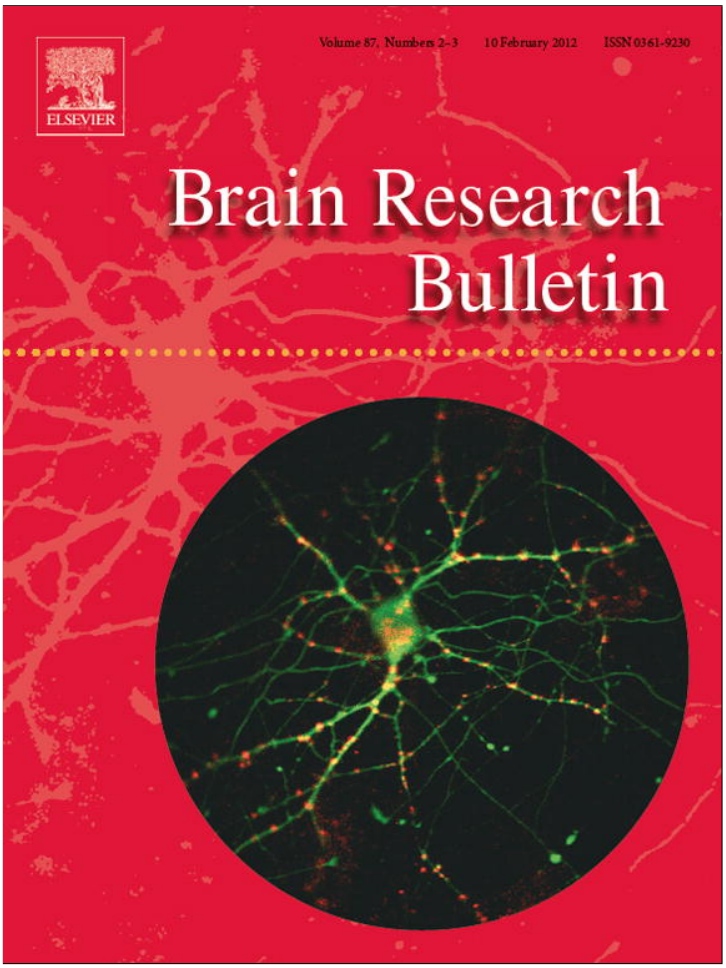

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Research report

Immediate and persisting effect of toluene chronic exposure on hippocampal cell loss in adolescent and adult rats

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ABSTRACT

Abuse of toluene-containing volatile inhalants has become widespread among adolescents. Besides, because toluene is usually used as an industrial solvent in manufacturing of chemical pharmaceuticals and multiple commonly used household and commercial products, it has high potential for abuse for adults also. Long-term exposure to toluene vapor has a severe impact on the central nervous system, resulting in numerous neurological, neurobiological and behavioral impairments. Recently in the hippocampus some molecular and biochemical changes as a result of toluene chronic exposure were described. Such data point out the involvement of this area in the toluene addiction. However it remains uncertain whether toluene provokes structural alterations in the hippocampus. In this study we exposed male Wistar rats to 2000 ppm inhaled toluene for 40 days in rats at ages P 28–32 (adolescents) and P 70–75 (adults). The immediate and delayed effects of toluene chronic exposure (immediately after the end of toluene chronic inhalation and 90-day after the end of toluene chronic inhalation, correspondingly) on pyramidal cell loss in adolescent and adult rats was investigated. The results reveal that (i) chronic exposure to 2000 ppm of toluene chronic exposure alters the structure of hippocampus in adolescent and adult rats provoking both, immediate and delayed effects; (ii) the character of structural alterations depends upon the postnatal age of testing of the animals.

1. Introduction

Abuse of toluene-containing volatile inhalants has become widespread among adolescents, young people and children. Besides, because toluene is usually used as an industrial solvent in manufacturing of chemical pharmaceuticals and multiple commonly used household and commercial products, such as gasoline, glue, rubber cement or paints, it has the highest potential for abuse for adult workers also [1–3]. Experimental and clinical data demonstrate that long-term exposure to toluene vapor has a severe impact on central nervous system of children, adolescents and adults, resulting to numerous, sometimes long-lasting neurological, neurobiological and behavioral impairments as well as diffuse changes in white matter [4–8].

