明した。BDNF20μg/day 投与では、対照群と比較して動物の活動性に大きな変化は認めなかった。

外傷群に対して受傷直後よりミニポンプを埋め込み BDNF20μg/day を投与したところ受傷 2 日では行動性が非外傷群に対して軽度低下していた（path 80.3±10.6%）が、1 週、2 週の時点では BDNF 非投与群に比して著しい活動性の増加を示し（1 週後 path 120±35.9%、2 週後 path 139±23.9%）非外傷群と比べてもむしろ行動量が増加していた(Fig. 7, 8)。動物の行動は field の周囲を回る動きが中心であり、正常動物に認められるパターンであった(Fig. 9)。

脳損傷を加えることにより動物の体重は 2 週後をピークとして約 10%減少する（2 週後 88.7%）が 4 週後には前値に復した。BDNF 脳室内持続投与により体重現象は増強され、非投与群に比し著しく低下した（2 週後 67.6%）。 (Fig. 10)。

D. 考察

前年度は、慢性脳損傷モデルの作成と形態学的解析を行った。本年度は、このモデルを用いて行動解析ならびに治療実験を試みた。

受傷動物は肉眼的観察によっても明らかに自発活動が低下しており、本研究による定量的解析においても肉眼的観察結果が実証された。open field での走行距離は 2 日、1 週、2 週でそれぞれ前値の約 43%、53%、55%に低下した。また行動開始までの時間も 2 日後 394%、1 週後 485%と延長し、2 週後には 204%と軽度の改善傾向を示したが依然として受傷前とは著しい差異が認められた。

BDNF は、発生期における神経細胞の生存維持、分化に作用しているが、成熟脳でも広く豊富に存在している。神経細胞の生存を維持するとともに、近年の知見によれば順行性に軸索を輸送されシナプス伝達の増強に重要な作用を行っているとされている。後者の重要な系として青班核よりの中枢ノルアドレナリン投射系が注目されている。そこで、免疫染色により青班核神経細胞における損傷後の BDNF 染色性を検討した。脳損傷を加えていない対照群では、青班核神経細胞は BDNF 免疫染色において軽度に陽性を示していたが、受傷後 1 日では同部神経細胞の BDNF 染色性が増強された。さらには青班核よりの投射繊維においても BDNF 染色が強く陽性に認められた。1 週、2 週、4 週後の標本においては BDNF 染色性は低下しほとんど認められなかった。損傷後早期の BDNF 軸索輸送の停滞とその後の産生低下が示唆され、外部よりの補充により何らかの治療効果が期待し得ると考えられた。

そこでミニポンプを用いて BDNF を髄腔内に持続投与を行った。受傷 2 日では行動性が非外傷群に対して軽度低下していた(path 80.3±10.6%）が、対象群との差はなく、1 週、2 週の時点では BDNF 非投与群に比して著しい活動性の増加を示し（1 週後 path 120%、2 週後 139%）非外傷群と比べてもむしろ行動量が増加していた。さらには、動物の行動は正常動物に認められるパターンであった。この行動改善効果の機序としては、投与された BDNF によるシナプス伝達の増強などが考えられる。このような行動改善効果が投与終了後も長期に維持されるかどうか今後さらに検討を加える必要がある。

BDNF 投与による副作用としては、体重減少があげられる。脳損傷を受けた動物は 2 週後に約 10％の体重減少を呈するが、BDNF 脳室内投与群では 68%もの体重減少が生じた。過去の文献にも BDNF により体重減少を来すことが報告されており、本研究の結果は合致するものである。視床下部での

セロトニン、ドーパミンなどの変動との関係が示唆されている。今回の行動改善効果も種々の神経伝達物質の作用が増強されたことによる可能性を示唆するものではないかと考える。これらの変動についても今後検索する必要がある。BDNF 投与群での受傷直後の死亡率は24%で非治療群と変化ないが、1週、2週での生存が悪く2週後では20%にとどまった。途中死亡した動物においても行動は活発であり死亡原因はいまだ特定されていない。文献的に BDNF 脳室内投与が致死的であったとの報告もなく、BDNF によるものか、他の要因によるものか今後究明する必要がある。

E. 結論

びまん性軸索損傷モデルにおける行動異常について open field 法で定量的に解析を行った。また、免疫染色により本モデル動物では BDNF の輸送停滞、産生低下が示唆された。外部より脳室内に BDNF を持続投与することにより動物の行動性が活発となることが定量的に確認された。今後の治療法として臨床応用に向けて安全性他の面でもさらに検討を加えていく必要がある。

F.研究発表

1）論文発表

Fujinaka T, Kohmura E, Yuguchi T, Yoshimine T: Shrinkage of locus coeruleus neurons in diffuse brain injury model. Advances in Neurotrauma Res Vol.10, 1998 (in press)

