Вісник Дніпропетровського університету. Біологія, екологія.
Vìsnik Dnìpropetrovs'kogo unìversitetu. Serìâ Bìologìâ, ekologìâ

Visnyk of Dnipropetrovsk University. Biology, ecology.
2013. 21(2)

ISSN 2310-0842

www.ecology.dp.ua

**УДК 577.391:612.8**

# The multipotent role of metallothionein in the nervous system

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We provide a commentary on current experimental and theoretical advances and frame our consideration in terms of the possible functions of MT I+II in the nervous system. Metallothioneins (MT) are a family of small cysteine rich proteins, which since their discovery in 1957 have been implicated in a range of roles including toxic metal detoxification, protection against oxidative stress, and as a metallochaperone involved in the homeostasis of both zinc and copper. The most well studied member of the family is the mammalian metallothionein, which consists of two domains: a β-domain with 9 cysteine residues and an α-domain with 11 cysteine residues. Despite over half a century of research, the exact functions of MT in the nervous system are still unknown. Our studies have shown that the distribution of MT-I+II in the brain after prolonged intoxication, inhalation of 0.1% $CdCl_2$ for 1 hour twice a week over 19 weeks, is dependent on the part of the brain. The metallothionein level declines more than 4 times in the hippocampus 3 weeks after continuous intoxication of 0.1% $CdCl_2$. The level of MT-I+II in the cerebral cortex decreased by 1.5 times compared with the control group and did not change significantly in the cerebellum and thalamus/hypothalamus. The results of an experimental model of postoperative pain indicated that injection with MT-II prevents the development of postoperative hyperalgesia in response to mild alteration of physiological activity. Activation of locomotory and exploratory activity, and decrease of anxiety in rats under MT-II treatment at 100 μg/rat manifests itself on the 4th day after surgery. Our experimental data indicate the multipotent function of MT I+II in the rat brain both as a metal detoxifier and as an inhibitor of postoperative pain.

*Keywords*: cadmium; zinc; metallothionein; brain; postoperative pain

# Мультипотентна роль металотіонеїну в нервовій системі

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Наведено коментар про сьогоденні експериментальні та теоретичні досягненя в рамках розуміння можливих функцій МТ I+II в нервовій системі. Металотіонеїни (МТ) – родина невеликих протеїнів, збагачених на цистеїн. Із моменту їх відкриття в 1957 році для цих протеїнів визначено функції різного діапазону, включаючи детоксикацію металів, захист від окислювального стресу, і як металошаперони вони беруть участь у гомеостазі цинку та міді. Найкраще вивчені металотіонеїни ссавців, які складаються з двох доменів : α-області з 11 залишками цистеїну та β-домену з 9 залишками цистеїну. Незважаючи на понад півстоліття досліджень, точні функції МТ у нервовій системі донині невідомі. Наші дослідження показали, що розподіл МТ I+II у мозку після тривалої інтоксикації (вдихання 0,1% $CdCl_2$ протягом години двічі на тиждень упродовж 19 тижнів) залежав від відділів мозку. Рівень металотіонеїну знижується більше ніж учетверо в гіпокампі через 3 тижні після безперервної інтоксикації 0,1% $CdCl_2$. Рівень МТ I+II у корі головного мозку знизився в 1,5 раза порівняно з контрольною групою, але не було значних змін у мозочку і таламусі / гіпоталамусі. Завдяки експериментальній моделі післяопераційного болю, отримані результати показали, що ін'єкції з МТ II запобігають розвитку післяопераційної гіперчутливості до болю відповідно до зміни фізіологічної активності. Активація рухової активності та зниження занепокоєння при застосуванні МТ II у дозі 100 мкг/щура вже визначалася на четверту добу після операції. Отримані експериментальні дані свідчать про мультипотентність функцій МТ I+II у мозку щурів: як детоксиканта, так і як інгібітора післяопераційного болю.

