1:400 (DAKO, Glostrup, Denmark) for 7 min in the micro oven. The positive reaction resulted in brown staining with the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB). After immunohistochemistry, autometallography method was performed as previously discribed. Then the sections were counterstained with hematoxylin.

Transmission electron microscopy

The spinal cords fixed in buffered 4% paraformaldehyde were fixed with 2.5% glutaraldehyde, rinsed in 0.1M phosphate buffer (pH: 7.4). Tissues were performed Autometallography method. And tissues were post fixed in 1% osmium tetroxide, dehydrated in alcohols and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-100CX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Gross findings

No significant changes were observed in any organs.

Histological findings

No significant changes were observed in the spinal cord.

Histological localization of mercury

No silver-mercury grains were detected in the control sections.

Spinal cord

After exposure to mercury vapor for 1 day, silver-enhanced mercury was not detected (Fig. 2-A, B). After exposure to mercury vapor for 3, 7 and 10 days, silver-enhanced mercury was detected in the neurons in the nucleus motorius (Fig. 2-C, D and 3). After exposure to mercury vapor for 14 days, silver-enhanced mercury was spread over the neurons in the nucleus intermediolateraris (Fig. 4-A, B). After exposure to mercury vapor for 21 days, silver-enhanced mercury was spread further on the neurons in nucleus proprius dosalis (Fig. 4-C, D). Then after exposure to mercury vapor for 28 days, silver-enhanced mercury was spread over the neurons in nucleus posteromarginalis (Fig. 5-A, B). The amount of mercury granules in the neurons showed time dependent increase (Fig. 7). From 14 days after exposure, silver-enhanced mercury was detected in the astrocytes (Fig. 6).

No mercury granules were detected in the endothelial cells of blood vessels (Fig. 5-C).

In electron microscopy, mercury granules like substances were detected in the lysosome like structures of neurons and astrocytes (Fig. 8).
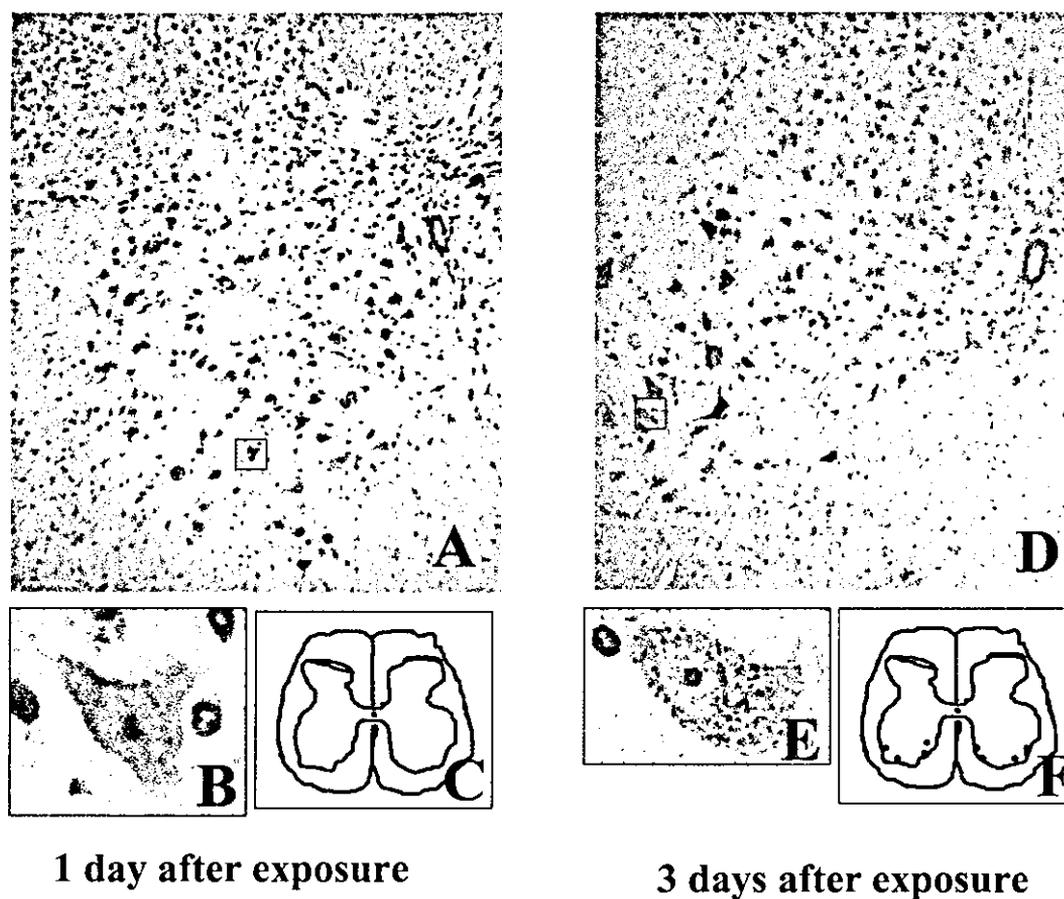
1 day after exposure　　**3 days after exposure**

Fig.2

Histological localization of mercury in the spinal cord 1 and 3 days after Hg⁰ vapor exposure.

One day after exposure, no mercury granules were detected in the spinal cord. Three days after exposure, silver-enhanced mercury was detected in the neurons in the nucleus motorius. Fig. 2-B and E show the neuron in the nucleus motorius. Fig. 2-C and F show shematic drawing of mercury distribution and amounts of mercury granules. Autometallography. Fig. 2-A ꞉ ×72　Fig. 2- B ꞉ ×1320　Fig. 2-D ꞉ ×120　Fig. 2-E ꞉ ×800