2）学会発表

藤中俊之、甲村英二、湯口貴導：び慢性脳損傷モデルにおける青斑核の変化. 第10回神経損傷の基礎シンポジウム、東京、1998

藤中俊之、甲村英二、湯口貴導：び慢性脳損傷モデルにおける中枢ノルアドレナリンニューロンの変化、第22回日本神経外傷学会、福岡、1999

藤中俊之、甲村英二、湯口貴導、埜中正博、西尾雅実、吉峰俊樹：び慢性脳損傷モデルにおける行動変化の定量的解析、第23回日本神経外傷学会、福島、2000

Fig. 1

Weight-Drop Device

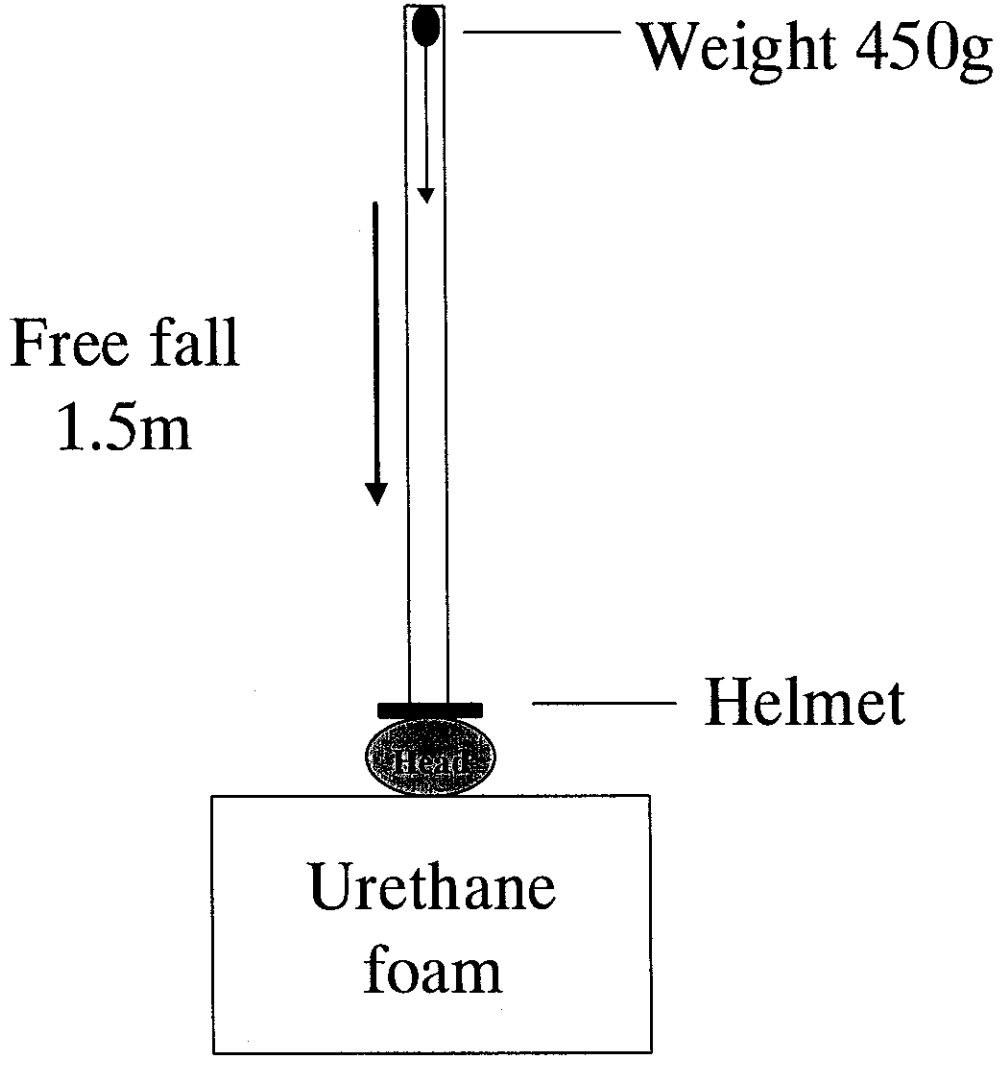


Fig. 2

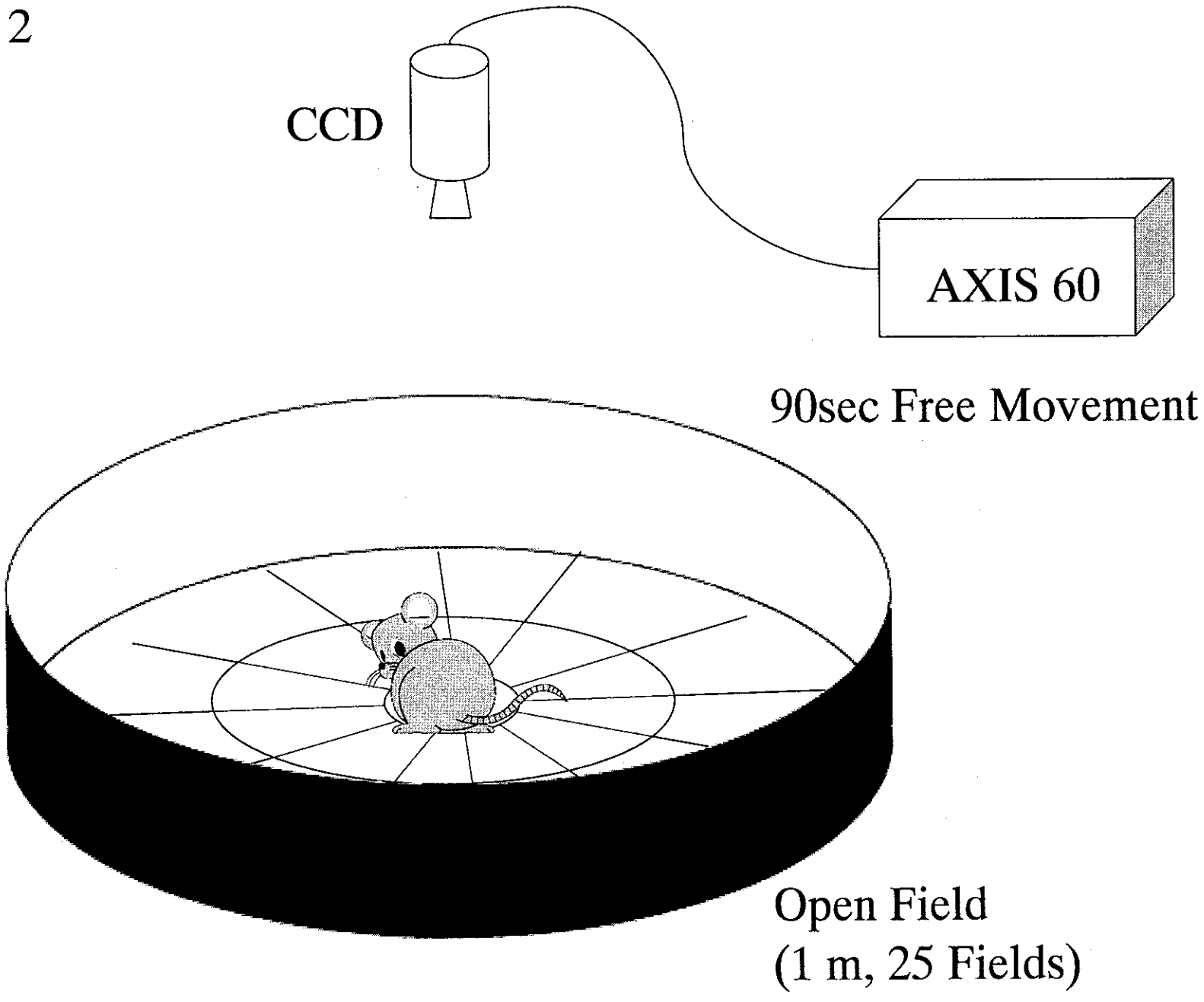


Fig. 3