It is clear that toluene share common cellular mechanisms and has similar actions to other drugs of abuse, namely, it activates the mesolimbic dopaminergic reward system—the major substrate of addiction [9–12]. But, compared with other abusive substances, relatively few experiments have been done exploring the alterations provoked by toluene at different levels of the organism of different age. Thus, while some studies have assessed outcomes of chronic misuse of toluene-containing substances in adults, there are few data on its effects in younger animals [5,12,13]. Besides the biggest part of investigations concerning toluene's addictive nature has focused on its immediate effect, while persisting effect (months or years after withdrawal) is not well known.

The importance of learning and memory in addiction is an area of increased interest. More and more studies indicate that presentation of addictive drugs to abusers is associated with the activation of brain regions that are involved both, in addictive and learning processes [14,20,15,16]. Based on these data, it was suggested that addiction and learning and memory can shares the nervous substrates. As potential substrates for both processes mesocorticolimbic system—including the hippocampus, have been identified.

Hippocampus, along with amygdala, is considered to be critical for cue-elicited drug-seeking taking [17,18,19]. Thus, recently it was shown that hippocampal pathways are highly implicated in drug-seeking that is elicited by contextual stimuli [15,20,21]; the hippocampus participates in strengthening connections in the areas that are involved in addiction, suggesting that drug-induced

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changes in the hippocampal function could produce long-lasting functional changes in these areas [19,20,22]; besides, manipulation of hippocampal cells alters dopamine levels and firing rates of dopaminergic cells in the areas of mesolimbic system—neural substrate that could contribute to the pathophysiology of drug abuse [23,10,24; Eisch and Harturg, 2006]. These properties of the hippocampus make this structure as an intriguing area in regard to addiction.

It is well-known that detailed neuroanatomical analysis can provide insight into alterations provoked by addictive drugs in the morphology of mesolimbic reward pathway. Significant structural disorders of this system, including hippocampal cell death, have been described as a result of chronic misuse of nicotine, alcohol, cocaine, methamphetamine and other drugs [21,25,26]. These alterations, considering as associated with modifications in hippocampal learning-related cell signaling present documentations of the importance of learning, memory and synaptic plasticity in addiction of these drugs [20,22,27,28]. Recently in the hippocampus some molecular and biochemical changes as a result of toluene chronic exposure were also described [1,12,25,29,30]. These data point out the involvement of this structure in the toluene addiction. However it remains uncertain whether such exposure provokes structural alterations in the hippocampus of animals of different age or, in the case of such alterations, is the structure of the hippocampus of adults and adolescents differentially susceptible to this exposure. We suggest that studying a unique profile of structural changes in organisms of different age, may provide valuable information regarding the mechanism of action of toluene.

The present experiments are designed to clarify immediate and persisting effect of toluene chronic exposure on the pyramidal cell loss (most overt form of brain damage) of hippocampus in adolescent and adult rats: question that was never reported before.

2. Materials and methods

2.1. Experimental design and animal groups

Male Wistar rats of two age group 28–30 days (weighting 100–120 g) as adolescent [26,31,32] and 90–100 days (weighting 200–220 g) as adult were used in this study. Each rat was placed separately in special chamber (30 °C) and was exposed to toluene vapors (2000 ppm) for 3–4 min, during 40 days. Thereafter animals of each age group were divided into two subgroups. For the purpose to clarify the immediate effect of toluene chronic exposure, the animals of first group were perfused on the next (41st) day after the end of toluene exposure, while for to clarify its persisting effect, rats of second group were perfused 90 days after the end of toluene exposure. The age-matched control rats were exposed for 3–4 min to fresh air in the same chamber during 40 and 90 days. As a consequence the following animal groups were studied:

- Adolescents:
  Control animals: immediate and persisting effects;
  Experimental animals: immediate and persisting effects
- Adults:
  Control animals: immediate and persisting effects;
  Experimental animals: immediate and persisting effects

Totally 32 animals (4 rats for each group) were studied. Experimental protocol was approved by Animal Studies Committee of Georgian Life Science Research Centre and was in accordance with guidelines of the EC Council Directives.

