylornithine (DFMO), markedly diminished stab lesion - induced increase of GFAP content in the striatum (Zini et al. 1990). However, such an effect was not confirmed in conditions of neurotoxic brain insult (O'Callaghan and Seidler 1992), showing that the obligatory role of ODC induction in activation of astrocytes after lesions does not hold for each lesion condition. This would be in line with a recent view that although astrogliosis represents a universal response to all types of CNS injuries, the astrocyte response to such insults may be induced and maintained through different mechanisms (see Norton et al. 1992). Yet another type of astrogliosis is represented by a remote response of astrocytes occurring in the areas distant from the insult site. Diffuse and/or remote astrogliosis is observed in most human neurodegenerative disorders (e.g., Beach et al. 1989, Delacourte 1990, Kushner et al. 1991, Nagy et al. 1994), and therefore understanding of the underlying mechanisms might have potential significance in the development of therapeutic strategies.

The first aim of the present study was to investigate the potential role of ODC activation in the induction of remote astrogliosis. To this end we transected lateral fimbria, which lead to upregulation of ODC activity (Skup and Grądkowska 1990)

and of GFAP content in the hippocampus (Oderfeld-Nowak et al. 1993), and on the top of that we applied an ODC inhibitor, DFMO.

Since GM1 ganglioside also attenuates lesion - induced GFAP increment following lateral fimbria transection (Oderfeld-Nowak et al. 1993), the question arose, whether this effect of GM1 involves interaction with polyamines. Polyamines have been suggested to play a permissive role in GM1 ganglioside trophic action exerted upon neuronal recovery after brain injury (Agnati et al. 1985b, Zini et al. 1986, Skup and Grądkowska 1990). The second aim of this study was to investigate the effect of a combined treatment of DFMO and GM1 ganglioside on lesion-induced GFAP content increment in the hippocampus in the same lesion model.

Adult, male Wistar rats, bred in the Nencki Institute, weighing 200-240 g were used. The animals had free access to food (standard pellets) and tap water. The animals were operated under sodium pentobarbital (Nembutal, a dose of 50 mg/kg, i.m.) anaesthesia, by bilateral knife-cut of the lateral fimbria performed stereotactically, essentially as described by Hefti et al. (1984). There were three groups of lesioned animals: operated - untreated, operated - DFMO treated, and operated - receiving a combined treatment with DFMO and GM1 ganglioside. DFMO (Eflornithine HCl·H₂O kindly provided by Dr. P.P. McCann, Marion Merrell-Dow Research Institute, Cincinnati, Ohio, USA, was dissolved in saline and injected intraperitoneally on the first postoperative day in four equal doses (500 mg/kg), administered every 6 h starting immediately prior to the surgery (20 min after injection of the anaesthetic). GM1 ganglioside (99% purity, kindly provided by Fidia Research Laboratories, Abano Terme, Italy), dissolved before injection in 0.01 M phosphate buffer pH 7.4, in the daily dose of 30 mg/kg, i.m., was injected, starting on the day of the surgery. This regimen was exactly the same as used before, when GM1 ganglioside treatment upon postlesion gliosis was investigated (Oderfeld-Nowak et al. 1993). Naive rats served as controls. The rats were sacrificed by decapitation one or seven days after operation, the brains

removed and the hippocampi dissected out on ice and divided into three parts: dorsal, medial and ventral - along the longitudinal axis of the structure. Tissue samples were homogenized in 8 volumes of ice-cold 25mM Tris-HCl buffer (pH 7.4), which contained pyridoxal 5'-phosphate, DTT and EDTA. Samples of 10 μ l were taken out from each aliquot for GFAP and protein estimation. Protein content was measured according to the method of Bradford (1976). The remaining aliquots from each hippocampus of control, operated and operated - DFMO treated rats, were pooled and centrifuged at 48,000 \times g, 20 min at 4°C. In the supernatants the ODC activity and protein content were estimated.

Ornithine decarboxylase activity was estimated by the radiometric method of Russel and Snyder (1968) modified according to Slotkin and Bartolome (1983) with the use of DL- [1- 14 C] ornithine as described in detail elsewhere (Skup and Grądkowska 1990).

