THINNER EXPOSURE DISTURBS NEUROSPECIFIC PROTEIN EXPRESSION AND LEADS TO MEMORY RETENTION

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Neural functions are very sensitive to different kind treatment from pharmacology to environmental pollutants substances. Neurospecific proteins are involved in learning and memory.

Thinner is a neurotoxic mixture which is widely used as an aromatic industrial solvent. This product has been shown to cause functional and structural changes in the central nervous system. We investigated the effect of exposure to high concentrations (3000 p.p.m.) of thinner for 45 days (1 hr/day) on cognitive functions and the levels of neural cell adhesion molecules (NCAM) and lipid peroxidation products (LPO) in the hippocampus, cortex and cerebellum of rats. The actions of melatonin on the effects produced by thinner exposure were also tested. Thinner exposure caused a dramatic increase in LPO (malondialdehyde and 4-hydroxyalkenals) in all brain regions. Melatonin administration significantly reduced LPO in these brain regions. NCAM (180 kDa) was significantly decreased in hippocampus and cortex of thinner-exposed rats. Furthermore, thinner-exposed rats showed cognitive deficits in passive avoidance and Morris water maze tasks, whereas in the rats chronically treated with melatonin these effects were reversed. This study indicates that treatment with melatonin prevents learning and memory deficits caused by thinner exposure possibly by reducing oxidative stress and regulating neural plasticity

Learning, lipid peroxidation, melatonin, neural cell adhesion molecules, thinner

Introduction. Thinner is a compound containing a mixture of toluene, benzene, acetone, methanol, hexane, and other substances and is a widely used aromatic solvent in textile, paints and solvent-based cleaning fluids. The major component is the neurotoxin toluene (60–70%). Toluene is an abused substance and a well-known neurotoxic agent [1]. The chronic abuse of solvents results in structural and functional impairment to a variety of organs. Thinner fume inhalation is an important cause of encephalopathy and may lead to irreversible brain damage. Particularly, toluene abuse has been shown to cause permanent changes in brain structures which correlate with neural dysfunction [2–4].

Exposure to toluene is known to cause hearing impairment and reduce birth weight in rats [5]. Furthermore, Mattia et al. [6] demonstrated that an intraperitoneal injection of toluene caused a significant elevation in the rate of reactive oxygen species (ROS) generation and a reduction in glutathione (GSH) levels in the brain. ROS, in turn, damage lipids, proteins and nucleic acids. This leads to neurodegenerative disorders which mediate behavioral changes. Acute and chronic effects of toluene on neurons have been well documented [6]. Toluene can cause CNS depression, loss of memory and progressive brain and nerve damage [7]. Exposure to toluene has been shown to

deplete spatial learning, as measured by the Morris water maze [8, 9]. There are limited data to explain the mechanism of the neurotoxic effects of solvents such as thinner and toluene on behavioral and functional structures of the CNS.

Neural cell adhesion molecules (NCAM) play important developmental and structural roles in the nervous system. Furthermore, NCAM are involved in learning and memory [10]. These molecules are also involved in cellular migration, axonal growth, and regeneration of peripheral axons [11, 12].

Melatonin, pineal-derived product, is a potent free radical scavenger [13, 14] which is known to reduce oxidation-based neurotoxicity [15]. In addition to its antioxidant effects, melatonin's ability to influence cognitive functions has also been studied. It has been demonstrated that melatonin modulates specific forms of plasticity in hippocampal pyramidal neurons [16]. Melatonin may be involved in structural remodeling of synaptic connections during memory and learning processes. Recently, we showed that melatonin modulates the expression of NCAM in brain areas concerned with cognitive function [17]. In the present study, we investigated the effects of melatonin on learning and memory impairment in rats induced by thinner exposure and we examined the possible mechanism involved.

2. Material and methods.

Animals and treatments

Male Wistar rats (weighing 200–250 g) were purchased from Animal Research Unit, Dnepropetrovsk national University. They were kept in a temperature- and light-controlled room with free access to food pellets and tap water. The animals were randomly divided into three groups each consisting of 40 animals. Two groups were exposed to inhalation of 3000 p.p.m. thinner 1 hr/day between 17:00 and 18:00 hr for 45 days. The exposure to thinner was performed in a whole-body inhalation chamber with glass walls. The control group was exposed only to fresh air. One of the thinner-exposed groups was given melatonin intraperitoneally in a dose of 10 mg/kg body weight once daily before thinner exposure over a period of 45 days. All protocols described were reviewed and approved by the Local Institutional Committee for the Ethical Use of Animals.