2.2. Histological procedure

Under pentobarbital injection (100 mg/kg), experimental and control animals underwent transcardiac perfusion with heparinized 0.9% NaCl, followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH-7.4 at a perfusion pressure 120 mm Hg. The brains were removed from skull, hippocampi were isolated, placed in 30% sucrose in 0.1 M PB until equilibrating, then were blocked, frozen and sectioned in the coronal plane (30 mm) with freezing microtome. 30 μm thick, consecutive coronal sections were collected between −2.28 and −3.48 mm from bregma [52]. Sections were kept in serial order, placed in 0.1 M PB, and every 3rd section was stained for Thionin in order to identify the spatial distribution of pyramidal cell loss in the CA1 and CA3 areas of the hippocampus. Using anatomical land marks totally 6–10 sections/animal of similar hippocampal levels within and between experimental groups were selected (Fig. 1).

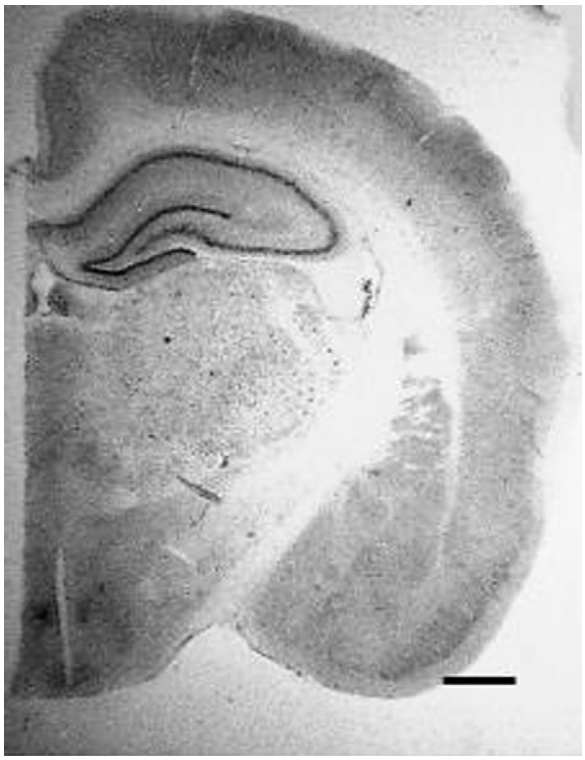

Fig. 1. Section of hippocampal sub region of control rat brain demonstrating one of the levels used for cell counting. Magnification 3.6×; scale = 1.4 μm.

2.3. Cell counting

For cell counting a systematic random sampling was employed and the neurons with distinct nucleus and nucleolus were counted in 5 non-overlapping fields with 2-dimensional counting grid (250 μm × 250 μm) in CA1 area and 3 fields in CA3 area at the magnification 400× in the hippocampal sections of each condition. Totally 30 fields per animal were analyzed and average of cell central profiles per field was used to assess anatomical damage of CA1 and CA3 hippocampal subregions in abovementioned groups of animals. The sections were analyzed with a microscope Leica MM AF.

2.4. Statistical analysis

To determine whether toluene chronic exposure provokes immediate or persisting effect on the number of principal cells of the hippocampus, the one-way ANOVA of quantitative data was performed separately in adolescent and adult rats. The results were presented as mean ± standard error (SE). A p-value less than 0.05 were considered as statistically significant. In the case of significant effect planned comparisons were carried out using t-tests.

3. Results

3.1. CA1 area

3.1.1. Adolescent rats

One-way ANOVA revealed effect of experimental conditions (immediate and persistent effects of toluene exposure) on the number of principal cells in the CA1 area of adolescent rat [$F(3,12) = 20.65$; $p = 0.001$] (Table 1). The significant pyramidal cell loss was observed immediately (25%, $p = 0.009$) and 90 days after the end of toluene chronic exposure (40%, $p = 0.01$) vs. control. No significant difference was observed between these experimental animal groups ($p > 0.05$) (Table 2.).