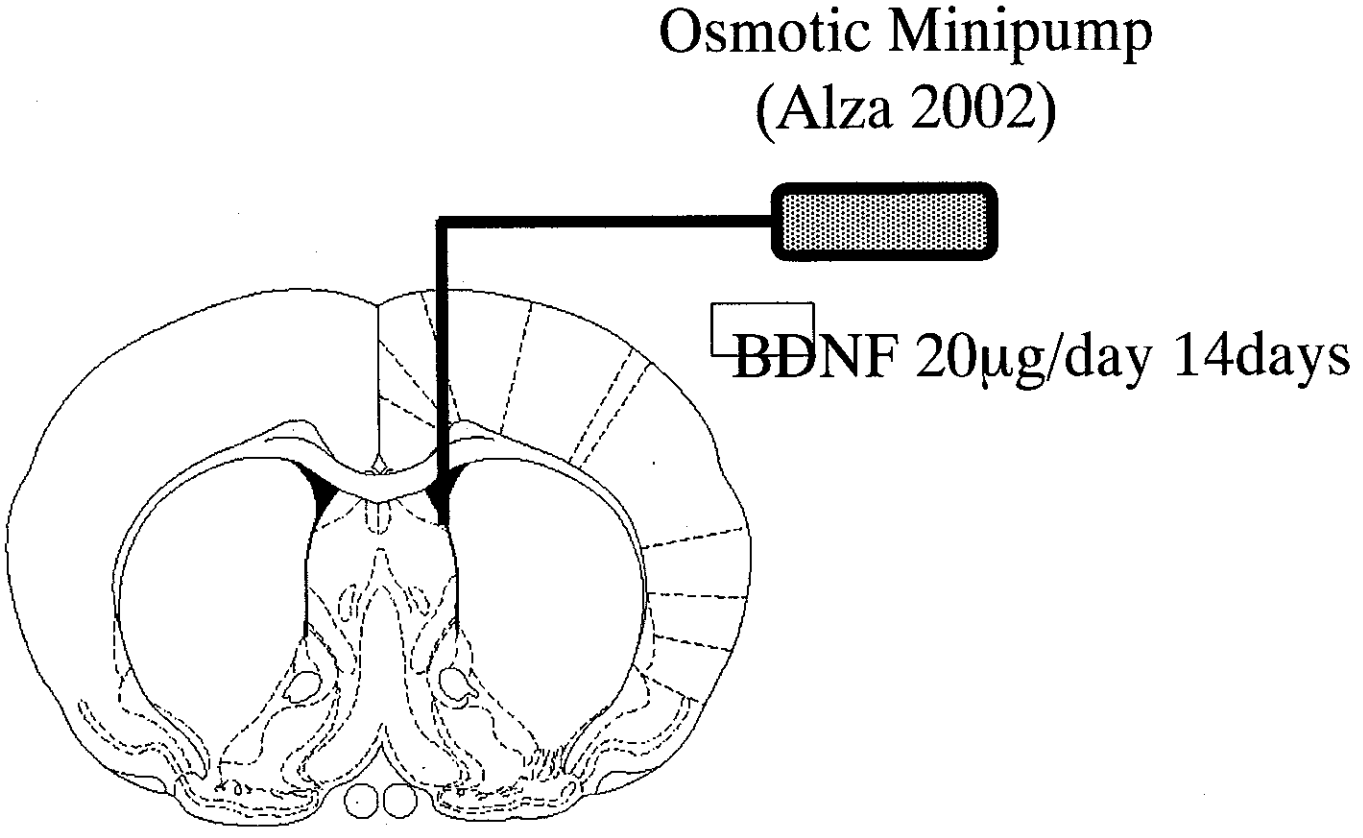


Fig. 4

Open Field

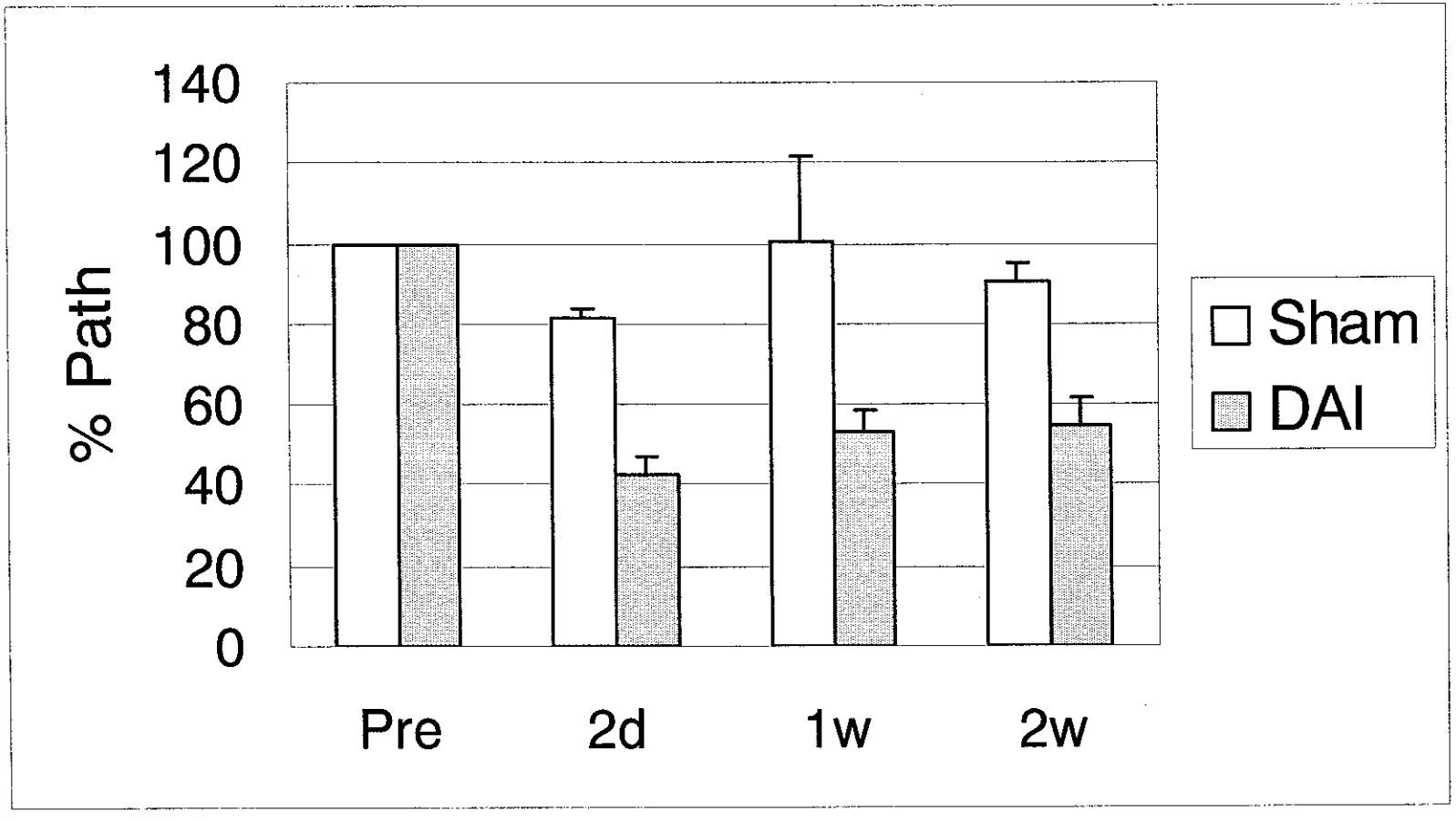


Fig. 5

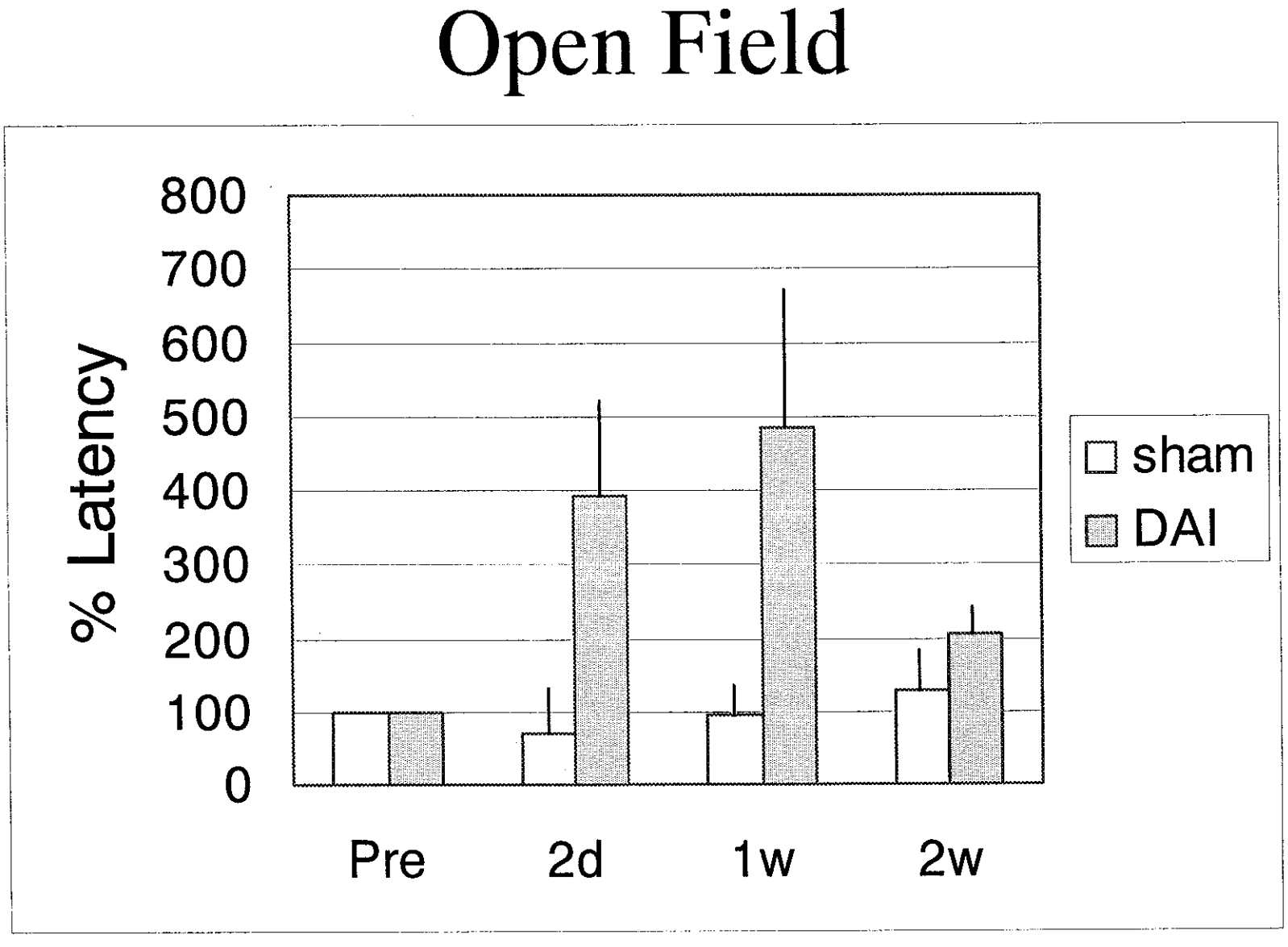
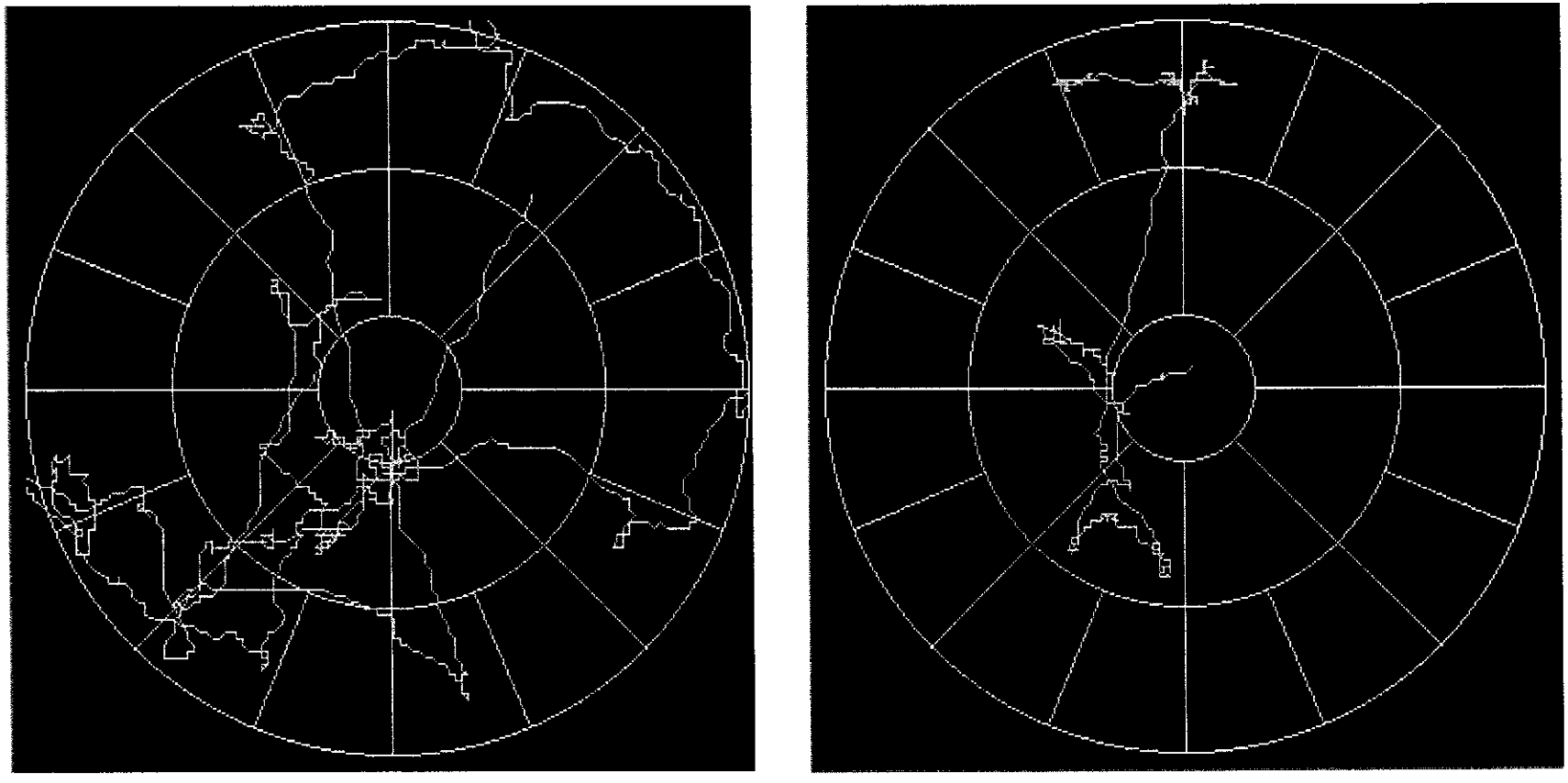