Passive avoidance test

A one-trial step-down type passive avoidance task was used to evaluate memory retention deficits in rats as previously described [18]. Training was carried out 24 hr after the last exposure to thinner. The apparatus consisted of two compartment dark/light shuttle box. The floor of the dark compartment consisted of a stainless steel shock grid floor. Electric shocks were delivered to the grid floor with a stimulator. For these trials, the rats were placed in a lighted compartment of the apparatus, as a rat was about to enter the dark chamber foot shock was delivered for a period of 5 s. The entrance latency to the dark compartment (step through latency) was recorded.

The retention test was performed 24 hr after the acquisition trial in a similar manner; latency time was measured by placing the animals in the light chamber and recording the interval to enter the dark compartment. Animals had free access to both compartments for 10 min. Short latencies indicate poor retention compared with significant longer latencies.

Morris water maze learning performance

The Morris water maze [19] was selected as a test of spatial learning and memory. A circular water tank (180 cm diameter, 70 cm wheight) was filled to a depth of 40 cm with water at 25 ± 1^0C and the tank was divided virtually into four equal quadrants, labeled N–S–E–W. The water was made opaque by the addition of semi-skimmed milk. A platform (10 cm diameter) was placed in one of the four maze quadrants (the target quadrant) and submerged 1.5 cm below the water surface. For each individual rat the position of the platform was fixed during the entire experiment. The rats were required to find the platform using only distal spatial cues available in the testing room. Cues were maintained constant throughout testing. The rats were trained to locate and escape onto the platform for four trials per day for five consecutive days. A different starting position was used on each trial (in a quadrant not containing the platform). A trial began by placing the rat into the water facing the wall of the pool at one of the starting points. The animals were allowed to swim freely to find the hidden platform within 60 s and after reaching the platform they were allowed to stay on it for 30 s and then returned to the cage, which was warmed with a heating pad, to await its next trial. There was a 30-s intertrial interval. If the rat had failed to escape, then the rat was directed to the platform by the experimenter and allowed to remain there for the same amount of time. The experimenter, who stood at the same location during each trial, recorded the latencies to reach the platform.

Probe trial

A probe trial was performed wherein the extent of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the rat was placed into the pool as in the training trial, except that the hid-

den platform was removed from the pool. The time of crossing the former platform quadrant and the total time of crossing all quadrants were recorded for 1 min. To test possible deficits in sensorimotor processes, rats were tested in the water maze with a visible platform on a new location on the final day of training [20]. For the visual test, the black target platform was placed inside the pool 1 cm above the water line. Latency times to reach the platform were recorded for each trial.

Immunoblotting

After decapitation, the brain was removed and the hippocampus, cerebral cortex and cerebellum were dissected. Samples were used fresh or kept at − 80^0 C. Fresh or frozen tissue samples were homogenized 1:10 (w/v) in buffer [10 mM Tris–HCl, pH7,4, 0.1 mMNaCl, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 5 μM soybean (Sigma, St. Louis, MO, USA) as trypsin inhibitor]. Homogenates were centrifuged at 60,000 g for 60 min. Pellets were washed and resuspended in homogenizing buffer and recentrifuged at 60,000 g for 60 min. The resulting pellets were washed and resuspended in buffer (25 mM Tris–HCl, pH 7,4, 0.1 mM PMSF and 2% Triton X-100). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 2% β-mercaptoethanol was added to the supernatants. Equal amounts of total protein were applied on each lane as described previously [17, 21]. Separated proteins were transferred to nitrocellulose filters (Schleicher and Schuell Inc., Keene, NH, USA) with an electro blotter. After saturation of the nonspecific sites of binding with 1% bovine serum albumin in 100 mM NaCl, 20 mM Na_2H-PO_4, and 20 mM NaH_2PO_4 at pH 7,2, the blots were incubated overnight at 4^0C with a polyclonal rabbit anti-rat NCAM (1:2000). The blots were washed and incubated for 1 hr with a secondary antibody, a goat anti rabbit Ig peroxidase conjugate (Sigma, Dorset, UK). Specific binding was detected using diaminobenzidine and H_2O_2 as substrates. The relative amount of immunoreactive NCAM isoforms on Western blots was quantified in arbitrary units by scanning blots using a computerized software program (LabWorks 4.0; UVP, Inc. Cambridge, UK).