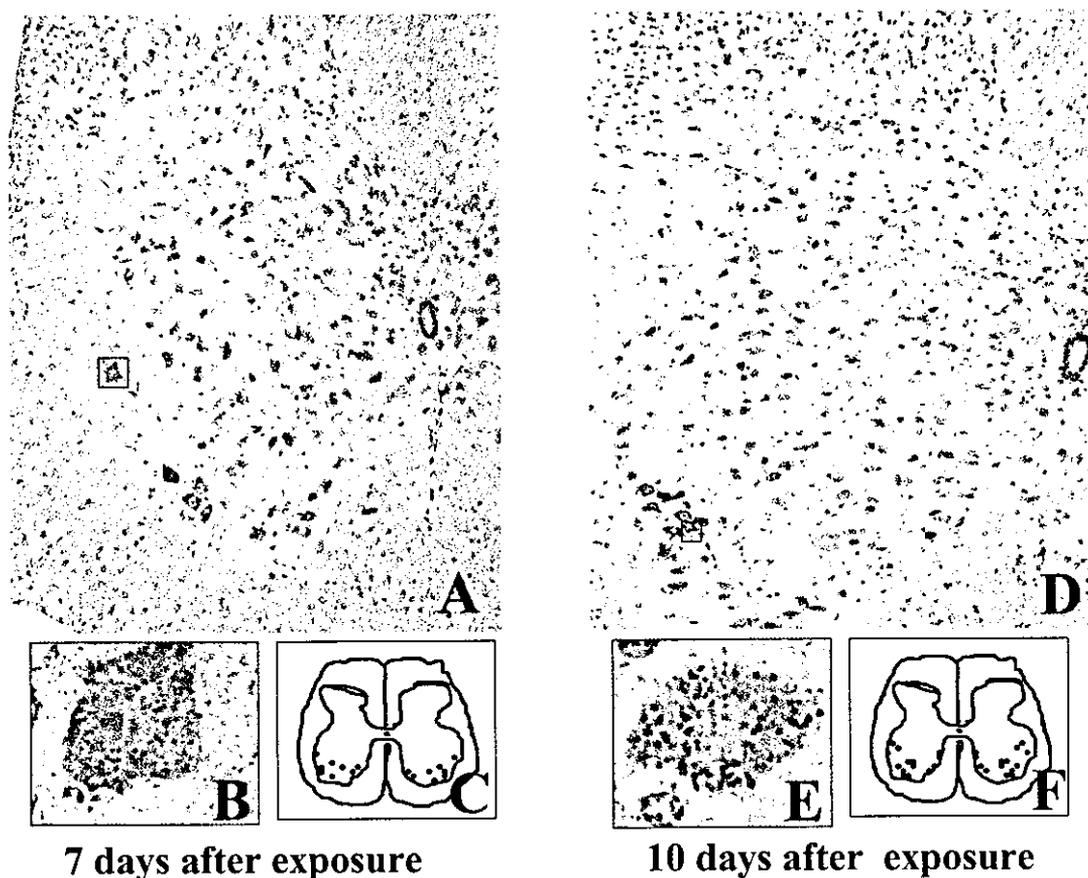**7 days after exposure**   **10 days after exposure**

Fig. 3
Histological localization of mercury in the spinal cord 7 and 10 days after Hg^0 vapor exposure.

Seven and ten days after exposure, silver-enhanced mercury was detected in the neurons in the nucleus motorius.   Fig. 3-B and E show the neuron in the nucleus motorius.   Fig. 3-C and F show schematic drawing of mercury distribution and amounts of mercury granules.   Autometallography.   Fig. 3-A and D ： ×96   Fig. 3-B ： ×800   Fig. 3-E ： ×1200

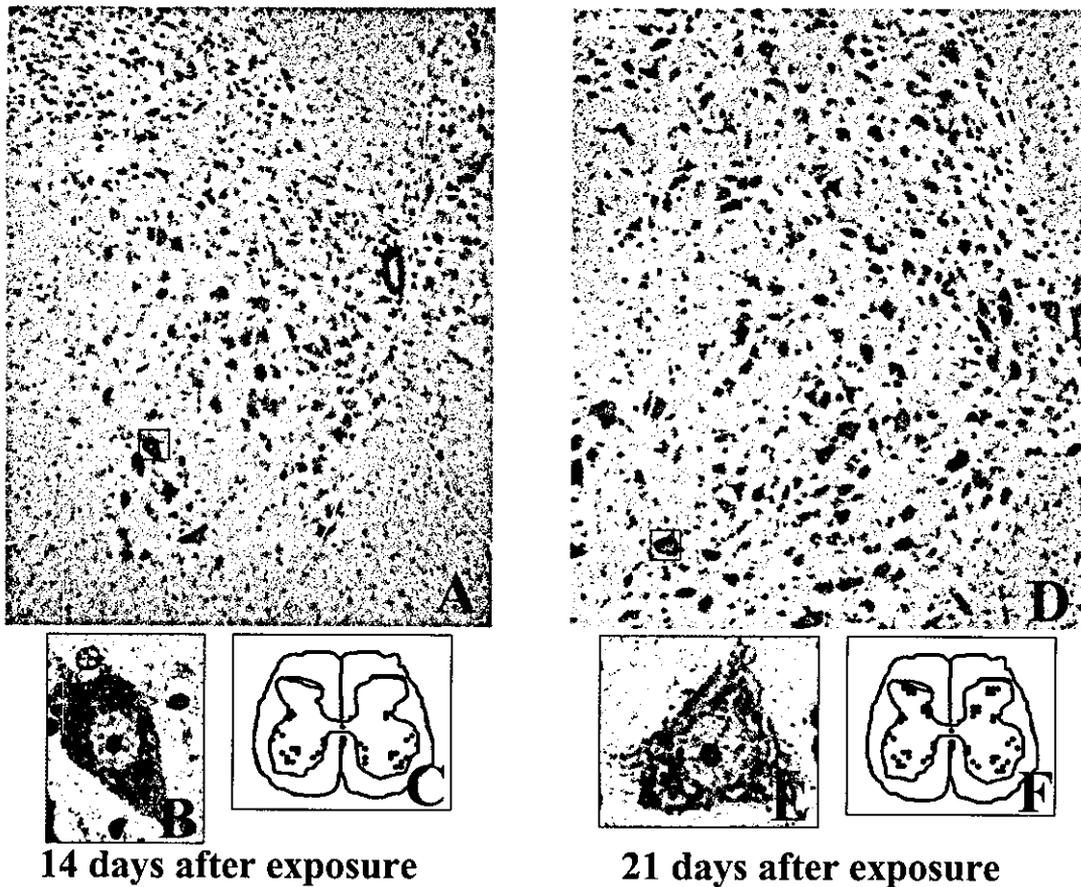**14 days after exposure**　　　　**21 days after exposure**

Fig. 4

Histological localization of mercury in the spinal cord 14 and 21 days after Hg⁰ vapor exposure.