Open Field
% Latency
800
700
600
500
400
300
200
100
0
Pre
2d
1w
2w
sham
DAI

Fig. 6

Open-Field Behavior



Before DAI

1W after DAI

Fig. 7

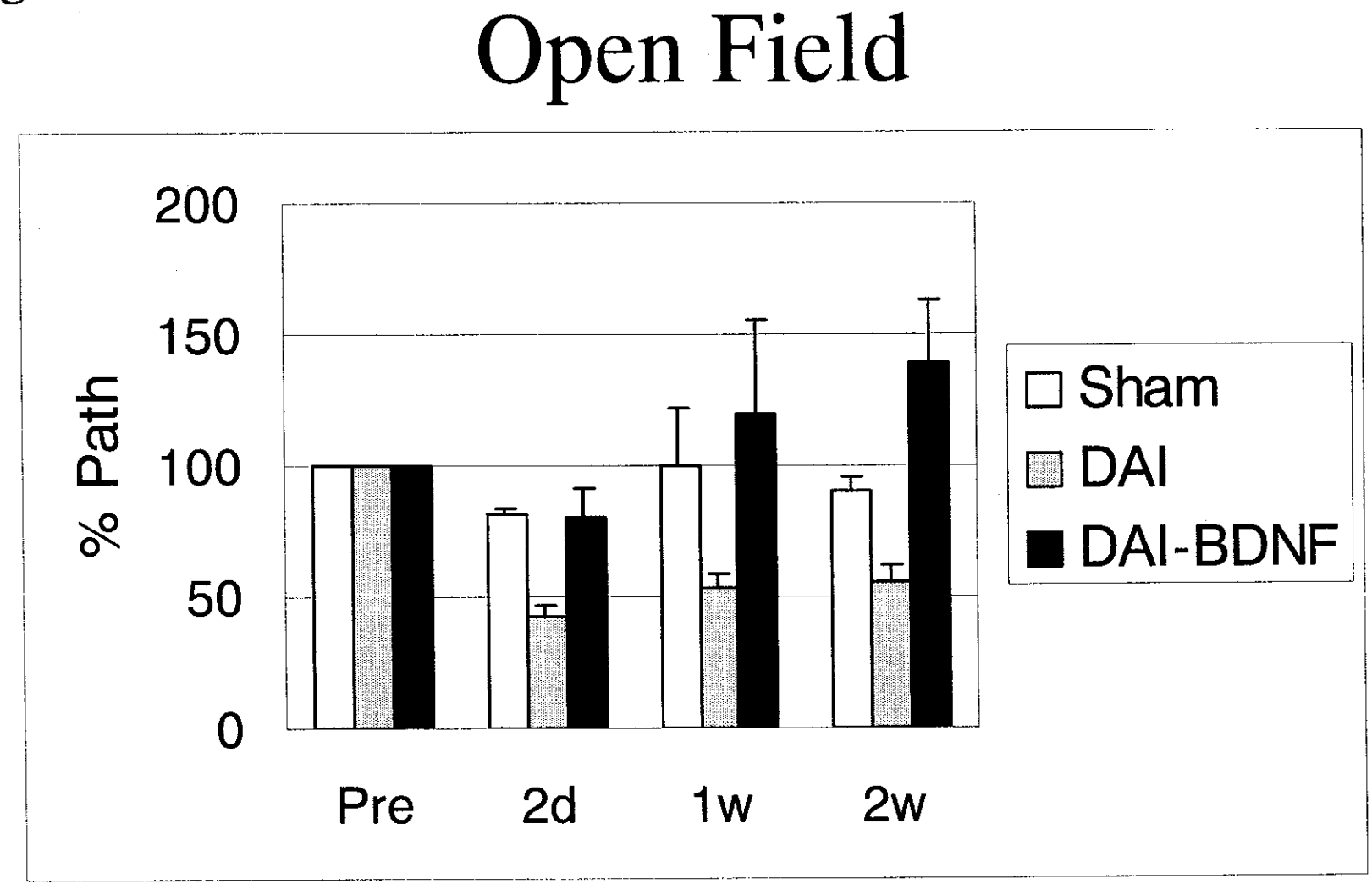


Fig. 8

Open Field