Protein, lipid peroxidation, and GSH assays

Total protein levels were measured according to Lowry et al. [22]. Tissue lipid peroxidation (malondialdehyde + 4-hydroxyalkenals: MDA + 4-HDA) was determined using an LPO-586 kit (Oxis International, Inc., Corvallis, OR, USA); the method is based on a reaction of N-methyl- 2-phenylindole with MDA + 4-HDA at 45^0 C. GSH levels were determined according to the method of Ellman [23].

Statistical analysis

Data are presented as mean ± S.E. Between-group differences in passive avoidance test and biochemical data were analyzed by one-way analysis of variance (ANOVA) with the post hoc Duncan's multiple range tests. Between-group differences in latencies were analyzed by analysis of variance for repeated measurements (ANOVAR) followed by Fisher's post hoc test for all groups.

3. Results and Discussion

Thinner exposure significantly increased the level of LPO, i.e. MDA and 4-HDA, in hippocampus, cerebellum ($P < 0,001$) and in cortex ($P < 0,01$) as compared with those in the control. Melatonin administration to thinner exposed rats significantly reduced the levels of LPO in brain tissues compared with thinner-exposed group (Fig. 1). There was no significant effect of thinner inhalation on the levels of GSH in any brain region. Melatonin treatment significantly increased GSH levels in thinner-exposed rats compared with the thinner group.

The ANOVA test indicated that before the acquisition trial, there were no significant differences in the step through latency between groups. Retention of passive avoidance response was different from the control group, the mean retention latency in thinner-exposed group was significantly less ($P < 0,01$) as compared with that of control rats. A reduction in retention latency indicates impairment in memory retention of the passive-avoidance task in thinner exposed rats. Chronic administration of melatonin to thinner-exposed rats ameliorated significantly the impairment of passive avoidance memory in rats exposed to thinner ($P < 0.05$; Fig. 2).

All rats showed a gradual reduction in the time taken to find the escape platform as training proceeded. The mean latencies in thinner-exposed and control rats were similar in the first trial, which suggests that their motor performance (ability to swim) was unaffected by the thinner

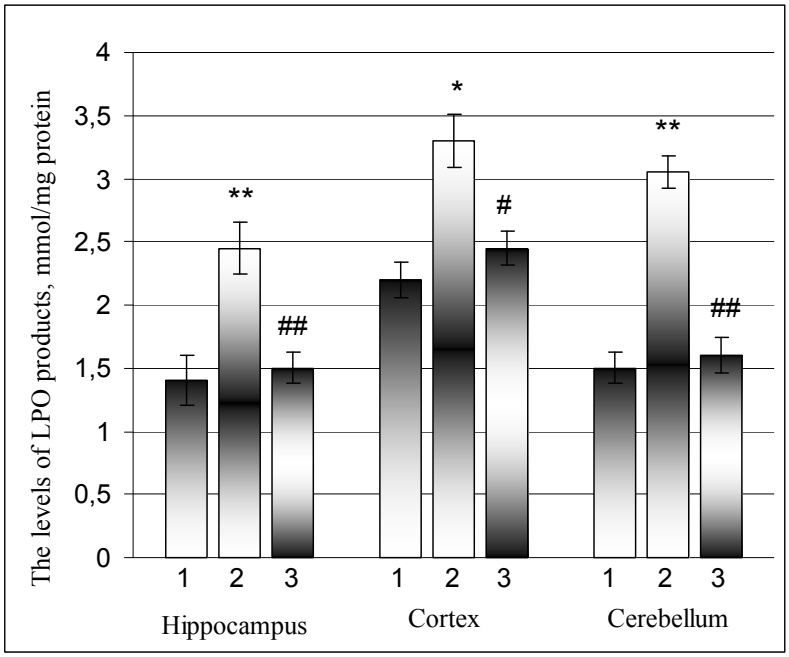

Figure 1. **The levels of lipid peroxidation products (as malondialdehyde+4-hydroxialkenals) in different brain regions (mean ± S.D.). 1 – control; 2 – thinner-exposed group; 3 – thinner+melatonin treated group**