*Ключові слова*: кадмій; цинк; металотіонеїн; мозок; післяопераційний біль

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## Introduction

The first health effect of cadmium (*Cd*) was lung damage, reported in workers already in the 1930's, while bone effects and proteinuria were reported in the 1940's. After World War II, a bone disease with fractures and severe pain, the itai-itai disease, a form of *Cd*-induced renal osteomalacia, were identified in Japan (Kazantzis, 2004). Subsequently, the toxicokinetics and toxicodynamics of *Cd* were described including its binding to the protein metallothionein. *Cd* is classified as a human carcinogen causing tumors of the lung, prostate, and other tissues. Human activity has markedly increased the distribution of *Cd* in the global environment. Food is the major source of *Cd* exposure for the general population, and cigarette smoking significantly adds to the burden of *Cd* on the body. Occupational exposures are mainly from *Cd* fume inhalation, the cadmium-nickel battery industry, electroplating, and paint pigments (Liu et al., 2007).

In search of the component which is responsible for the natural accumulation of cadmium in the kidneys of mammals, in 1957 Margashes and Vallee discovered metallothioneines (Margoshes and Vallee, 1957). This family of low-proteins is localized in the cytoplasm and nucleus. Today, many results about metal-binding proteins have been obtained, but these data are still not enough to fully understand the molecular mechanism of participation of these proteins in normal and damaging factors. International warnings of health risks from cadmium pollution were issued in the 1970's (Nordberg et al., 1975). WHO, 1992, identified renal dysfunction as the critical effect and a crude quantitative evaluation was presented (Groten et al., 1994). In the 1990's population groups in China exposed to *Cd* via rice were studied and new information on skeletal, renal and reproductive toxicity of *Cd* was obtained in the ChinaCad project. There was a decrease in Bone Mineral Density (BMD), an increased prevalence of fractures and an increased urinary content of marker proteins of renal dysfunction among persons with long term exposure to *Cd*. The development of such biomarkers can be seen as a result of applied 'proteomics' research. Variations in metallothionein gene expression were related to development of renal dysfunction, supporting the usefulness of this 'genomic' approach. The ongoing rapid development of 'genomics' and 'proteomics' technologies will improve possibilities for molecular epidemiology studies in the future, providing even a better basis for preventive action. In many countries, *Cd* exposures are now under better control than in the past. The target for the 21st century is to achieve a totally acceptable exposure situation without adverse health effects from *Cd*. More details about cadmium and health in the 21st century are presented in the review by Nordberg (2004).

Metallothionein gene expression is transcriptionally regulated by dietary zinc too (as by cadmium) and thus could serve as an assessment parameter based on zinc-dependent function. Zinc status is difficult to evaluate in humans. Sullivan and Cousins used semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to establish that MT mRNA is increased in a human monocytic cell line by addition of zinc to the medium (Sullivan and Cousins, 1997). The RT-PCR data show that there was a significant increase in monocyte MT mRNA in subjects within 6 d of zinc supplementation, which remained elevated at d 15 of supplementation. In contrast, plasma zinc was greater at d 6 of zinc supplementation, but by d 15 of supplementation, while still elevated, was close to control levels. These data suggest that monocyte MT mRNA levels respond to zinc supplementation and that the response could serve as a more useful assessment variable than plasma zinc for the measurement of zinc status in humans.

## Materials and methods

Two different experiments were performed using adult Wistar rats weighting 250–280 g. Animals were kept in the animal house under standard conditions with consumption of water and food ad lib during whole experimental period. All experimental protocols and handling of the animals were approved by the local authorities (Dnipropetrovsk, Ukraine).

The model of long-lasting intoxication with cadmium was induced by inhalation of 0.1% $CdCl_2$ dispersed in air during 19 weeks, twice a week, 1-h-long sessions ($n$ = 7). The control animals ($n$ = 7) were in the same condition without inhalation.

The experimental model of postoperative pain was developed due to Brennan (Brennan et al., 1996). Operated rats were anaesthetized with ether. The plantar aspect of the hindpaw was prepared in a sterile manner with iodine solution. A 1 cm longitudinal incision was made with a SM65 blade through the skin and fascia of the plantar aspect of the foot, starting 0,5 cm from the proximal edge of the heel and extending towards the toes. After haemostasis with gentle pressure, the skin was apposed with 2 mattress sutures of 3 nylon on an FS-2 needle. The wound site was covered with iodine solution and a mixture of polymixin B and neomycin ointment. After surgery, the animals were allowed to recover in the cages. The study has been approved by animal protection authorities. Wistar rats were divided into 4 groups: 1 – control, without any treatment ($n$ = 8); 2 – incision ($n$ = 8); 3 – injection by 0.2 ml physiological solution containing 48 µg MT-I+II per animal during 2 days after incision ($n$ = 8); 4 – injection by 100 µg MT-I+II per animal during 2 days after incision ($n$ = 6).