Fourteen days after exposure, silver-enhanced mercury was spread over the neurons in the nucleus intermediolateraris. Twenty-one days after exposure, silver-enhanced mercury was spread over the neurons in nucleus proprius dosalis. Fig. 4-B and E show the neuron in the nucleus motorius. Fig. 4-C and F show shematic drawing of mercury distribution and amounts of mercury granules. Autometallography. Fig. 4-A ： ×96　Fig. 4-B ： ×780　Fig. 4-D ： ×138　Fig.4-E ： ×800

Vascular endothelial cells
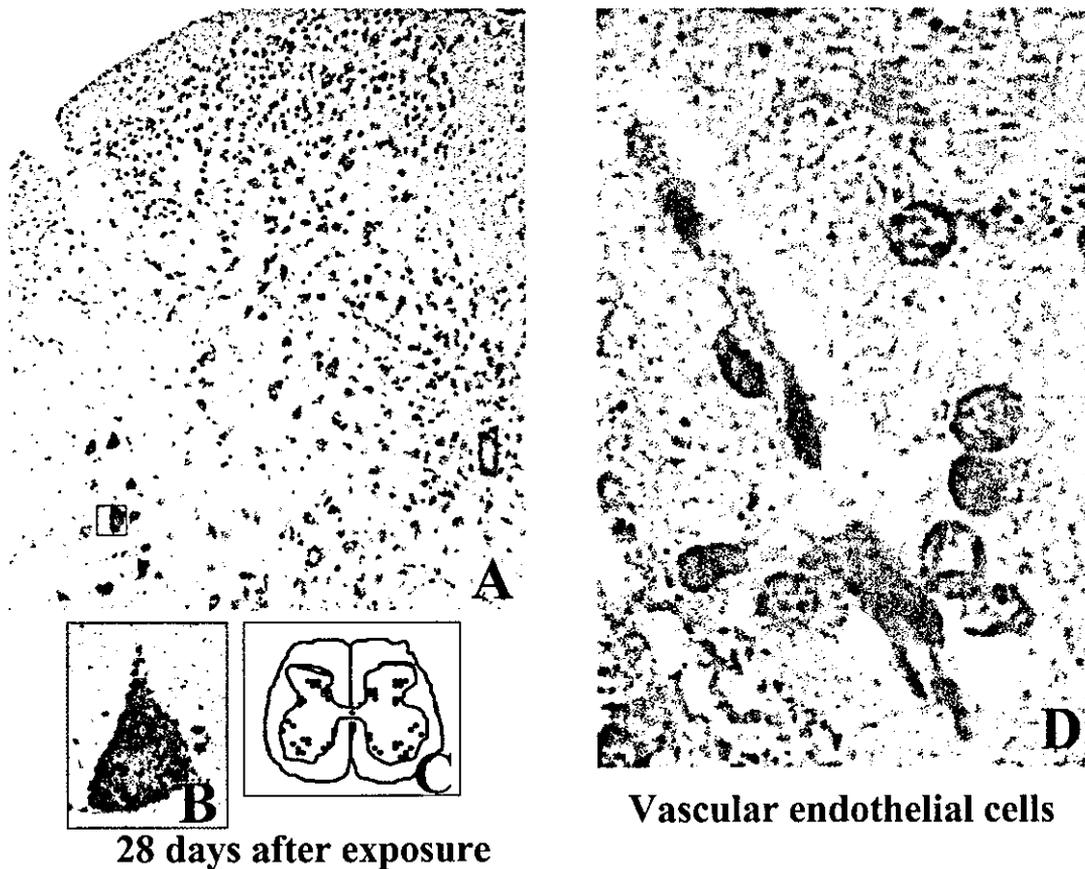
28 days after exposure

Fig. 5

Histological localization of mercury in the spinal cord 28 days after Hg^0 vapor exposure.

Twenty-eight days after exposure, silver-enhanced mercury was spread over the neurons in nucleus posteromarginalis. Fig. 5-B shows the neuron in the nucleus motorius. Fig. 5-C shows shematic drawing of mercury distribution and amounts of mercury granules.

Fig. 5-D : Vascular endothelial cell did not have mercury granules. Autometallography. Fig. 5-A : ×84　Fig. 5-B : ×720　Fig. 5-D : ×1600
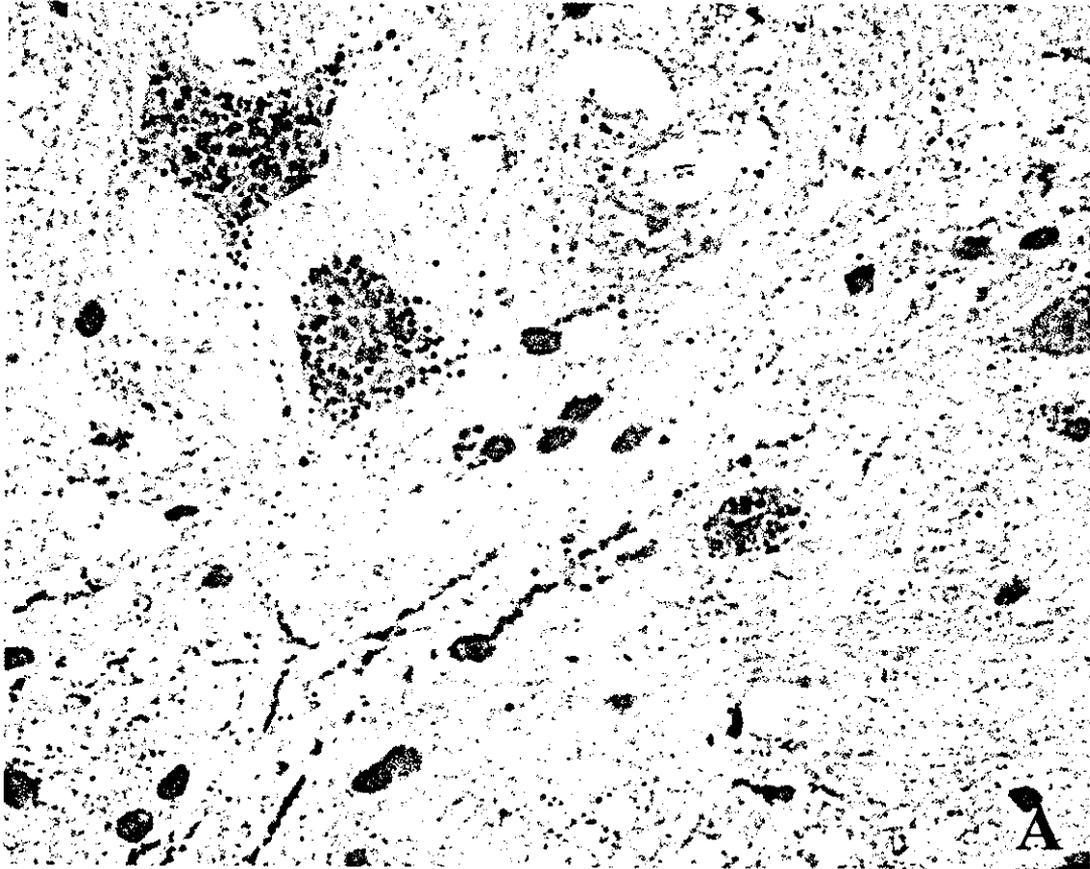
Fig. 6

Double staining for immunohistochemistry (GFAP) and autometallography in the spinal cord after Hg⁰ vapor exposure.