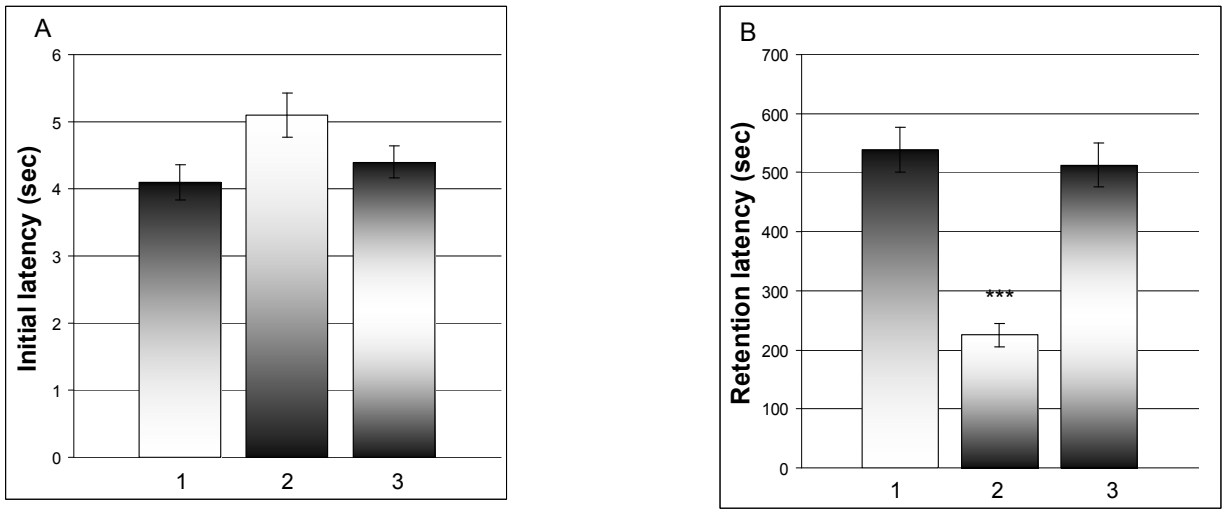

Figure 2. **Effects of thinner inhalation on the initial latency (A) and retention latency (B) in the passive avoidance test. 1 – control; 2 – thinner-exposed group; 3 – thinner+melatonin treated group. (***P < 0,001 vs. control)**

inhalation; however, the thinner-exposed group tended to use more time than controls in following trials. The overall rate of learning was significantly higher in control group than in thinner-exposed rats ($P < 0,02$). Although, melatonin administered rats learned the task faster than thinner-exposed rats ($P < 0,05$), the learning performance of control rats was better than melatonin-treated group ($P < 0,05$). The performance of thinner exposed and control rats in the trial with the visible platform were not different (latencies: 9,2 ± 0,8, 10,2 ± 1,2 and 10,1 ± 1,0 s in control, thinner-exposed and thinner + melatonin-treated rats respectively). Melatonin treatment largely prevents the impaired performance induced by thinner inhalation in the water maze.

Data from the probe trial of the Morris water maze study, which measures how well the animals had learned and consolidated the platform location during the 7 days of training, indicated significant

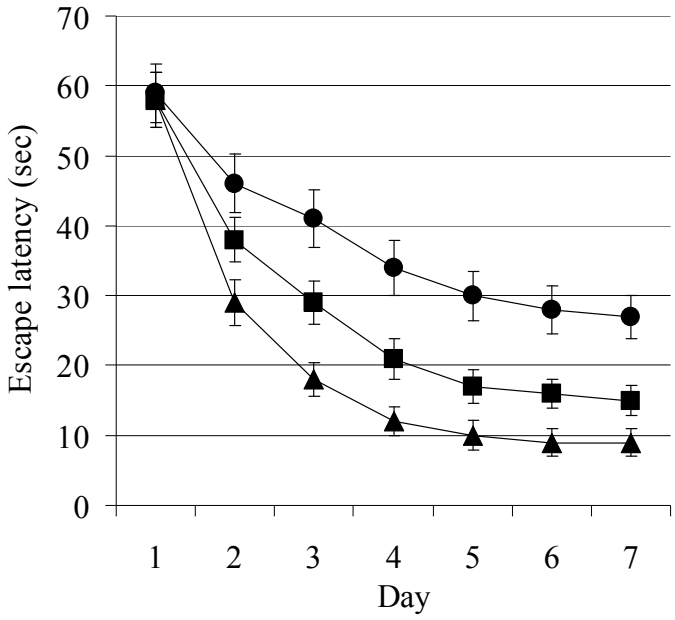

Figure 3. **Effects of chronic thinner inhalation on the acquisition of spatial learning in the Morris water maze. ▲ – control; ● – thinner-exposed group; ■ – thinner+melatonin treated group**

differences between the groups ($P < 0,001$; Fig. 3). Thinner-exposed rats spent 39,6% more time in the target quadrant than the control group. On the contrary, the rats treated with melatonin spent significantly less time in the target quadrant than the thinner-exposed group in the probe test ($P < 0,05$). There was a greater significant difference between control and thinner groups ($P < 0,001$) than melatonin and thinner groups ($P < 0,05$).