Behavioural testing was performed by the open field test (Koob et al., 2006).

Isolated different brain parts were homogenized in 10-times volume of buffer containing 25 mM tris-*HCl*, *pH* 7.4, 1 mM EDTA, 2 mM ditiotreytol, 0.2 mM PMSF and 0.01 M mertiolate. All procedures were performed at +4 ºC. The homogenates were centrifuged at 100 000 g during 60 minutes. Supernatants were used to analyse the MT-I+II with solid phase competition immuno-enzyme analysis with monospecific polyclonal antisera against MT-I+II (DAKO, Denmark) and highly purified MT-I+II (Sigma) as a marker. Optical density was measured with the help of Anthos-2010 absorbance reader (Anthos Labtec Instruments, Austria).

Statistical analysis was performed using Statistica software (version 5, StatSoft, Tulsa, OK, USA). The two-

tailed nonparametric Kolmogorov-Smirnov test and parametric Student's t-test were used to assess the differences between samples ($P < 0,05$ was considered to indicate statistical significance).

## Results and discussion

### The structure and nomenclature of metallothioneins (MTs)

Metallothioneins consisting of 61–62 amino acids without aromatic amino acids and histidine, cysteine residues are 25–30%. The molecular weight of these proteins is 6.5–7.0 kDa. MTs show a considerable level of polymorphism while they possessed an exceptional number of homology, which shows a high conservative primary structure. Metallothioneins have been identified in many organisms: fungi, plants, spiders, animals, including humans (Simpkins, 2000). Using the method of polymerase chain reaction (PCR) the gene sequence of MT was isolated. This gene has two introns (575 and 602 bp) and three exons (22, 77 and 78 bp) (Fig. 1).

In humans, the MT genes are tightly clustered in the q13 region of chromosome 16 (West et al., 1990), consisting of 7 functional MT-I genes (MT-1A, -B, -E, -F, -G, -H and -X) and a single gene encoding each of the other MT isoforms, namely MT-II (the MT-2A gene), MT-III and MT-IV. High metallothionein conservative structure in the evolution and wide distribution shows the importance of fundamental physiological functions, despite the fact that the nature of MT is still discussed.