Table 1
Summary of one-way ANOVAs results: *F*-variance ratio from one-way ANOVA; *P*-probability.

	Adolescents		Adults	
	$F_{3,12}$	*P*	$F_{3,12}$	*P*
CA1 area				
Pyramidal layer (PL)	20.65	0.000	32.56	0.000
CA3 area				
Pyramidal layer (PL)	9.59	0.006	5.26	0.031

3.1.2. Adult rats

As in the hippocampus of adolescent rats, in adult rats one-way ANOVA revealed significant effect of experimental conditions (*immediate and persistent effects of toluene exposure*) on the number of principal cells in [$F(3,12) = 32.56$; $p = 0.0001$] (Table 1). Like in the adolescents, in adult animals significant cell loss was observed both, immediately (21%, $p = 0.008$) and 90 days after cessation of toluene chronic exposure (40%, $p = 0.003$). Significant difference was observed between animals investigated immediately after the end of toluene exposure and 90 days after) (25%, $p = 0.034$ *t*-test) (Table 2).

Thus: In the CA1 area of adolescent and adult rats both, immediate and persistent effects of toluene chronic exposure were observed (Fig. 2a and b).

3.2. CA3 area

3.2.1. Adolescent rats

One-way ANOVA revealed effect of experimental conditions (*immediate and persisting effects of toluene exposure*) on the number of principal cells [$F(3,12) = 9.59$; $p = 0.006$] (Table 1). As in the CA1 area, in the CA3 area significant loss of pyramidal cells was observed immediately (23%, $p = 0.037$) and 90 days after cessation (28%, $p = 0.008$) of toluene exposure. In the CA3 area both effects were almost the same (no difference between aging groups was observed) (Table 3; Fig. 3a and b).

3.2.2. Adult rats

According to one-way ANOVA [$F(3,12) = 5, 26$; $p = 0.031$] (Table 1), in adult animals the outcome of experimental conditions on the principal cell loss is less expressed. Statistically significant effect is observed only after 90 days of toluene exposure (25%, $p = 0.039$) vs. control. The significant difference is also revealed between both experimental groups [immediate effect vs. persisting effect (18%, $p = 0.049$) (Table 3).

Thus: in the CA3 area of adolescent and adult rats the toluene chronic exposure provokes different effect than in the CA1 area: (i) in adolescent rats the immediate and persistent effects were lesser than in the CA1, (ii) in adult rats only persistent effect was observed (Figs. 3a, b and 4a, b).