The levels of NCAM were examined in different brain parts of thinner-exposed and control rats. The antibody against NCAM allowed the detection of bands at 120, 140 and 180 kDa in rat brain. Although there is no significant change in the expression of NCAM 120 and NCAM 140 levels, a significant reduction was found in NCAM 180 level in thinner-exposed rats. Melatonin administration significantly elevated the levels of the three NCAM isoforms both in hippocampus and cortex (Fig. 4).

Thinner is thought to generate ROS which induce oxidative damage in lipids, proteins and nucleic acids. Toluene has been shown to produce ROS both in vivo and in vitro in many tissues including brain [5]. Individuals working with paint thinner have been shown to have increased levels of MDA in their serum compared with unexposed subjects [24]. Recently, we showed [25] that thinner-exposure increases LPO in different brain regions and these elevations are inhibited by antioxidant melatonin.

Consistent with the previous studies herein we demonstrate that thinner exposure significantly elevated the levels of LPO in several brain regions. Generation of ROS can lead to cell and tissue damage and alterations in functions of the brain resulting in cognitive deficits. In the present study, thinner exposure-related effects on cognitive functions were observed in both the Morris water maze and passive avoidance tests in rats. We found that exposure to high level of thinner caused spatial learning deficit, compared with control animals. These findings are in agreement with the results of other investigators who showed that toluene exposure stimulates ROS formation [5, 6, 24] and that memory is impaired by oxidative stress [26, 27]. The retention latency in the passive avoidance test was correlated with increased LPO levels in cortex and hippocampus ($r = -0,50$, $P < 0,05$; $r = -0,55$, $P < 0,01$ respectively). Furthermore, increased LPO levels within the cortex and hippocampus were also correlated with the swim time spend in the target quadrant $r = -0,62$, $P < 0,01$; $r = -0,67$, $P < 0,001$ respectively). These findings indicate that oxidative stress induced by thinner exposure may be an important pathophysiology mechanism underlying learning and memory deficits.

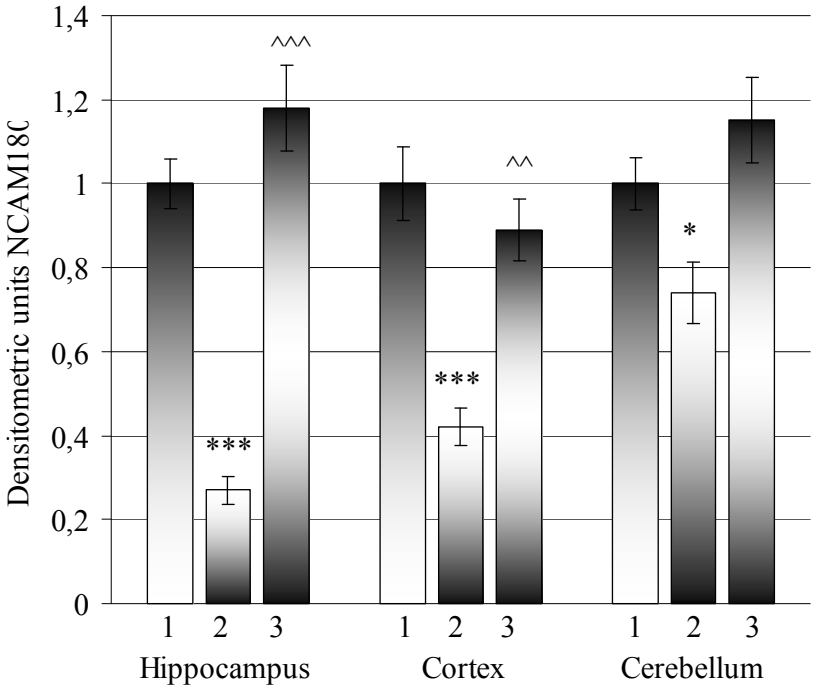

Figure 4. **Relative densitometric analysis of NCAM 180 kDa from control and thinner-exposed rats. 1 – control; 2 – thinner-exposed group; 3 – thinner+melatonin treated group.**