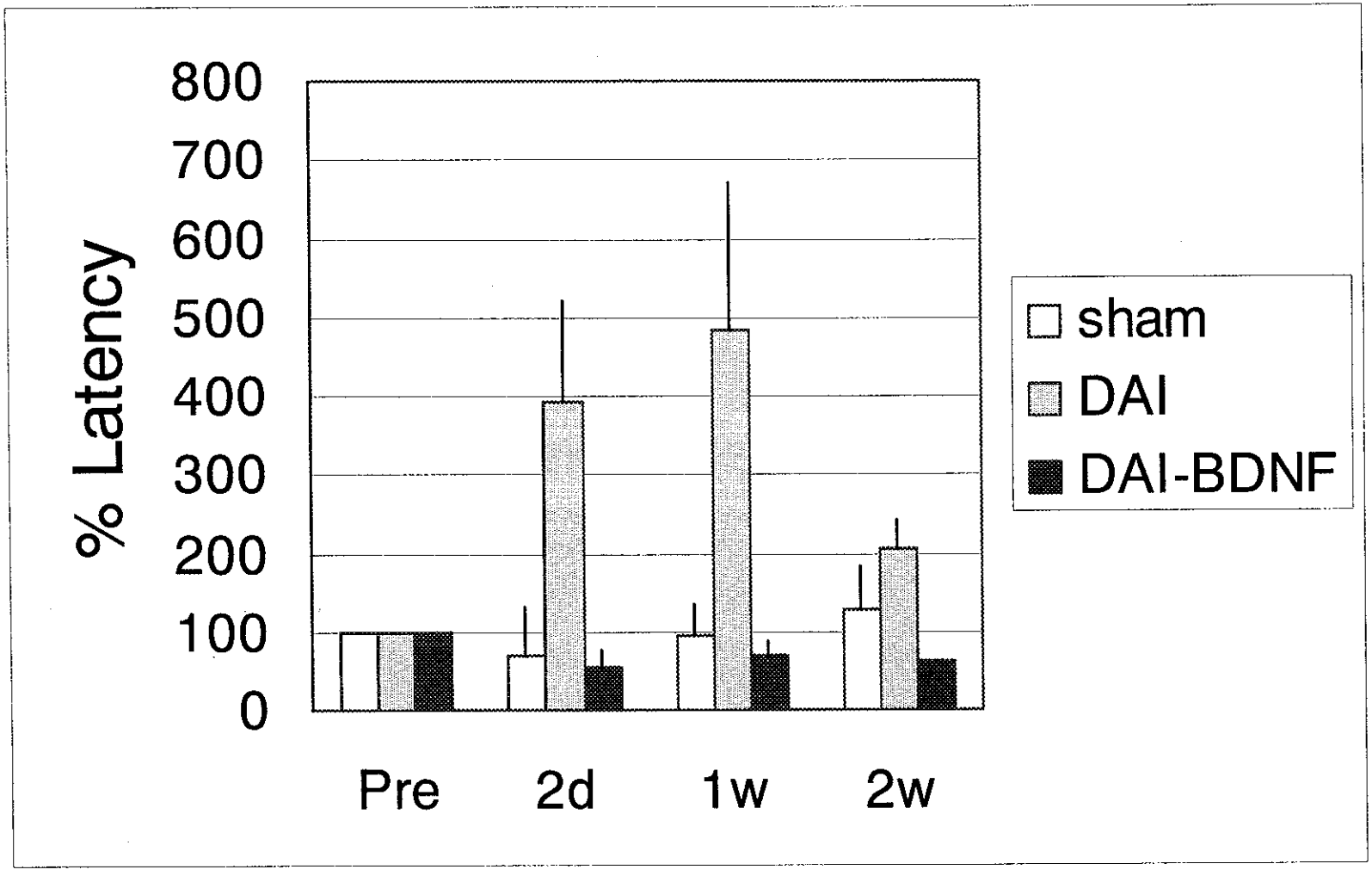
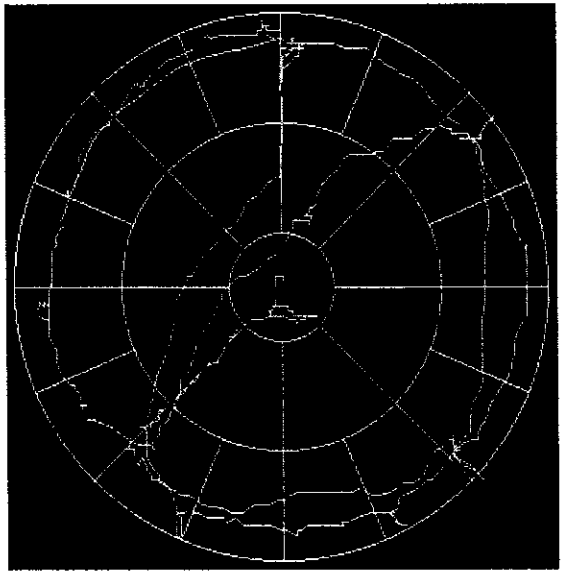


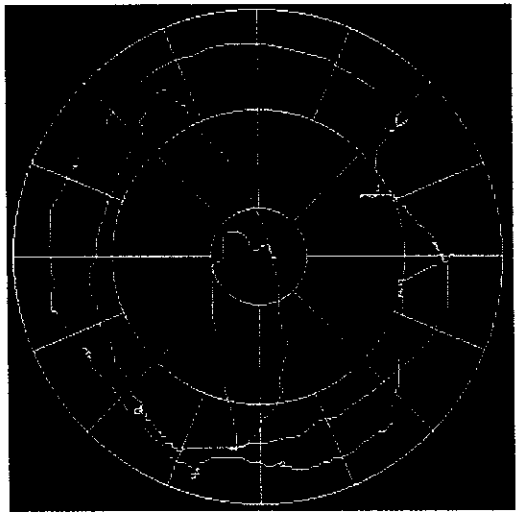
Fig. 9



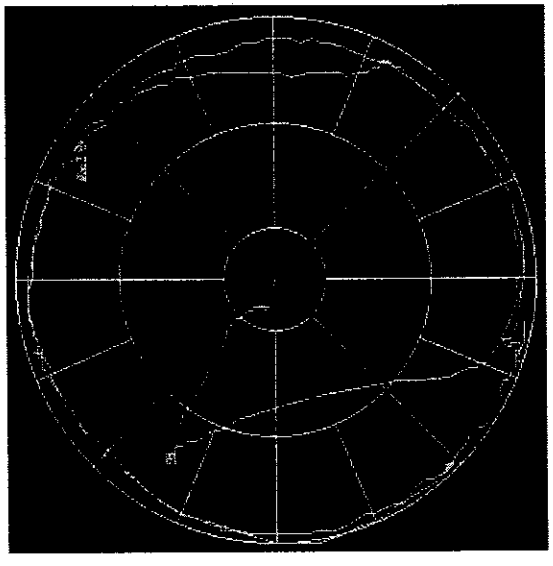
Before

BDNF

(+)