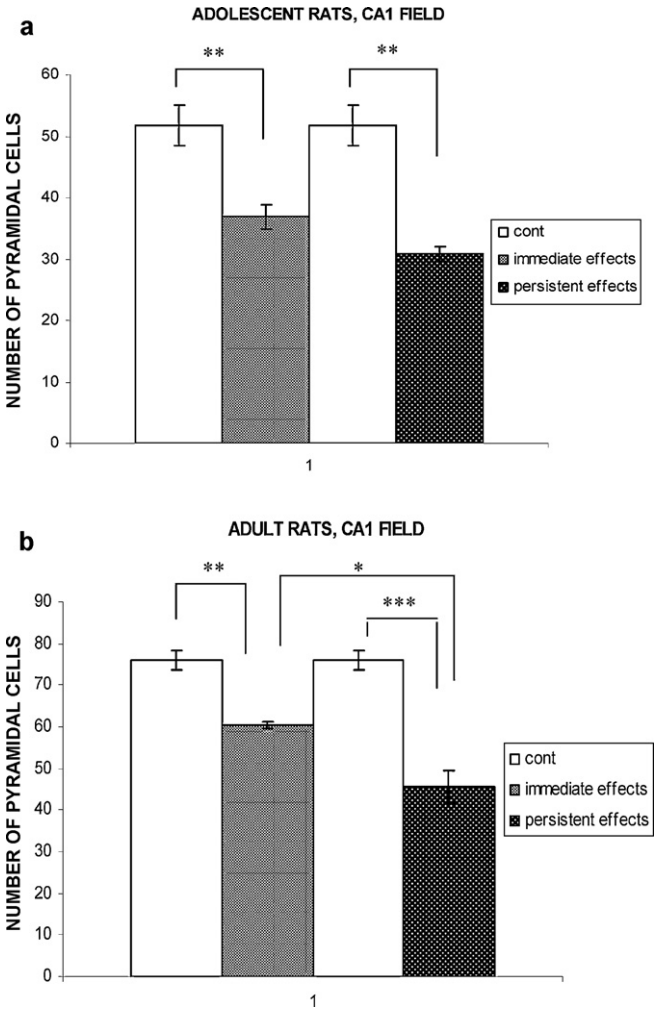

Fig. 2. (a) Measurement of pyramidal cell number in the CA1 area of adolescent rats after toluene chronic exposure: immediate and persistent effects. Data are given as mean ± SE. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$. (b) Measurement of pyramidal cell number in the CA1 area of adult rats after toluene chronic exposure: immediate and persistent effects. Data are given as mean ± SE. $^{**}p \leq 0.01$; $^{***}p \leq 0.005$.

4. Discussion

The abuse of toluene-containing volatile inhalants by adolescents and adults is a significant public health problem. To fully understand the effect of toluene addiction on the developing brain, studies need to be done where comparisons between adult and adolescent responses are made. Recent data indicate that in addition to the intrinsic reinforcing effect of addictive drugs, other factors may contribute to addiction. One such factor is learning or rather the

Table 2
The immediate and persisting effect of toluene chronic exposure on the number of pyramidal cells in the hippocampal CA1 area in adult and adolescent rats: data are given as mean ± SE; † indicates vs. control group; ‡ indicates vs. immediate effect group. C: control group of animal; EXP: experimental group of animal.

CA1 area								
	Adolescents				Adults			
	Immediate effect		Persisting effect		Immediate effect		Persisting effect	
	C	EXP	C	EXP	C	EXP	C	EXP
Mean	51.74 ± 3.3	36.92 ± 2.0	51.74 ± 3.3	30.90 ± 1.2	76.05 ± 2.3	60.43 ± 0.88	76.05 ± 2.3	45.46 ± 3.9
T-value		3.80†		5.87† 2.54‡		6.38†		6.71† 0.003‡
p-value		0.019†		0.01† 0.064‡		0.008†		3.70† 0.034‡

Table 3
The immediate and persisting effect of toluene chronic exposure on the number of pyramidal cells in the hippocampal CA3 area in the adolescent and adult rats: data are given as mean ± SE; † indicates vs. control group; ‡ indicates vs. immediate effect group.

CA3 area								
	Adolescents				Adults			
	Immediate effect		Persisting effect		Immediate effect		Persisting effect	
	C	EXP	C	EXP	C	EXP	C	EXP
Mean	32.74 ± 2.3	25.08 ± 1.4	32.74 ± 2.3	23.30 ± 0.68	45.22 ± 3.3	41.20 ± 2.2	45.22 ± 3.3	33.83 ± 1.8
T-value	2.81†			3.92† 1.12‡		1.01†		3.02† 2.58‡
p-value	0.037†			0.03† 0.32‡		0.358†		0.039† 0.049‡

ability of addictive drugs to alter learning and memory processes that may underlie addiction. More evidence is pointing towards the role of the hippocampus in drug addiction because of its importance in learning and memory [15,22,33]. The ability of addictive substances to alter the neural substrate of these processes would explain the capacity of these substances to produce long-lasting and maladaptive behavioral and cellular changes that maintain addiction [15,34–36].