Values are mean ± S.D.; *P <0,05, ***P <0,001 vs. control; ^^P <0,01, ^^^P <0,001 vs. thinner group

Herein we also showed that the administration of melatonin significantly reduced the levels of LPO in hippocampus and cortex and considerably improved cognitive performance. This strongly indicates that thinner exposure impairs memory, an effect probably mediated by oxidative stress as treatment with the antioxidant melatonin prevented learning and memory deficits. Melatonin is highly effective in reducing oxidative damage in the central nervous system; this efficacy derives from its ability to directly scavenge a number of free radicals and to function as an indirect antioxidant [13–15]. Furthermore, consistent with the previous studies melatonin significantly increased GSH levels in brain regions. As learning and memory deficits are associated with increased brain oxidative stress after thinner exposure, and its reversal by antioxidants [28, 29], our results suggest that, at least partly, the effects of melatonin in the improving cognitive deficits could be due to its antioxidant actions. This is in agreement with the previous studies which indicated that anti-amnestic effects of melatonin is related to its antioxidant action [30].

The second important observation of the current study are the changes in the expression pattern of NCAM in different brain regions of thinner-exposed and melatonin administered rats. To our best knowledge this is the first report that documents an effect of melatonin on the expression of NCAM in thinner-exposed rats. NCAM contribute to the structural organization of the nervous system during brain development and also participate in synaptic modification in the mature brain. Furthermore, it has been shown that NCAM may contribute to the neural regeneration. In accordance with learning, expression of NCAM was impaired in the thinner-exposed rats compared with controls. Several recent studies indicate a role for NCAM in learning and establishment of long-term memory [11, 31]. In the present study, we found that chronic exposure to thinner induced a significant reduction especially in the expression of NCAM 180 in both hippocampus and cortex.

The mechanism by which thinner exposure alters the pattern of NCAM expression is not known. Chronic stress exposure induces a considerable degree of structural plasticity in the adult brain, especially in the hippocampus where these changes are accompanied by impairments in cognitive performance [32]. It is postulated that chronic stress interferes with the mechanisms involved in the ex-

pression of NCAM [33]. Thinner exposure to rats provides a relevant example of chronic stress which generates ROS and induces structural and functional changes in the nervous system [5, 6, 24]. Supporting this hypothesis, a significant elevation of lipid breakdown products was found in hippocampus, cortex and cerebellum. Loss of NCAM 180 has been found to be accompanied by cell injury following ototoxicant trimethyltin chloride, which involves ROS generation, exposure and to be related to cytoskeletal alterations and destabilization of cellular contacts [34, 35]. We have previously indicated that NCAM is down-regulated in the hippocampus of rats under constant light leading to generation of ROS [17, 36]. Thus, we speculate that generation of ROS induced by thinner exposure alters the pattern of NCAM expression and suggests that modification of NCAM could affect cognitive functions in rats. We found here that thinner exposure, like ototoxicant trimethyltin chloride, reduced the levels of NCAM 180 in cortex and hippocampus.

The changes in the expression of NCAM may underlie the impaired cognitive performance induced by thinner exposure. One possible explanation for the learning and memory deficits in the thinner-exposed rats is that synaptic remodeling and plasticity require optimal NCAM concentrations that are permissive for activity-dependent synaptic sprouting [37]. Thus, thinner exposure causes down-regulation of NCAM 180 in hippocampus and this in turn inhibits the formation of new synapses required for learning and memory. NCAM 180 is the main isoform of NCAM and it is crucial for the stabilization of cell binding at synaptic sites [38]. NCAM 180, but not NCAM 140, is known to interact with spectrin via its cytoplasmic domain. This interaction was proposed to be important in signal transduction [39]. Augmented NCAM expression in the hippocampus and cerebral cortex by pharmacological doses of melatonin indicates that these brain areas may be sites at which this indole modulates cognitive processes. In the brain, melatonin-binding sites have been found in regions implicated in cognition and memory [40]. Argyriou et al. [41] showed that melatonin facilitated short-term memory and that endogenous melatonin administration exerted a permanent facilitatory effect on memory processes.