From fourteen days after exposure, silver-enhanced mercury was detected in the cytosol and dendrites of astrocytes. Immunohistochemistry (GFAP) and Autometallography. Fig. 6-A : × 1000
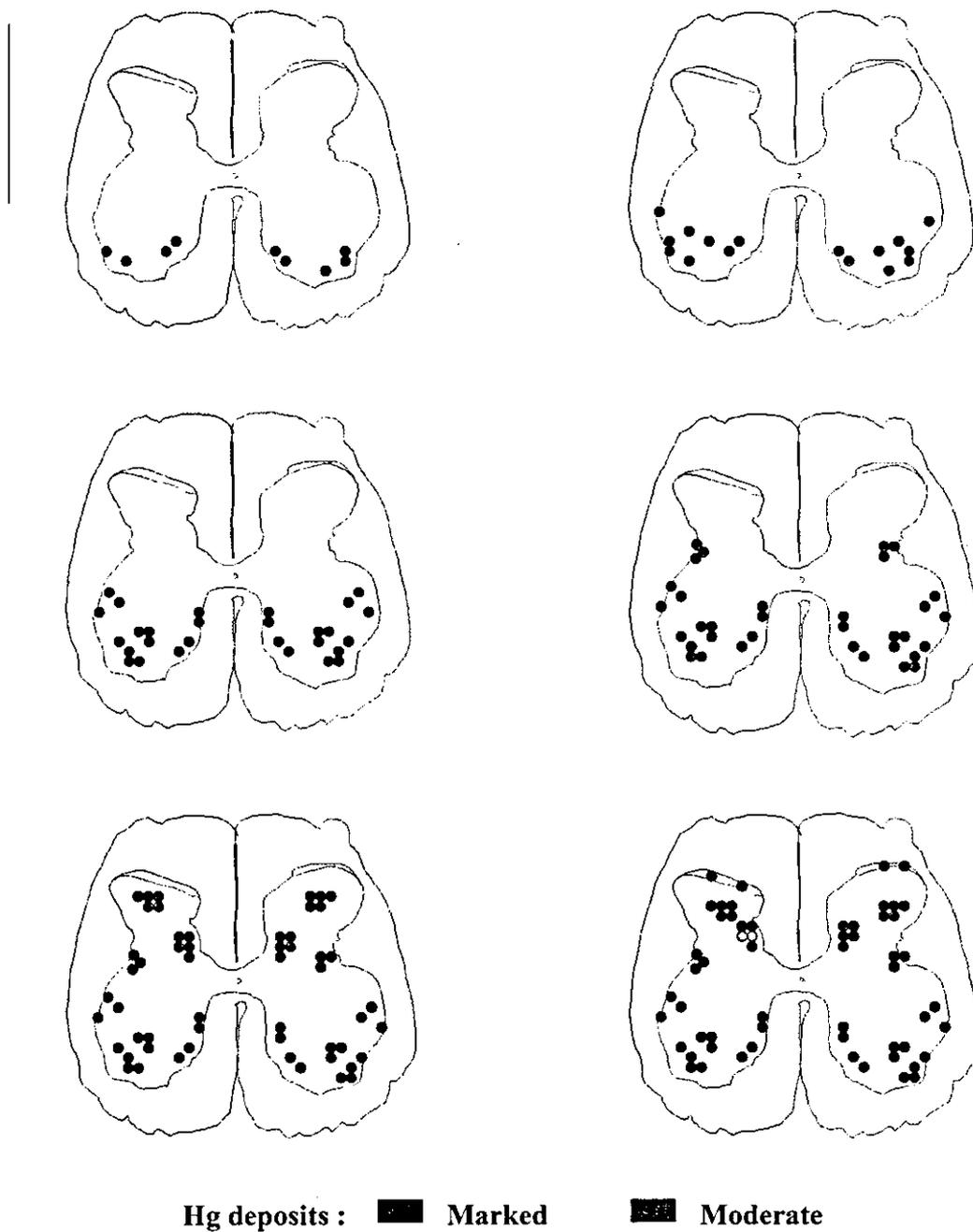
Hg deposits : ■ Marked ▦ Moderate

Fig. 7

Shematic drawings of transverse section of spinal cord of mouse after Hg^0 vapor exposure.

Mercury distributions were first detected in the motor neurons in ventral horn and spread out neurons in the dorsal horn following to substancia intermedia. The amount of mercury granules in the neurons showed time dependent increase.
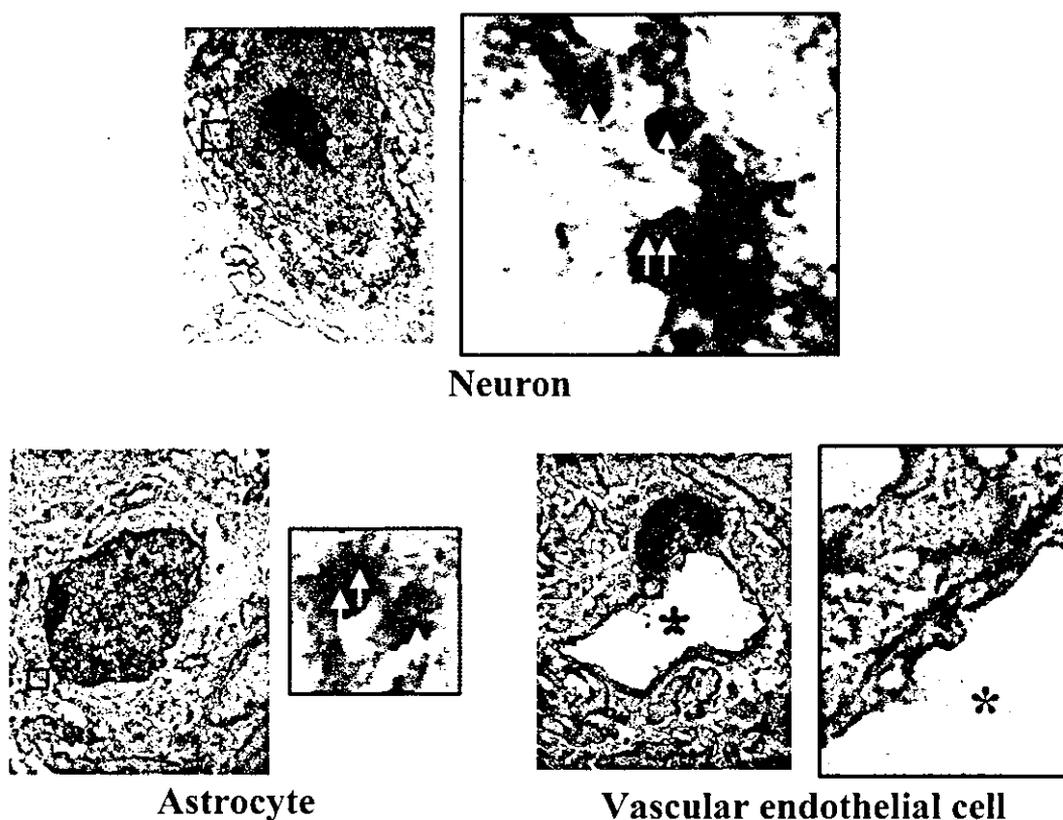
Neuron

Astrocyte **Vascular endothelial cell**

Fig. 8
Histological localization of mercury in the spinal cord after Hg^0 vapor exposure for electron microscopy.
Mercury like substances were detected in the lysosome like structures in the neurons and astrocytes.
No mercury granules were detected in the vascular endothelial cell.