While the neurobehavioral and neurotoxic effects of toluene have been studied extensively [1,9,28], the corresponding structural alterations are described only in a few numbers of studies [30,37,38]. However we suggest that studying a unique profile of structural changes in organisms of different age, may provide valuable information regarding the mechanism of action of toluene. In the present research we describe (in our knowledge) for first time, the immediate and persisting effect of toluene chronic exposure on the structure of hippocampus in adult and adolescent rats.

It is well-known that all alterations provoked by toluene chronic exposure and observed on clinical and experimental levels are dose-dependent. The dose used in our study is 2000 ppm. The Occupational Safety and Health Administration considers toluene levels of 2000 ppm as dangerous to life and health. Clinically, this dose is comparable to the inhaled exposure which produces euphoria in humans. Thus, euphoria usually appears at levels near

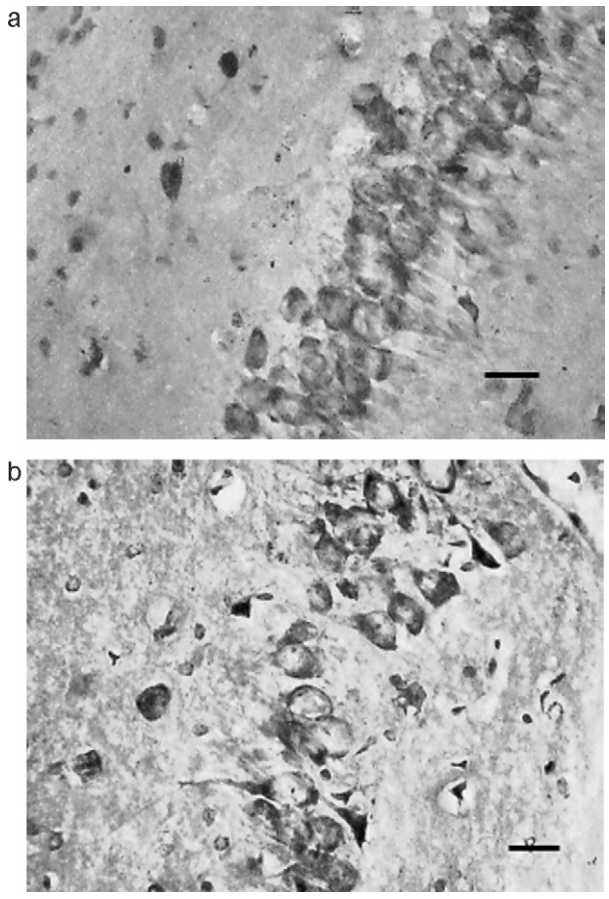

Fig. 3. (a) Representative photomicrograph demonstrating the CA3 area of control adolescent rat. Magnification 200×; scale = 25 μm. (b) Representative photomicrograph demonstrating depletion of pyramidal cell layer in the hippocampal CA3 area of adolescent rat. Persistent effect of toluene chronic exposure (90 days after withdrawal). Magnification 200×; scale = 25 μm.