Electrophoresis and immunoblotting of GFAP were performed as described in detail earlier (Oderfeld-Nowak et al. 1993, Jegliński et al. 1995). The aliquots of homogenates were analyzed by sodium dodecyl sulfate (SDS) - polyacrylamide gel (PAGE) electrophoresis in 6 - 15 % acrylamide gradient slab gel according to Laemmli et al. (1970). Samples (15 - 20 μ l) of homogenates containing 5 μ g of hippocampal proteins were loaded onto each line of the gel. Homogenates from all investigated groups of animals and purified GFAP standards were run (each in duplicate) on the same gel. Electrophoresis was carried out at 30 mA for 45 min followed by 45 mA for 2.5 h in a vertical gel unit. After electrophoresis proteins were transferred onto nitrocellulose sheet as described by Towbin et al. (1979) at 90 mA for 24 h in Tris-glycine buffer, pH 8.3 containing 20% methanol. Blots were stained with 0.2% Ponceau Red S for 1 min for visualization of transferred proteins. After checking the effectiveness of the transfer the sheet was destained in distilled water. GFAP was detected immunochemically with the use of monoclonal mouse anti - rat GFAP antibody (Boehringer, Germany) at a dilution of 1 : 1,000. The immunochemical reaction

product was visualized with the use of avidin-biotin horseradish peroxidase kit (Vectastain) with 0.06% 3,3-diaminobenzidine and 0.01% H₂O₂ as a substrate. All washes between subsequent steps were performed in Tris - buffered saline (pH 7.5) containing 0.1% Tween 20. Immunoblots were analysed using computer assisted image analysing system with CCD video camera. The mean gray level of each stained band and its area was measured to give the value in pixels. The results were then plotted against the GFAP standard curve and the amounts of GFAP in analysed tissue samples were calculated and expressed as μ g of GFAP per mg of total protein.

Statistical analysis was performed using two-tailed Student's *t* - test.

Confirming our earlier data (Oderfeld-Nowak et al. 1993) an uneven increase of GFAP content along the longitudinal axis of the hippocampus was found 7 days after bilateral transection of lateral fimbria, amounting from about 20% to about 50% of the control values, in the dorsal and ventral parts, respectively (Fig. 1). No increase in GFAP content in any of the investigated hippocampal part was found one day after operation. Also confirming the previous data (Skup and Grądkowska 1990), obtained using the same experimental model, ODC activity was increased in the hippocampus after one day from the surgery, amounting to about 200% of the control value. Seven days from the operation, however, only an insignificant increase in the activity of this enzyme was noted. DFMO treatment attenuated early ODC induction in the hippocampus of operated rats, diminishing it by about 50%; no attenuation of ODC activity in the hippocampus was observed after 7 days. In the DFMO treated rats 7 days after operation attenuation of the lesion-induced increase in GFAP content in all parts of the denervated hippocampus was observed (Fig. 1, densely hatched columns). Attenuation of GFAP increment by DFMO was approximately the same magnitude in all three hippocampal parts ranging from 20 - 25%. As a result the GFAP increase was totally abolished in dorsal and medial parts and reduced by about 50% in the ventral part where was originally the strongest. Interestingly, the pattern of