1W after DAI



2W after DAI

Fig. 10

Body Weight

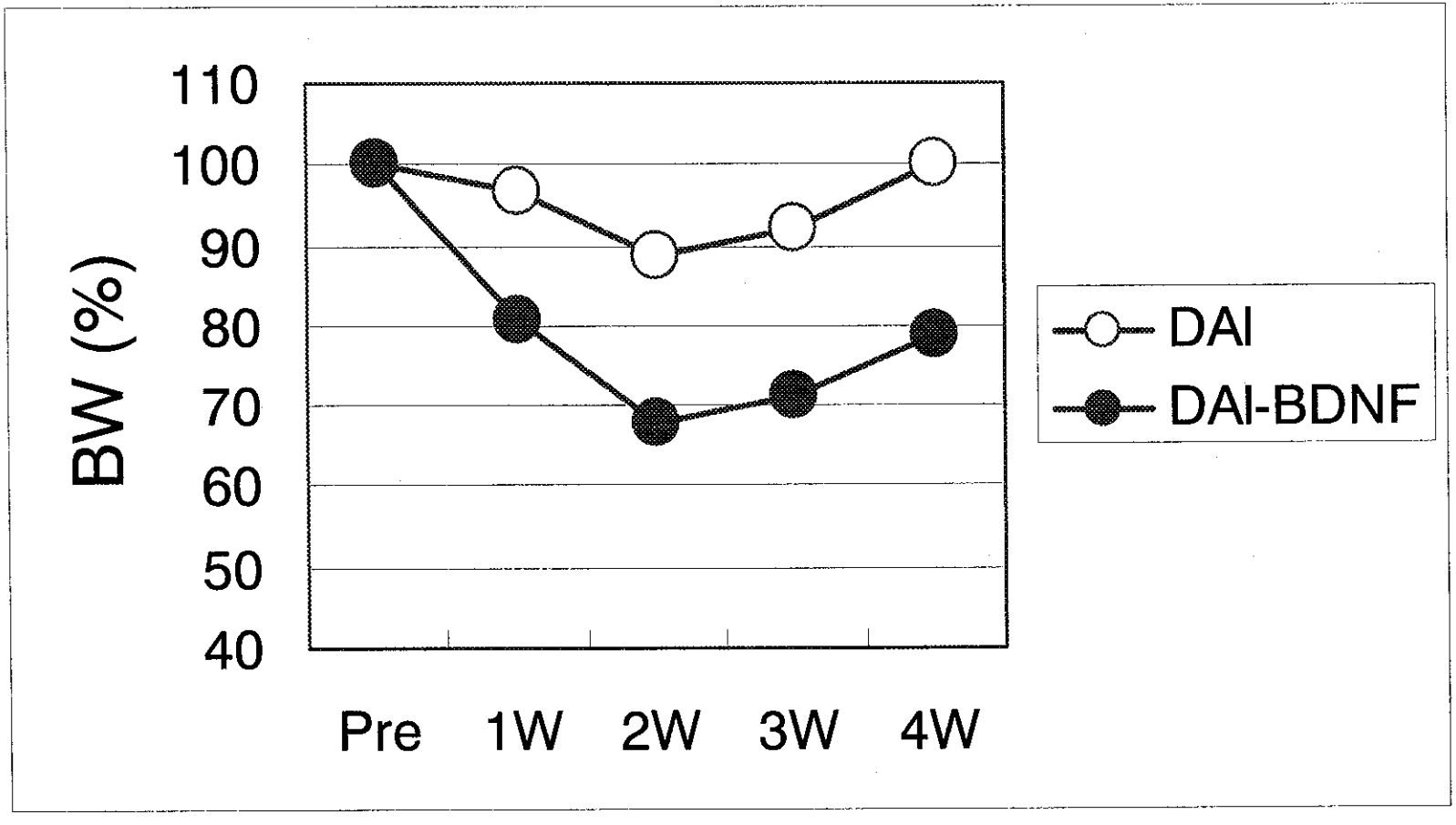


Table 1

Diffuse Brain Injury Model

- Survival rate
  - Immediate 80.5%
  - 2 days 77.9%
  - 7 days 52.0%
  - 14days 50.7%

(n=77)

- Seizure
  - Survived group 62.8%

Accumulation of BDNF protein in locus coeruleus neurons in diffuse brain injury model

Toshiyuki Fujinaka, Eiji Kohmura, Takamichi Yuguchi, Masahiro Nonaka, Masami Nishio, Toshiki Yoshimine

Department of Neurosurgery, Osaka University Graduate School of Medicine

Address correspondence to : Toshiyuki Fujinaka, M.D.
Department of Neurosurgery, Osaka University Graduate School of Medicine
2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN
Tel: +81-6-6879-3652 Fax: +81-6-6879-3659

Introduction

It is known that brain-derived neurotrophic factor (BDNF) regulates the survival and differentiation of the target neurons. And it have been recently reported that BDNF is anterogradely transported and released from the terminals of noradrenergic neurons.[1,2,3,6] We previously reported morphological change of locus coeruleus (LC) neurons, the center of noradrenergic system, in an impact-acceleration brain injury model.[5]

In this study, alteration of BDNF protein in locus coeruleus neurons in a diffuse brain injury model was examined to understand the effect of the injury.

Materials and Methods

Diffuse Brain Injury Model

The diffuse brain injury model in rats developed by Marmarou et al.[4,7] was used with slight modifications. Adult male Sprague-Dawley rats weighing 500-550 g were anesthetized with chloral hydrate (350 mg/kg, i.p.) and placed in prone position on a foam bed with spontaneous breathing. Dropping a brass weight (450 g) freely by gravity from a height of 1.5 m onto a metallic helmet (20 mm in diameter and 1.5 mm thick) fixed to the skull vertex, impact-acceleration brain injury was produced. The rat was moved away immediately to prevent a second impact and then observed for several minutes. After removing the helmet, scalp was sutured and the rat was returned to its cage. As a control group, rats were treated as described above except receiving impact.

Brain Fixation and Immunohistochemistry

At 24 hours, 48 hours, 7 days, 14 days, 28 days and 56 days after injury, two animals each from the experimental and control groups were anesthetized and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). The brains were removed and postfixed with the same fixative for 24 hours. After immersed in 30% sucrose in 0.01M phosphate buffered saline (PBS), the brains were embedded in Tissue-Tech and stored at -80℃. Fixed brains were sliced into 12 μm serial coronal sections from 8 mm to 11 mm posterior to the bregma including LC on a freezing microtome and processed for immunohistochemistry.

Immunohistchemistry was done for BDNF, dopamine-β-hydroxylase and neurofilament. Sections were washed in PBS and permeabilized with acetone at -20℃ for 10 minutes, and then incubated with 0.3% hydrogen peroxide for 30 minutes to quench innate peroxidase. After washes in PBS, blocking of nonspecific biding was achieved with 2% normal horse serum in buffer (0.1% Triton X-100 and 5% sucrose in PBS) for 20 minutes, and sections were finally incubated overnight at 4℃ with primary antibody. The following antibodies were used for the immunohistochemical studies : Anti rat BDNF rabbit monoclonal antibody diluted 1:50, Wako Pure Chemical Industries, anti 68-kD neurofilament mouse monoclonal antibody diluted 1:50, Boehringer Mannheim Biochemica, anti dopamine-β-hydroxylase rabbit polyclonal antibody diluted 1:1000, Eugene Tech International. VECTASTAIN Elite ABC Kit (Universal) was appropriated to visualize immunolabeled structures according to the manufacturer's recommendation. The precipitate formed by DAB was enhanced with $NiCl_2$.

Result

Fifty rats underwent diffuse brain injury, and immediate mortality was 22%. Post traumatic seizure was observed in 36 (72%) animals through the experiment and in 26 (67%) survivors. Recovery from anesthesia was protracted in injured animals compared with control animals. Spontaneous motility in the survivors was reduced immediately after recovery from anesthesia and was gradually improved. Gross pathological observation showed subarachnoid and intraventricular hemorrhages commonly. No skull fracture nor contusion were observed in any animals. Immunohistochemistry for anti-neurofilament 68-kD showed extensive axonal injury particularly in the brain stem at one or two days after injury.

Neuronal cell bodies in LC were partially positive for BDNF immunoreactivity and faint immunostaining was rarely seen in noradrenergic fibers in control animals. At 24 hours after injury, cell bodies of LC neurons were strongly positive for BDNF immunoreactivity and significant immunostaining could be seen in swollen axons. At 7, 14 and 28 days after injury, however, BDNF immunostaininng was weak compared with control animals, and restored in 56 days. (Fig.1, 2)

Discussion

Neurotrophic factors are traditionally thought that they are produced by target cells and promote neuron survival through their retrograde transport and signaling to the cell body. Recent studies have shown that BDNF is anterogradely transported through axons from dopamine, norepinephrine or epinephrine-containing neurons and regulates the survival and differentiation of its innervating neurons.[2,3,6] Furthermore, anterograde transport of BDNF suggests a possible neurotransmitter-like role modulating synaptic transmission.