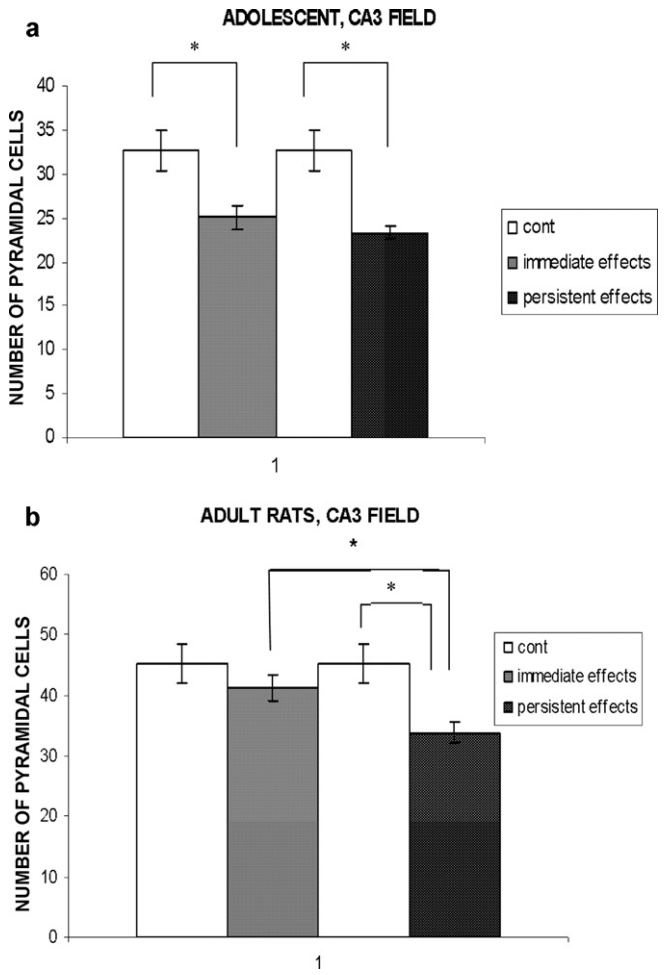

Fig. 4. (a) Measurement of pyramidal cell number in the CA3 area of adolescent rats after toluene chronic exposure: immediate and persisting effects. Data are given as mean ± SE; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.005$. (b) Measurement of pyramidal cell number in the CA3 area of adult rats after toluene chronic exposure: immediate and persisting effects. Data are given as mean ± SE. $^{*}p \leq 0.05$.

800–1200 ppm but some people may be more or less sensitive to the effect of inhaled toluene. Because euphoria is the desired effect, individuals with toluene abuse deliberately expose themselves to toluene levels of at least 800–1200 ppm and far higher. For instance, the person who has used toluene by sniffing glue can achieve levels of exposure estimated at 500–1200 ppm. Several authors consider the 2000 ppm as low level toluene inhalation (comparably to 5000–12,000 ppm—dose, usually used by chronic abusers). However this dose is known to alter the brain neurotransmitter levels (for example: to gradually decrease acetylcholine and increase GABA) [29,25] and some types of behavior (for example, diminishes avoidant behavior) [39].

Our data indicate that 40 days exposure to 2000 ppm of inhaled toluene in adolescent and adult rats to simulate human toluene abuse, in addition to abovementioned biochemical and behavioral changes is associated with alterations in the structure of hippocampus. Such alterations were observed both, immediately after the last day of exposure (immediate effect of toluene chronic inhalation) and after 90 day's abstinence (late-emerging or persisting effect of toluene chronic inhalation) herewith the age-dependent difference was revealed. Specifically, in the CA1 and CA3 of adolescent animals the significant cell loss observed by the day the following toluene chronic exposure does not progress significantly during abstinence period, while in adults the delayed effect (observed after 90 days withdrawal) was more substantial than immediate effect. Taking together, these data indicate that as a result of toluene chronic misuse, the level of hippocampal structural changes depends upon the postnatal age of testing animals.

The age-differences of behavioral (such as development of sensitization to the locomotor stimulant effect, risk-taking or exploratory activities) and biochemical alterations in the case of other drugs of abuse (such as amphetamine, cocaine, methylphenidate or inhalants) in adolescent rodents, as compared to adults, have been reported before [23,35,36,40,41]. In a few number of studies such age-dependent difference was revealed in the case of toluene misuse also. Thus, recent data point to less sensitization and attenuation of neurochemical responses in adolescents in comparing to adults during toluene chronic exposure [4,13] or differential effects of inhaled toluene on locomotor activity in adolescent and adult rats [42]. The age-difference in toluene-pharmacokinetics was also shown. Hence, levels of hepatic enzymes, responsible for toluene metabolism to benzyl alcohol and o- and p-cresol vary across age in drug-naïve rats [43–45], suggesting that younger rats might metabolize toluene more quickly. However some facts (for example: adolescent animals are differentially sensitive to the acute effect of other CNS depressants that have other pharmacokinetic mechanism than toluene) argue against such position [5; Bowen and McDonald, 2009]. Therefore, additional multidirectional investigations are necessary to evaluate the factors that may play a role in these differences.