4. Conclusions.

Astrocytes play the key role in the regulation of neuron microenvironmental. Presented data show that melatonin can defend the neurons from toxic compounds through the protection of astrocyte surviving. Especially, melatonin may promote effectiveness neuroprotective effect of astrocytes by its antioxidant activity.

The cellular and molecular mechanisms by melatonin modulates cognitive functions are as yet unclear. The findings of the present study show that melatonin alters NCAM expression in the hippocampus, cortex, and cerebellum. Loss of NCAM 180 has been found to be accompanied by cell injury following toxicant exposure and to be related to cytoskeletal alterations and destabilization of cellular contacts. Thus, melatonin could stabilize neuronal connections by preventing the reduction of NCAM 180 expression.

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ВОЗДЕЙСТВИЕ ТОЛУЕНА НА ЭКСПРЕСИЮ НЕЙРОСПЕЦИФИЧЕСКИХ БЕЛКОВ И СОХРАНЕНИЕ ПАМЯТИ

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Нервные функции чрезвычайно чувствительны к действию различных факторов от фармакологических до загрязнителей окружающей среды. Нейроспецифические белки принимают участие в процессах обучения и памяти. Растворители являются нейротоксичными смесями, которые широко используются в качестве органических промышленных сольвентов. Показано, что такие соединения могут вызывать функциональные и структурные изменения в центральной нервной системе. Мы изучали эффекты действия высоких концентраций (3000 р.р.т.) растворителя на протяжение 45 дней (1 час/день) на познавательные функции, уровни молекулы адгезии нервных клеток (NCAM) и перекисного окисления липидов (ПОЛ) в гиппокампе, коре и мозжечке крыс. Было выявлена также действие мелатонина на эффекты вызванные растворителем. Действие растворителя вызывало значительное возрастание продуктов ПОЛ во всех исследуемых регионах мозга. Введение мелатонина существенно снижало уровень ПОЛ в этих регионах. Уровень NCAM (180 кДа) был так же значительно сниженный в гиппокампе и коре группы крыс, которые получали ингаляции растворителя. Более того, в этой группе крыс показан познавательный дефицит в тесте пассивного избегания и водном тесте Морриса. В это же время, в группе крыс, которые дополнительно получали инъекции мелатонина эффект растворителя был снижен. Исследование показывает, что введение мелатонина предупреждает развитие дефицита обучения и памяти вызванное воздействием растворителя, что может быть связано с снижением оксидативного стресса и регуляцией нейрональной пластичности

***Ключевые слова**: обучение, перекисное окисление липидов, мелатонин, молекула адгезии нервных клеток, растворитель*

ВПЛИВ ТОЛУЕНУ НА ЕКСПРЕСІЮ НЕРВОВОСПЕЦИФІЧНИХ БІЛКІВ ТА ЗБЕРЕЖЕННЯ ПАМ'ЯТІ

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Нервові функції дуже чутливі до дії різних чинників від фармакологічних до забруднювачів довколишнього середовища. Нервовоспецифічні білки залучаються до процесів навчання і пам'яті. Розчинники є нейротоксичними сумішами, які широко використовуються в якості органічних промислових сольвентів. Показано, що такі сполуки можуть спричиняти функціональні і структурні зміни у центральній нервовій системі. Ми досліджували ефекти дії високих концентрацій (3000 р.р.т.) розчинника протягом 45 днів (1 год/доба) на пізнавальні функції, рівні молекули клітинної адгезії нейронів (NCAM) і перекисного окиснення ліпідів (ПОЛ) у гіпокампі, корі та мозочку щурів. Була визначена також дія мелатоніну на ефекти викликані розчинником. Дія розчинника викликала значне зростання продуктів ПОЛ в усіх досліджених регіонах мозку. Введення мелатоніну суттєво знижувало рівень ПОЛ в цих регіонах. Рівень NCAM (180 кДа) був також значно знижений у гіпокампі і корі групи щурів, які отримували інгаляції розчинника. Більш за те, в цій групі щурів показаний пізнавальний дефіцит у тесті пасивного уникання і водному тесті Моріса. В той же час, в групі щурів, які додатково отримували ін'єкції мелатоніну ефект розчинника був знижений. Дослідження показує, що введення мелатоніну попереджає розвиток дефіциту навчання і пам'яті спричинений через вплив розчинника, що може бути пов'язано із зниженням окисного стресу і регуляцією нейрональної пластичності

***Ключові слова**: навчання, перекисне окиснення ліпідів, мелатонін, молекула адгезії нервових клітин, розчинник*