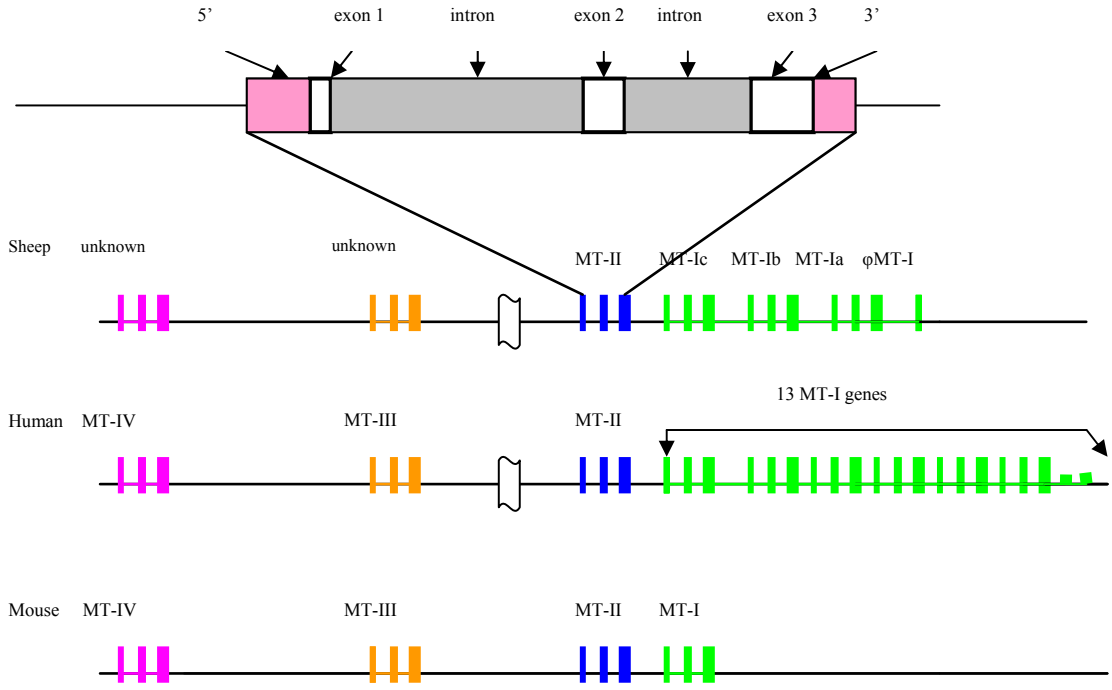


**Fig. 1. Metallothionein genes distribution in mammalians**

Metallothionein domains of different origins, exhibiting distinct, highly conserved cysteine positions, show differences in metal-cysteine coordination and reactivity. Munoz et al. (2000) showed the possible influence of (1) the position of the cysteine residues and (2) the steric and electrostatic effects of neighboring amino acids on the folding and stability of MT clusters have been examined with the native lobster beta *C* and beta *N* domains, each having nine cysteines and binding three $M^{2+}$ ions, and a modified domain beta $C \rightarrow N$, in which the cysteines of the C-terminal domain are relocated so they are spaced as in the *N*-terminal domain. Each has been synthesized and characterized by UV, CD, $^{113}Cd$ NMR, and $^{1}H$ NMR spectroscopies. The synthetic native domains ($Cd_3$ beta *C* and $Cd_3$ beta *N*) displayed spectroscopic properties, metal-binding affinities, and kinetic reactivity similar to those of the holo protein. In contrast, the modified $Cd_3$ beta $C \rightarrow N$ domain was unusually reactive and, in the presence of Chelex, a metal-ion chelating resin, was converted to a $Cd_5$(beta $C \rightarrow N)_2$ dimer. These differences in structure and reactivity demonstrate that the requirements for formation of a stable type-B, $Cd_3S_9$, beta cluster are more stringent than simply the sequential positions of the cysteines along the peptide chain and include specific interactions with neighboring amino acids. Molecular mechanics calculations suggest that changes of even a single amino acid in lobster $Cd_3$ beta *N* toward lobster $Cd_3$ beta $C \rightarrow N$ or in mammalian MT1 or MT2 toward $Cd_3$ beta-MT3 (GIF) can destabilize their structures. Now many studies are being conducted to understand the mechanism of metal binding MT which is important to know the exact structure of the active site of this protein.

There are two main types of metallothioneins isoform: MT-I and MT-II. They differ in amino acid composition and are separated by DEAE-ion exchange chromatography or gel-penetrating HPLC. Separation of DNA gives us information that there are two types or more genes for metallothioneins that can be presented as iso-MT, which were identified in tissue extracts (Danielyan et al., 2007). For to-

day, there are four known MT isoforms: MT-I, MT-II – in epithelial tissues (skin, intestinal mucosa, liver), MT-III – in the brain, MT-IV – epithelium of tongue, gastrointestinal tract (Quaife et al., 1994). More than 17 subtypes of metallothioneins have been selected by chromatography, but only ten of them supposedly "function" in the human body (Vasak and Hasler, 2000).

The biosynthesis of metallothionein is controlled by complex processes. One of these processes – the action of metals. Apparently, copper and other metals with zinc replacing existing microtubules can promote the genes of this protein. The other process is a control diet. When the dietary supply of zinc and copper is increased enough to induction the MT occurred (primarily in liver and intestine), metallothionein is synthesized quickly and resists the toxic effects of zinc (detoxication). It is generally accepted that the expression of MT-I/II proteins is highly inducible in response to a range of stimuli, including metals, hormones, cytokines, oxidative agents, inflammation and stress. Mt-1 and Mt-2 genes are co-ordinately regulated in mice by metals and glucocorticoids. Metal induced synthesis is mediated through the action of short cis-acting DNA sequences known as metal responsive elements (MREs), which are present in the promoter region of all mammalian MT genes (Radtke et al., 1993), and is mediated mainly by metal response element-binding transcription factor (MTF-1), a zinc sensitive trans-acting factor (Andrews, 2000).