DISCUSSION

In previous studies, two transport pathways of the inorganic mercury to the CNS were reported. One is that inorganic mercury is transported by bloodstream to the CNS [10, 11, 17-19, 28, 32, 33]. The details of system are that elementary mercury (Hg^0) readily crosses the lung alveoli due to its high difusibility and lipid solubility and is taken up by erythrocytes, in which catalases oxidize the elemental mercury to divalent ionic mercury (Hg^{2+}) [17,18,28]. Although the ionic mercury does not readily pass through the

blood-brain barrier, a fraction of the metallic mercury is transferred from the bloodstream into tissues, including the CNS, where it is trapped by oxidation [17, 18, 28]. The other is that inorganic mercury (Hg^{2+}) is taken up in nerve terminals in skeletal muscles (neuromuscular junction) and then transported retrogradely along the motor neurons to the cell bodies in the spinal cord and dorsal root ganglia [3·5, 13, 27]. However, the details of the mechanism by which mercury is taken up at the nerve endings are not known [2]. In this study, mercury granules were first detected in motor neurons of the spinal cord after 3 days exposure. These results suggest that the mercury was taken up in nerve terminals in skeletal muscles (neuromuscular junction) and then transported retrogradely along the motor neurons to the cell bodies in the spinal cord as same as studies previously performed.

We hypothesized to two transport pathways of mercury granules within the spinal cord. One is transneuronal transport, the other is transport through the blood brain barrier. Structurally, nerve tissue consists of two cell types: neurons and glial cells [25]. Most neurons consist of three parts: cell body, dendrites and axon [25]. The cytoplasm-filled dendrites, which are multiple, elongated processes, receive and carry stimuli from other neurons to cell [25]. The axon, which is a single cytoplasm-filled process, is specialized for generating and conducting nerve impulses away from the cell body to other nerve cells [25]. In this study, the mercury deposits were first detected in the motor neurons in ventral horn and spread out the neurons in the dorsal horn following to substancia intermedia. No mercury granules were detected in the endothelial cells of blood vessels in the all sections examined. According to these findings and basic neuronal function, transport pathways of mercury

granules within the spinal cord were suggested that transneuronal transport rather than the pathway through the blood brain barrier (Fig. 9).

Mercury transport pathway within the spinal cord
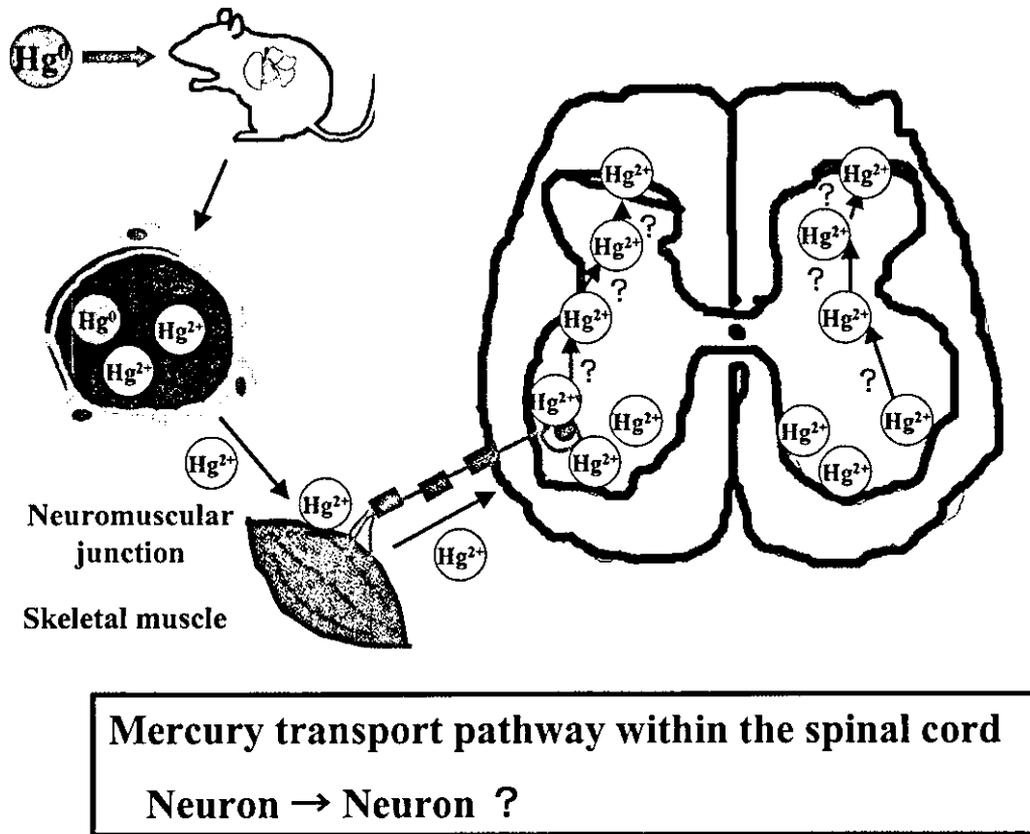
Neuron → Neuron ?

Fig. 9

Hypothesis of mercury transport pathway to and within the spinal cord.

The transport pathway of the inorganic mercury to the spinal cord was suspected to retrograde axonal transport as same as studies previously performed according to mercury distribution. Furthermore, it was suggested that transneuronal transport was one of the ways of mercury granules spread within the spinal cord.

Generally, astrocytes have many processes, some reaching to the surface of the neurons, and still others filling most of the intercellular space of the CNS

[25]. The astrocytic linkage between the blood vessels and the neurons may provide a transport mechanism for the exchange of oxygen, carbon dioxide and metabolites [25]. Astrocytes together with the tightly joined endothelial cells of the capillaries in the CNS, contribute to what is called the blood brain barrier [25]. Blood brain barrier is used to emphasize the impermeability of the nervous system to large or potentially harmful molecules [25]. Double staining for immunohistochemistry (GFAP) and autometallography showed that mercury granules were detected in cytoplasm and dendrites in the astrocytes from 14 days after exposure in this study. Mercury depositions of astrocytes were following to its depositions in neurons. Although, the functions of astrocytes in mercury toxicity are not known, these results suggest that astrocytes passively receive excessive mercury (Hg^{2+}) from neurons. However, we cannot deny the mercury transport through the blood brain barrier in the present study.