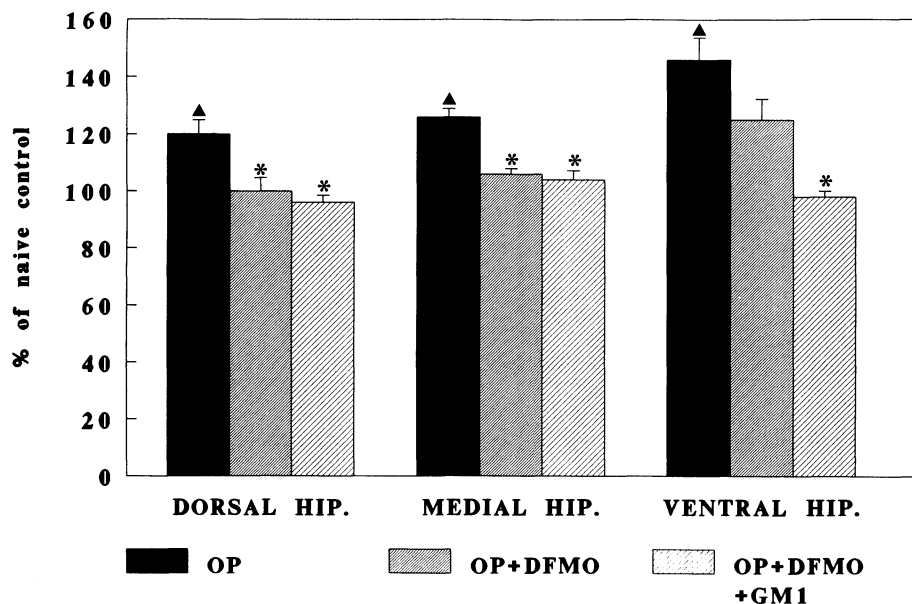


Fig. 1. Effects of DFMO treatment alone and combined treatment with DFMO and GM1 ganglioside on the GFAP content increase induced by lateral fimbria transection in the dorsal, medial and ventral hippocampus 7 days after surgery. The GFAP content in the respective hippocampal parts of naive control rats was taken as 100% and amounted to 6.83 ± 0.440 , 6.36 ± 0.626 and 6.11 ± 0.582 $\mu\text{g}/\text{mg}$ of total protein, in dorsal, medial and ventral parts, respectively. Data are given as the mean \pm SEM, for $n=8$. * $P < 0.05$ vs. operated untreated, $\blacktriangle P < 0.05$ vs. control.

DFMO-induced diminution of lesion - evoked astrogliosis along the longitudinal axis of the hippocampus strikingly resembles that induced by GM1 ganglioside treatment seen earlier in the same experimental model (Oderfeld-Nowak et al. 1993). The combined treatment with DFMO and GM1 ganglioside elicited a more profound effect on induced GFAP content, than the single treatment, which is evident in the case of ventral hippocampal part (Fig. 1, ventral HIP. - hatched column as compared with a densely hatched one). In fact, single treatments cut by half the lesion induced increase in GFAP (see earlier data for GM1 ganglioside effect alone, in Oderfeld-Nowak et al. 1993), but the combined treatment lead to a total abolition of increased GFAP (Fig. 1).

The present results demonstrate that remote lesion - induced astrogliosis is attenuated by DFMO treatment. This result is in line with earlier immunocytochemical data, obtained on the stab - wound model (Zini et al. 1990). Thus, our results provide a support for the hypothesis of an obligatory role of ODC/polyamine pathway in astrogliosis induced by mechanical injury, whether direct or remote. As judged from complete recovery of ODC activity at 7 days postlesion, the here observed attenuating effect of DFMO on astrogliosis in the hippocampus, reflects inhibition of early induction of ODC activ-

ity. Interesting is contrast of the present data and those of Zini et al. (1990), with those of O'Callaghan and Seidler (1992) who reported no ODC activation and lack of effect of DFMO treatment upon astrogliosis evoked by neurotoxic insult (MPTP injection). Mechanical lesions compromise the blood - brain barrier, allowing the entry into the brain of blood - born inflammatory cells participating in reactive gliosis (see Norton et al. 1992) which would not be the case in conditions of exposure to MPTP unaffected the blood - brain barrier.

Another interesting finding of the present study is the intensification of the DFMO-induced down regulation by combined action with GM1 ganglioside. The striking similarity of single effects of DFMO (present results) and GM1 found in the same experimental regimen in the hippocampus (Oderfeld-Nowak et al. 1993), implied a possibility of a common mechanism of action of both agents on attenuation of astrogliosis. However, this does not seem to be the case since a common mechanism would imply a nonadditive effect. The additive effect of both agents indicates that the attenuation of astrogliosis by GM1 ganglioside does not involve the inhibition of activation of ODC/polyamine pathway, and thus the action of the two may be independent. However, the combined inhibitory effect of two agents observed in this study may

indicate another, as yet not unravelled, mechanism by which polyamines mediate the effect of GM1 ganglioside on astrogliosis.

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This paper is dedicated to Professor Stella Niemierko on the occasion of her 90th birthday, with esteem and admiration