Metallothioneins are relatively rapidly degrading proteins. Twenty-four hours is enough to reduce the pool of MT-bound zinc to insignificant levels. It is known that cadmium and zinc-binding (2 : 1) MT is 3.5 half-day period. *Zn*-MT is more resistant to degradation, rather than *Cd*-MT. Foremost is the lysosomal degradation of MT (Bremner, 1987).

**The role of metallothioneins in the nervous system**

The previous studies have suggested that zinc deficiency may decrease, whereas the addition of zinc or cadmium can increase the expression of metallothionein in the brain, as well as MT-I mRNA and MT-III mRNA in the hippocampus, more stress can provoke even greater increase in the expression of these proteins. Chen et al. (2005) studied the effects of different doses of zinc on the expression of metallothionein isoforms in the hippocampus of stressed rats. In the zinc deficiency group, plasm zinc content was decreased, while in zinc complementarity group it was slightly increased. On the one hand, the expressions of metallothionein in the brain and MT-1 mRNA, MT-3 mRNA in the hippocampus were downregulated in zinc deficiency group, however, their expressions were evidently enhanced in stressed zinc deficiency group. On the other hand, inductions of metallothionein and its mRNAs in zinc complementarity group were increased, furthermore, the stressed zinc complementarity group has a more significant yield of metallothionein and it's mRNAs. In addition, the levels of plasma cortisol, IL-6, IL-1 and *NO* were increased clearly in the zinc deficiency group and stressed zinc deficiency group. These results suggested that zinc deficiency may decrease while zinc complementarities increase the expressions of metallothionein in the brain and MT-1 mRNA, MT-3 mRNA in the hippocampus. Moreover, stress can increase their expressions dramatically. The impairment of stress on the body may be involved with the nutrition status of zinc, and zinc deficiency can lower the body's resistivity to stress.

In the brain, the main source of expression of MT-I+II are astrocytes (Aschner et al., 2002). Choroids plexus epithelial cells, endothelial cells and membranes can also produce this protein. Recent data show that in physiologically normal conditions, MT-I+II is distributed both inside and outside the cell (protecting neurons) in low concentration, its level increases during postnatal development and during the induction of metal ions (Ushakova and Kruchinenko, 2009).

However, our studies have shown the distribution of MT-I+II in the brain after prolonged intoxication, inhalation by 0.1% $CdCl_2$ 1 h twice a week for 19 weeks is dependent on the part of the brain. These results do not show a sharp increase in the expression of MT-I+II in the studied brain regions (Fig. 2).

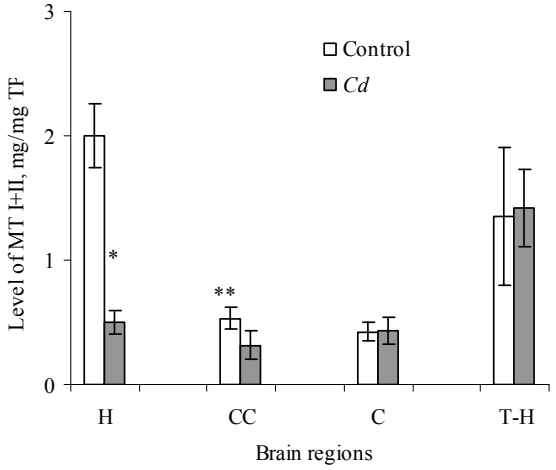


**Fig. 2. The distribution of metallothionein-I+II in various brain regions of rats under normal conditions and after prolonged cadmium intoxication, inhalation of 0.1% $CdCl_2$ during 1 h twice a week for 19 weeks:**
H – hippocampus, CC – cerebral cortex, C – cerebellum, T-H – thalamus/hypothalamus, TP – total protein; $n = 7$, * – $P < 0.001$, ** – $P < 0.05$

The metallothionein level declines by more than 4 times in the hippocampus 3 weeks after continuous intoxication of 0.1% $CdCl_2$. The level of MT-I+II in the cerebral cortex decreased 1.5 times compared with the control group and did not change significantly in the cerebellum and thalamus/hypothalamus. The data obtained suggest that long-term cadmium intoxication may overwhelm the biosynthetic resources of MT-I+II or lead to a reorganization of the protective mechanisms in astrocytes depending on the structure of the brain. Further detailed study of the morphology of astrocytes and the distribution of astrocyte-specific proteins, along with other metal-binding proteins, will provide an opportunity to understand the phenomenon of reducing the expression of MT-I+II in the hippocampus after chronic cadmium intoxication.