Investigations with electron microscopy have demonstrated that these granules correspond to lysosomes and prelysosomal structures [3, 4, 15, 24, 28-31]. Exogenous macromolecules that are taken up by nerve endings by unspecific mechanisms will accumulate in lysosomes of neurons after retrograde axonal transport, and as a rule will then undergo enzymatic degradation [15, 30]. Previous studies have shown that mercury can persist in the cell body of the motor neuron for long periods of time where it is stored in lysosomes [7]. Intralysosomal Hg^{2+} is not metabolized or excreted by the lysosomal enzymes and can remain in the organelle indefinitely [2]. Provably the mercury inside lysosomes is relatively inert biologically, perhaps due to its binding to selenium, a binding known to reduce the toxicity of Hg^{2+} in animals

[20]. And excessive accumulation of mercury in lysosomes of nerve cells could possibly damage the neuron because [12, 24]. In this study, mercury granules like substances were detected in lysosome like structures of neurons and astrocytes in the spinal cord. So it was suspected that the same phenomenon had been occurred in this study.

In conclusion, this study indicated that the transport pathway of the inorganic mercury to the spinal cord was retrograde axonal transport as same as studies previously performed. Furthermore, it was suggested that transneuronal transport was one of the ways of mercury granules spread within the spinal cord.

REFERENCES

1. Arvidson, B. 1987. RESEARCH NOTE. Retrograde Axonal Transport of Mercury. Experimental Neurology. 98: 198-203

2. Arvidson, B. 1990. Accumulation of mercury in brainstem nuclei of mice after retrograde axonal transport. *Acta. Neurol. Scand.* 82: 234-237

3. Arvidson, B. 1992. Accumulation of inorganic mercury in lower motoneurons of mice. *NeuroToxicol.* 13: 277-280

4. Arvidson, B. 1992. Inorganic mercury is transported from muscular nerve terminals to spinal and brainstem motoneurons. *Muscle & Nerve* 15: 1089-1094

5. Arvidson, B. 1994. A review of axonal transport of metals. *Toxicology* 88: 1-14

6. Carpenter M.B. 1996. Core text of Neuroanatomy, fourth edition: 65-91

7. Danscher, G. and Mϕller-Madsen, B. 1985. Silver amplification of mercury sulfide and selenide: a histochemical method for light and microscopic localization of mercury in tissue. J. *Histochem. Cytochem.* 33: 219-228.

8. Eide, R. and Wesenberg, R. 1993. Mercury contents of indicators and target organs in rats after long-term, low-level, mercury vapor exposure. *Environmental Res.* 61: 212-222

9. Frans W. Sørensen, Jytte O Larsen, Rune Eide and Jørgen Drasbæk Schiønning 1999. Neuron loss in cerebellar cortex of rats exposed to mercury vapor: a stereological study. *Acta Neuropathol.* 100: 95-100

10. Goering, P. L., Morgan, D. L. and Ali, S. L. 2002. Effects of mercury vapor inhalation reactive oxygen species and antioxidant enzymes in rat brain and kidney are minimal. *J of Applied Toxicol.* 22. 167-172

11. Hursh, B. J., Cherian, M. G., Clarkson, T. W., Vostal, J. J. and Mallie, R. V. 1976. Clearance of mercury (^{197}Hg, ^{203}Hg) vapor inhaled by human subjects. *Environ. Health* 31: 302-309.

12. IPCS 1991. International program on chemical safety. Environmental health criteria vol.118, World Health Organization, Geneva.

13. Juan, V., Rocio, M., Auxiiadora, A. and Damaso, C. 1999. Accumulation of mercury in neurosecretory neurons of mice after long-term exposure to oral mercuric chloride. Neuroscience Letters. 271: 93-96

14. Kimura N. 1971. Electron microscopic studies on the rat cerebellum in experimental inorganic mercury poisoning. *Psychiatria et neurologia Japonica.* 74(4): 312-325

15. Kristensson K. 1978. Retrograde transport of macromolecules in axons. *Ann. Rev. Phramacol. Toxicol.* 18: 97-110

16. Lindstedt, G. and Stkerfiving, S. 1972. Method of analysis. Pp. 3-13. In Mercury in the Environment.(friderg, L. and Vostal, J ed.), CRC press, Cleaveland, OH.

17. Magos, L., Halbach, S., Clarkson, T.W. 1977. Role of catalase in the oxidation of mercury vapor. *Biochem. Pharmacol.* 27: 1373-1377

18. Morgan, D. L., Chanada, S. M., Price, H. C., Liu, J., Brambila, E., O'Connor, R. W. and Barone, S. Jr. 2002. Disposition of inhaled mercury vapor in pregnant rats: maternal toxicity and effects on developmental outcome. *Toxicol. Sci.* 66: 261-273.

19. Möller-Madsen, B. 1992. Localization of mercury in CNS of the rat. V. Inhalation exposure to metallic mercury. *Arch. Toxicol.* 66: 79-89

20. Nordlind, K. 1990. Biological effects of mercuric chloride, nichel sulphate, and nichel chloride. In: G.P. Ellis and G.B. West, (Eds), *Progress in Medical Chemistry,* Amsterdam, Elsevier Science Publishers, vol. 27. 190

21. Roger Kevin Stankovic, Victer Lee, Murat Kevic and Clive Harper 2003. The expression and significance of metallothioneins in murine organs and tissues following mercury vapour exposure. *Toxicol. Pathol.* 31: 514-523

22. Pamphlett, R. and Coote P. 1998. Entry of low doses of mercury vapor into the nervous system. *Neurotoxicology* 19: 39-47

23. Pamphlett, R. and Kum-Jew, S. 2000. Mercury vapor uptake into the nervous system of developing mice. *Neurotoxicol. and Teratology* 23: 191-196

24. Pamphlett, R. and Waley, P. 1996. Motor neuron uptake of low dose inorganic mercury. Journal of the Neurological Sciences. 135: 63-67

25. Porth, C. M. 2002. Pathophisiology: concepts of alterd health steates, sixth edition

26. Schiønning, J. D. and Møller-Madsen, B. 1991. Autometallographic mapping of mercury deposits in the spinal cord of rats treated with inorganic mercury. *Acta Neuropathol.* 81: 434

27. Schiønning, J. D. 1993. Retrograde axonal transport. In: A. Lajtha, (Ed), *Handbook of Neurochemistry,* vol. 5, Plenum, New York, pp. 381-404

28. Schiønning, J. D. 1993. Retrograde axonal transport of mercury in rat sciatic nerve. *Toxicol. Appl. Phamacol.* 121: 43-49

29. Schiønning, J. D., Rune Eide, Möller-Madsen B., and Erik Ernst. 1993. Detection of Mercury in Rat Spinal cord and Dorsal Root Ganglia after Exposure to Mercury Vapor. Experimental and Molecular Pathology 58: 215-228