As far as we know, the age-differences of structural alterations provoked by toluene chronic exposure have been never reported. What accounts for these age-dependent differences is unclear. The existing literature data also could not contribute to the clarification of underlying mechanisms. First at all, it is the lack of comparative studies of toluene effects on adolescent and adult brain. At the given stage some assumptions are only possible. Thus, this phenomenon is most likely related to multiple factors one of which could be the unique developmental profile of the adolescent brain (in rat: 2-wk period P 28-P 42) [31,32]. The adolescence as developmental transition is characterized by a set of neurobehavioral, biochemical and structural changes (such as increased preference for novelty, increased risk-taking, increased orientation towards and interaction with peers) developmental changes in receptors, neuronal pruning and brain re-organization [32]. Hippocampus is one of the structures that undergo multilateral developmental changes during adolescence. Therefore, the differences in the effects of toluene in adolescents, as compared to adults are not surprising. Such difference could be related with its interaction with multiple neurotransmitter systems of the hippocampus, first at all, with dopamine system and GABA (A) or NMDA receptors, that still undergo developmental changes during adolescence and specific alterations of which are considered to be one of the common mechanisms of action for abused inhalants [3,25,29,31,46].

The identification of toluene induced cell loss pathway (apoptosis, necrosis) in adolescents and adults and the age dependent differences in corresponding mechanisms should also help to understand different effect of toluene chronic inhalation at the animals of different age. The potential of toluene to induce apoptosis and/or necrosis is demonstrated by several authors; the character of cell death depends on brain region, cell type, developmental stage of animal, duration of exposure and dosage [47,51,48,30]. For example, the expression of several peptides that participate in apoptosis and necrosis differs in adult and adolescent brain. Some of such peptides, for instance, the $p75^{NTR}$, the member of the TNF receptor superfamily, are shown to participate in toluene-induced cell death [49]. It is very likely that unique character of cell death in adolescents and adults as a result of toluene misuse is related to the difference in expression of the $p75^{NTR}$ and other peptides participating in the toluene-provoked apoptosis and necrosis. Therefore, comparative studies in several directions, such as identification of the cell death pathway, clarification of factors responsible for cell death and determination of the level of their expression in adolescent and adult animals in concrete conditions of toluene intoxication, are considered as future direction of our research.

Another our finding observed in both aging groups is the greater vulnerability of the CA1 area to toluene chronic exposure in comparing with the CA3. Among hippocampal regions, the CA1 is known to be the most susceptible to multiple experimental treatments and disorders. Several structural and molecular peculiarities (for example, the presence in this area of comparatively large number of electric synapses along with chemical forms or specific assortment of Ca+2-binding proteins) can only partially explain such vulnerability. However to fully understand the nature of toluene addiction the contributing factors of CA1 area vulnerability should be determined.

Earlier we have revealed the age-difference of immediate and delayed effect of toluene chronic exposure on the behavior of adolescent and adult rats in the maze: adult rats made slightly more mistakes and spent more time for maze passing than adolescents [50]. Taking together our data indicate that as a result of toluene misuse, structural disorganization of the hippocampus is associated with alterations in the hippocampus-dependent learning processes and both structural and behavioral alterations are age-dependent.

In this study we are focused on pyramidal cells of the hippocampus. In previous research we investigated the immediate and late-emerging effects of toluene chronic exposure on hippocampal radial and oriental interneuron cells [50]. The comparison of our data strongly indicates that in both aging groups pyramidal cell loss is more substantial. Thus, we suggest that in the rats of different age the special population of hippocampal neurons – principal cells – is more vulnerable/susceptible to toluene chronic exposure. What peculiarities of hippocampal pyramidal neurons provoke such susceptibility is the subject of further investigation.

5. Summary

Present results are additional evidence that neural substrate of learning and memory may contribute to pathophysiology of toluene abuse. Data indicate that toluene chronic exposure provokes some disorder in the structure of the rat hippocampus and the

level of this disorder depends on the age of animal. Future evaluation of such age-dependent difference is needed to fully understand the nature of toluene addiction.

Conflict of interest

The authors declare that there are no conflicts of interest.

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