During last decade many results highlight the important role of MT-I/II in coping with brain damage. Many studies have clearly demonstrated a similar essential role in coping with damage elicited by kainic acid induced seizures, the gliotoxin 6-aminonicotinamide, 6-hydroxydopamine, mutated *Cu,Zn*-superoxide dismutase, multiple sclerosis models, traumatic brain injury (Giralt et al., 2002), and transgenic

IL-6-induced neuropathology. The overall results obtained in these studies are compatible with a role of MT-I/II in decreasing oxidative stress, inflammation and apoptosis in the CNS, which is in accordance with the results in other tissues (Ushakova and Kruchinenko, 2009). It is worth noting again that besides being potent antioxidant proteins, MT-I/II are induced by oxidative stress (Andrews, 2000), MT induction in response to amyloid plaques/production has been demonstrated in three different mouse models of Alzheimer disease, and thus there are suitable models for analyzing the putative neuroprotective role of MTs.

In our study we assessed the physiological and biochemical changes that develop in response to noxious peripheral stimulation after incision (postoperative pain) under metallothionein-II effect.

The mechanical sensitivity of a surgical incision is the most important property that provides both peripheral and central pathways of pain mechanisms. Damage to peripheral tissue and injury to nerves typically produce persistent pain and hyperalgesia. The peripheral component is believed to be associated with peripheral inflammation due to different substances released in response to surgical trauma. That is connected with activation of second messenger systems and triggering of the immune system. The central pathways of pain are induced by sensitization of dorsal horn neurons by noxious stimuli. Today we have a good characterized mechanism of neuron sensitization under pain condition, the role of specific receptors, there is a molecular structure, type of agonists and antagonists (Yajima et al., 2005). However, if the peripheral inflammation caused by surgical incision plays a crucial role in postoperative pain, it is logical to speculate that synthesis induction (or additional injection) of the endogenous agent that provides antiinflammation reaction is one of the alternative ways to decrease pain without strong and prolonged treatment by direct receptors blockader to avoid the development of drug tolerance.

Following this line, our interest was focused on mammalian metallothioneins (MTs), cystein-rich small metal-binding proteins that are induced not only by heavy metals (*Zn*, *Cd*) and other divalent cations (*Ca*, *Mg*, *Mn*) but also by specific agents as catecholamines, glucocorticoids, interleukin-6, glucagon, tumor necrosis factor, acute phase response agents (Di Silvestro and Joseph, 1995). Many indicated agents take part in the peripheral and central pathways of postoperative pain.

In our study the experimental model of postoperative pain was developed due to Brennan (Brennan et al., 1996). During the time of the experiment time all animals were kept with a normal food and water intake. The weight of the control animals and rats before surgery was on average 324 ± 25 g. There was a small significant decrease in weight during the first 3 days after incision (283 ± 16, $P < 0.05$) for rats without additional treatment. The weight of the rats that obtained MT-treatment after surgery did not change compared with the control animals during the whole studied postoperative period.

The tests of locomotion, exploration activity and anxiety provide us with the indicators of underlying pain. Over the 6 days of testing the control rats (1) showed an unchanged level of locomotion, exploration and anxiety (Fig. 3).