30. Schiønning, J. D. and Danscher Gorm 1999. Autometallographic mercury correlates with degenerative changes in dorsal root ganglia of rats intoxicated with organic mercury. APMIS 107: 303-310

31. Schwab M., Thoenen H. 1983. Retrograde axonal transport. In: A. Lajtha, (Ed), *Handbook of Neurochemistry,* vol. 5, Plenum, New York, pp. 381-404

32. Warfvinge, K., Hua, J., and Berlin, M. 1992. Mercury distribution in the rat brain after mercury vapor exposure. *Toxcol. Appl. Pharmacol.* 117: 46-52

33. Warfvinge Kalin and Bruun Anitha 2000. Mercury distribution in the squirrel monkey retina after *in utero* exposure to mercury vapor. *Environmental Res. Section A* 83: 102-109

分担研究報告

胎児期における低濃度水銀曝露（単独：蒸気水銀、メチル水銀）（複合曝露：蒸気＋メチル水銀）の生後の影響（病理組織）

分担研究者　島田章則　鳥取大学農学部獣医病理学教授

研究要旨
　メチル水銀毒性の修飾要因と想定した水銀蒸気との複合曝露が行動にもたらす影響については、別項で吉田氏が報告したが、用いた検討条件に関する限り，複合曝露の場合に毒性が増強するとは言えず、各単独曝露の場合と同様の行動影響が検出された。本研究では、複合曝露の影響を病理学的に評価し、各単独曝露の場合と比較した。評価方法としては、HE 染色、GFAP 免疫染色、および水銀沈着の組織学的検出を試みたが、単独曝露・複合曝露を問わず、病理的な陽性所見は得られなかった。また、水銀沈着の程度についても、系統差は認められなかった。したがって、行動学的に検出された異常に対応するような病理所見を発見することはできなかった。

・はじめに
　本プロジェクトでは、メチル水銀毒性の修飾要因の一つとして、水銀蒸気を考え、両者の複合曝露がもたらす影響を、単独曝露の場合と比較した。行動影響については、別項で吉田氏が報告した通りで、用いた検討条件において、両者の著しい毒性増強を見ることはなかった。本研究では、複合曝露の影響を病理学的に検索することによって、両者の相互作用を検証することを目的とした。

・病理解析の方法（観察項目）：間脳レベル（海馬含む）および小脳
1. HE 染色
　器質的変化の有無（神経細胞の変性・脱落、グリオーシス、加齢性変化（リポフスチン

沈着など））
2. オートメタログラフィー：水銀顆粒沈着の有無
3. 免疫組織化学：GFAP（星状膠細胞の反応性変化、グリオーシス）

【実験1】加齢マウス（胎生期から出産10日までメチル水銀投与）

(1) HE 染色

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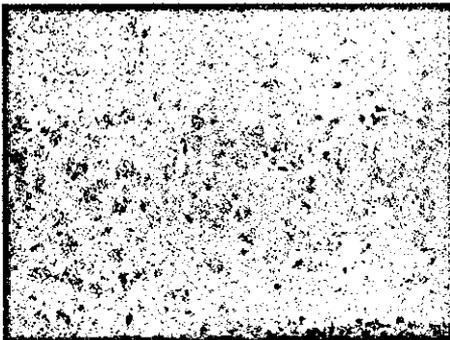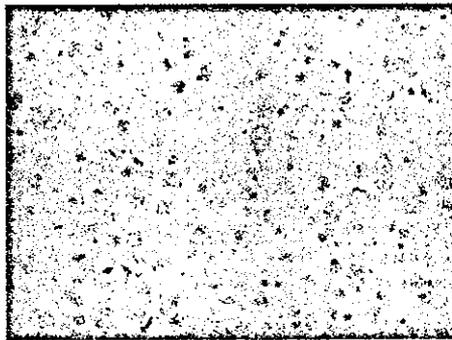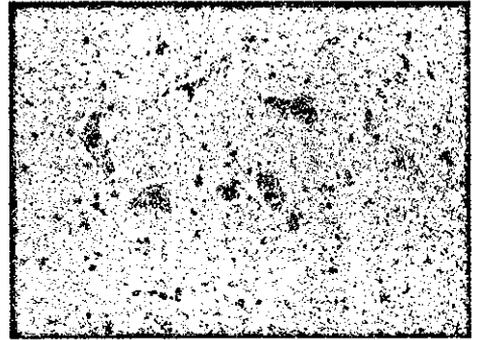
| 海馬 | 大脳皮質 | 延髄 |

MT－群
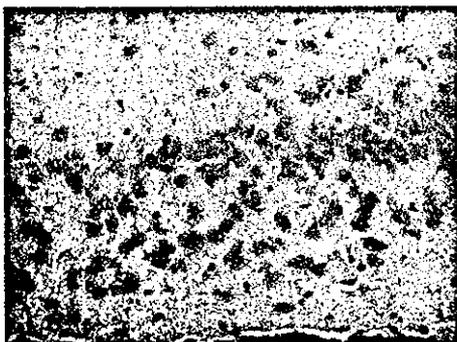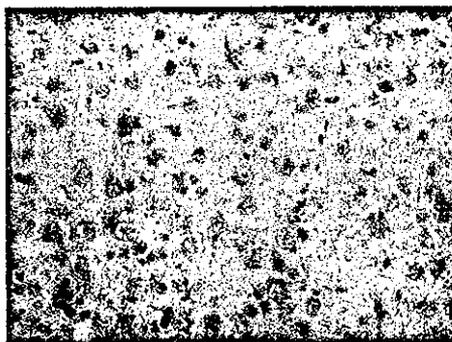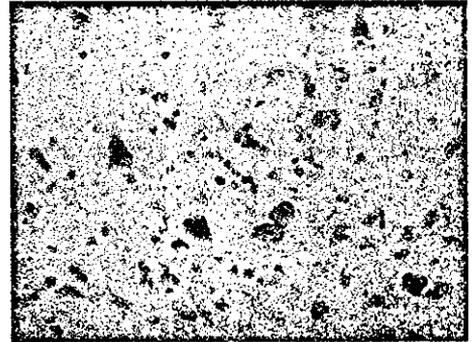
| 海馬 | 大脳皮質 | 延髄 |