The present study revealed alteration of BDNF protein expression in LC neurons in a diffuse brain injury model. At 24 hours after injury, neuronal cell bodies in LC were strongly positive for BDNF immunoreactivity , and at 7, 14 and 28 days after injury, BDNF immunoreactivity was reduced and restored in 56 days. Axons of noradrenergic neurons were swollen and significant immunostaining for BDNF could be seen at 24 hours after injury.

Increase of BDNF immunoreactivity in the early stage probably indicated the accumulation and stagnancy of BDNF in neuronal cell bodies and axons caused by the impairment of axonal anterograde transport of BDNF due to axonal damage. This hypothesis is compatible with anterograde neurotrophic factor theory. Decrease of BDNF immunoreactivity in the later stage may have been caused by the dysfunction of neuronal cell bodies or by the active downregulation. Further studies should be made about the translational level and the change in other regions of brain.

Acknowledgment

This work was supported by grant for Research on Brain Science from Ministry of Health and Welfare.

Reference

1) Castren E, Thoenen H, Lindholm D (1995). Brain-derived neurotrophic factor messenger RNA is expressed in the septum, hypothalamus and in adrenergic brain stem nuclei of adult rat brain and is increased by osmotic stimulation in the paraventricular nucleus. Neuroscience 64, 71-80.

2) Conner JM, Lauterborn JC, Yan Q, et al (1997). Distribution of brain-derived Neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: Evidence for anterograde axonal transport. J. Neurosci 17, 2295-2313.

3) Fawcett JP, Bamji SX, Causing CG, et al (1998). Functional evidence that BDNF is an anterograde neurotrophic factor in the CNS. J. Neurosci 18, 2808-2821.

4) Foda MA, Marmarou A (1994). A new model of diffuse brain injury in rats, Part II: Morphological characterization. J Neurosurg 80, 301-313.

5) Fujinaka T, Kohmura E, Yuguchi T, et al (1998). Shrinkage of locus coeruleus neurons in diffuse brain injury model. Adv Neurotraum Res 10, 63-66.

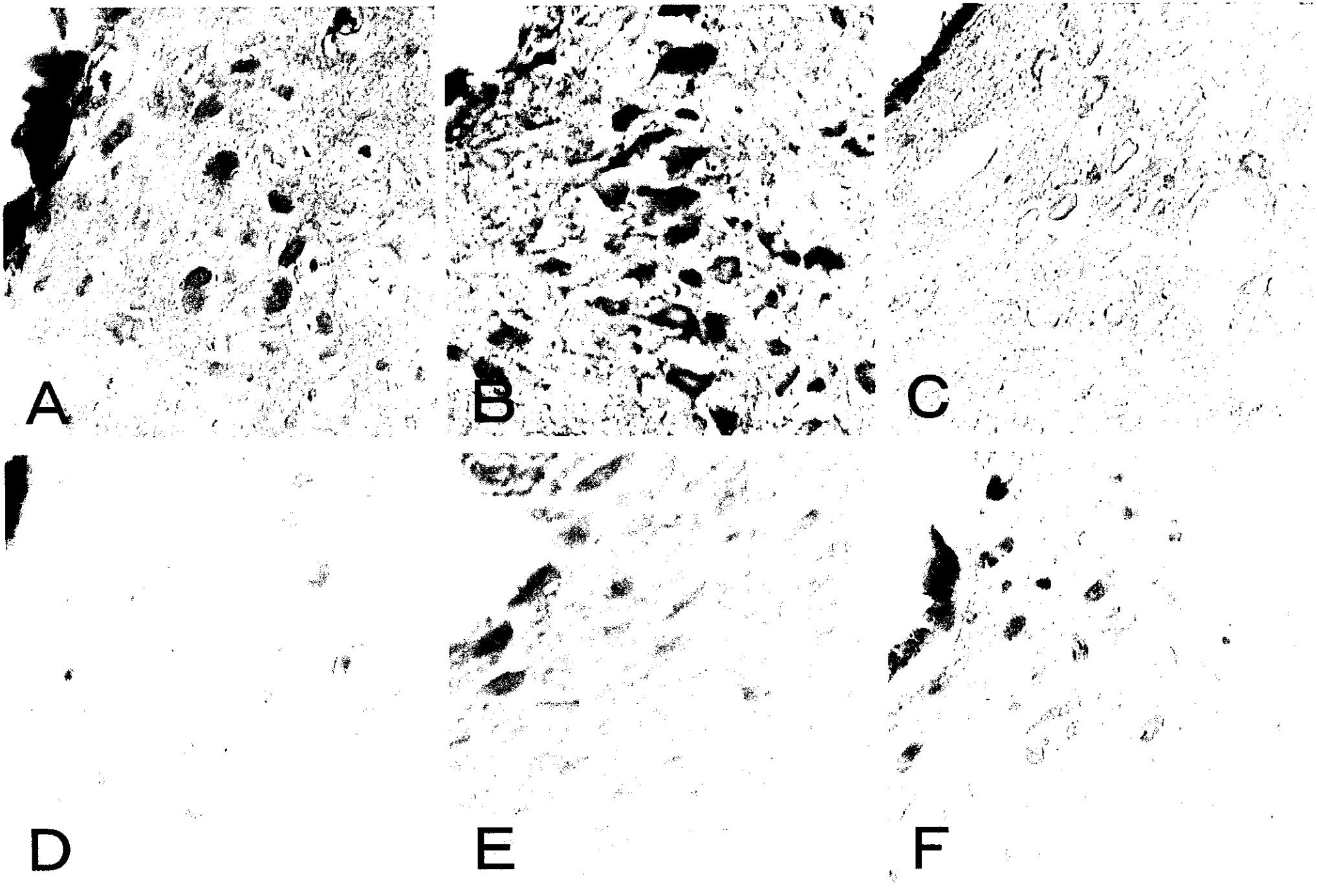
6) Kokaia Z, Andsberg G, Yan Q, et al (1998). Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: Evidence for increased synthesis and anterograde axonal transport. Exp. Neurol 154, 289-301.

7) Marmarou A, Foda MA, Van Den Brink W, et al(1994). A new model of diffuse brain injury in rats, Part I: pathophysiology and biomechanics. J Neurosurg 80, 291-300.

Figure legends

Fig.1. Photomicrographs of brain sections immunostained with anti-BDNF at 24 hours after injury. Neuronal cell bodies in LC were strongly positive for BDNF immunoreactivity (left). Diffuse axonal swelling and significant immunostaining for BDNF was observed in the reticular formation of upper pons (right).

Fig.2. Photomicrographs of neuronal cell bodies in LC immunostained with anti-BDNF in control animal (A), 24 hours after injury (B), 7 days after injury (C), 14 days after injury (D), 28 days after injury (E), 56 days after injury (F). At 24 hours after injury, neuronal cell bodies in LC were strongly positive for BDNF immunoreactivity. At 7, 14 and 28 days after injury, BDNF immunoreactivity was reduced, and restored in 56 days.


A
B
C
D
E
F