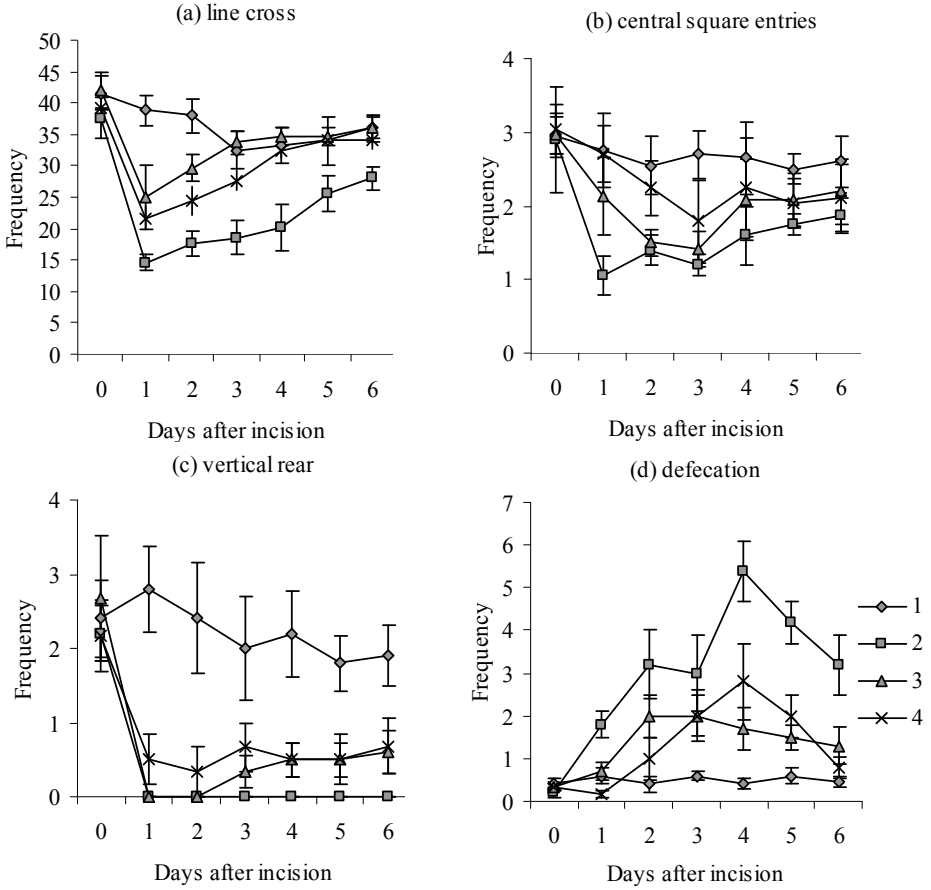


**Fig. 3. Frequency of physiological activity of rats in the open field test:**
1 – control, without any treatment ($n = 8$); 2 – incision ($n = 8$); 3 – injection by 0.2 ml physiological solution containing 48 μg MT-I+II per animal during 2 days after incision ($n = 8$); 4 – injection by 100 μg MT-I+II per animal during 2 days after incision ($n = 6$)

The highest significant difference was found for the all tests for rats (2) on day 1 after surgery. During next 5 days locomotive activity including frequency of line cross and central square entries returned gradually, however, the control level was not yet reached at day 6 after incision. The open field test revealed a high increase of anxiety during 6 days after surgery. The frequency of defecation was elevated to 3–5 times during week after incision. Injection of MT-II in the dose 48 μg/rat twice per day during 2 days after incision induced better recovery of locomotion of treated rats compared with the operated animals but there was not so much exploration activity and anxiety after incision. Much better results were shown for the rats under MT-II treatment at a dose of 100 μg/rat twice per day during 2 days after incision. The results obtained indicate that injection by MT-II prevents postoperative hyperalgesia according to mild alteration of all studied physiological tests. Moreover, we can see that recovery of locomotion and exploration activity, and decrease of anxiety under MT-II treatment by 100 μg/rat was already evidence by day 4 after surgery.

Many cellular events recognized by the system are believed to be the central event in the initiation of algesion after surgerical procedure, in which activation of NMDA receptors plays the key role. Previously it was believed that zinc inhibits NMDA receptors at two independent sites: 1) low-affinity inside zinc-binding site induces the voltage-dependent inhibition of NMDA-receptors; 2) high-affinity outside zinc-binding site promotes the voltage-independent inhibition. Later data suggest that tyrosine kinase Src potentiates NMDA-receptors currents by reducing the tonic inhibition of receptors composed of NR1 and NR2A subunits by extracellular zinc (Vander Jagt et al., 2009). The data obtained in our study link two modulator sites of NMDA receptors. Metallothioneins can help to regulate the zinc donation to provide efficiency of NMDA inhibition.

## Conclusion

The protective role of metallothioneins against *Cd* toxicity can change the level and character of MT distribution in the different brain regions subject to acute or chronic *Cd* poisoning.

Other proposed functions of MT, such as maintaining essential metal (zinc) homeostasis, regulating gene expression, tissue regeneration and inhibition of postoperative pain could contribute to protection by MT of the nervous system and the whole organism.

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*Надійшла до редколегії 19.11.2013*