(2) オートメタログラフィー（水銀染色）

MT＋群
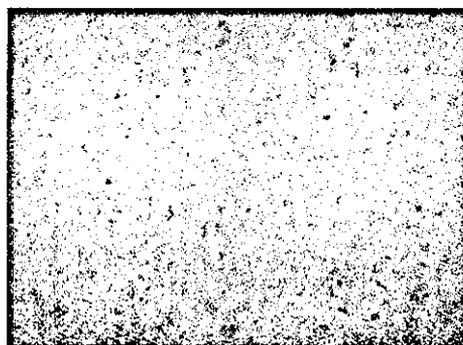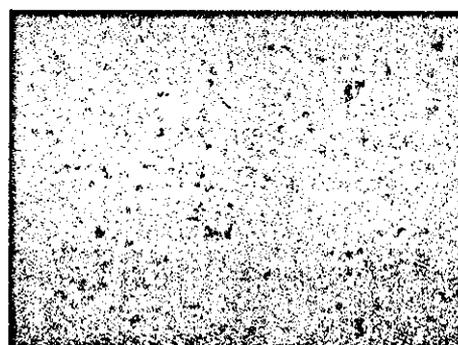
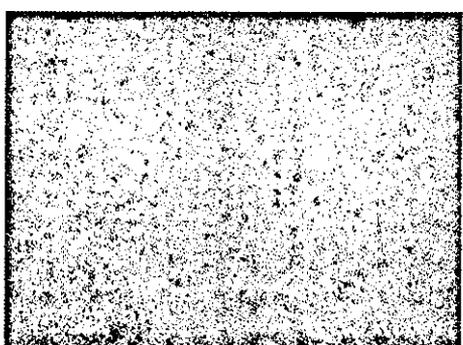海馬　　　　　　　　　　　大脳皮質　　　　　　　　　　延髄

MT－群
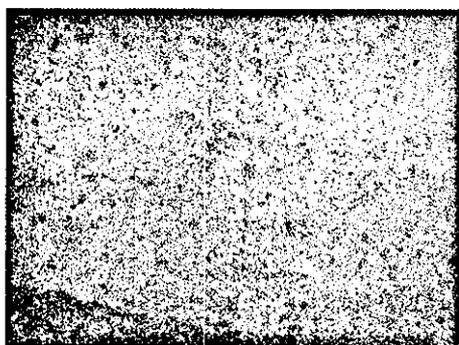
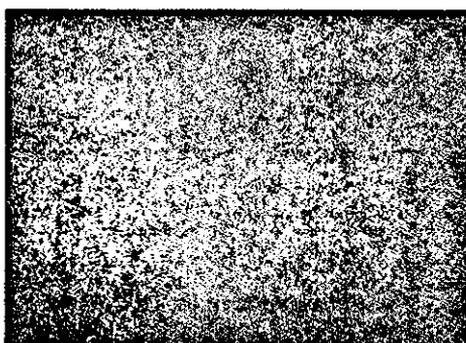海馬　　　　　　　　　　　大脳皮質　　　　　　　　　　延髄

(3) GFAP 免疫染色

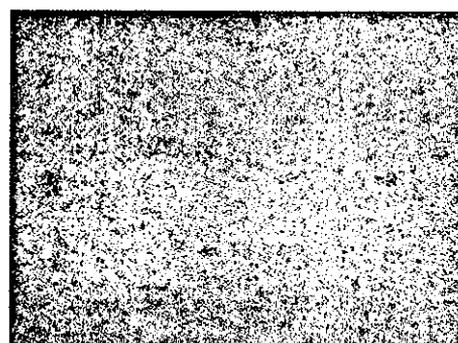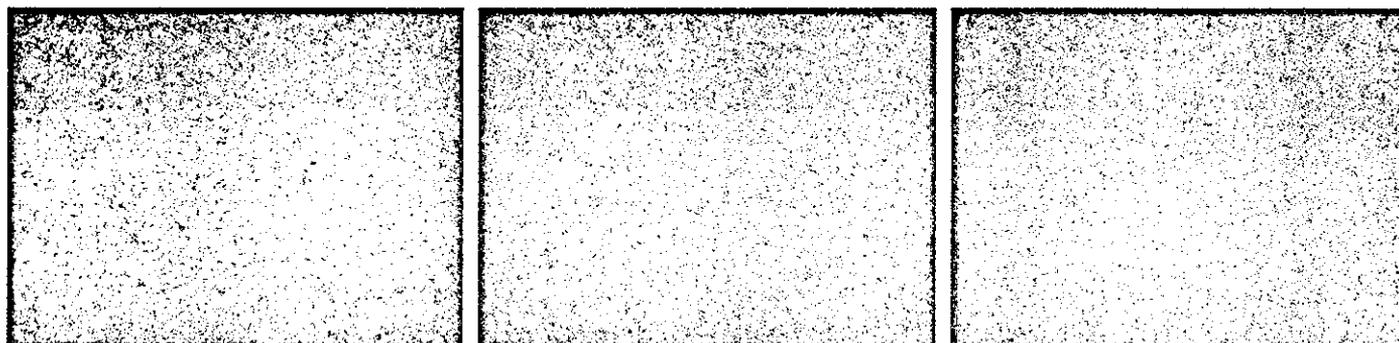MT＋群
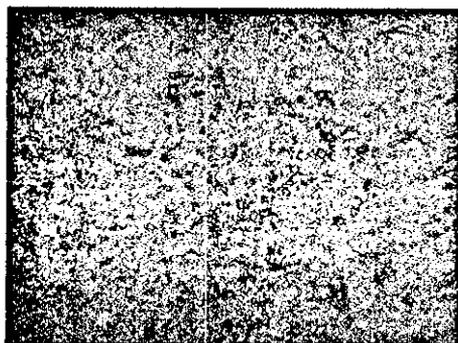
海馬　　　　　　　　　　　大脳皮質　　　　　　　　　　延髄

MT−群

| 海馬 | 大脳皮質 | 延髄 |

・結果のまとめ（上の図参照）

　HE 染色で、メタロチオネイン欠損および野生型マウスいずれにおいても、海馬、大脳皮質、大脳白質、小脳および延髄において著変は認められなかった。GFAP 免疫染色においても星状膠細胞の反応に両群に差は認められなかった。水銀染色では両群の延髄の神経核に神経細胞細胞質に陽性像が認められた。水銀顆粒陽性像の程度に関して、両群に差は認められなかった。

・考察

　行動解析により、野生型マウス（雌雄）において探索行動の低下が、雌性メタロチオネイン欠損マウスにおいて回避反応の低下および学習獲得能力の低下が認められているが、それらを裏付ける病理形態学的所見は認められなかった。また、メタロチオネイン欠損マウス、野生型マウス両群に水銀沈着の程度に差は認められず（延髄神経核）、メタロチオネインの有無と水銀沈着との関連を示唆する所見を得ることはできなかった。

【実験 2】若齢マウス
　　　　　単独曝露（蒸気水銀、メチル水銀）
　　　　　複合曝露（蒸気水銀＋メチル水銀）

・結果のまとめ

　単独曝露群、複合曝露群において、HE 染色および GFAP 免疫染色で特に著変は認められなかった。また、全ての実験群において水銀沈着の程度（延髄神経核）に差は認められなかった（次頁の図参照）。