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Progressive increase of lysosomal enzyme activities in hippocampus associated with reduction of population spike in a rat model of neurodegeneration

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Abstract

OBJECTIVES: Extensive effort has been made to identify early markers of neurodegeneration as late stages have no chance of treatment. Recently, many experimental models have been used to study hallmarks of neuronal injury. One of them is the model of trimethyltin (TMT)-induced damage associated with cognitive decline, thus called a model of Alzheimer-like disease. **OBJECTIVE AND METHODS:** Our aim was to study neuronal transmission in hippocampal slices of male Wistar rats affected with a single dose of TMT (7.5 mg/kg, *i.p.*) during the first three weeks of its action. The monitored time periods after TMT administration were days 1–3; 8–10 and 15–17. At the same time periods, right hippocampi were collected for determination of changes in specific activities of two lysosomal enzymes. Electrophysiological measurements were based on stimulation of Schäffer collaterals and registration of evoked responses in the *stratum pyramidale* and the *stratum radiatum* at the CA3–CA1 synapse. Specific activities of N-acetyl-β-D-glucosaminidase (NAGA) and cathepsin D (Cat D) were determined spectrophotometrically. **RESULTS:** During three weeks after i.p. TMT administration to rats, we found a time-dependent reduction of postsynaptic neuronal firing, expressed by diminished population spike (PoS) amplitude recorded in the stratum pyramidale accompanied with marked increase in specific activity of NAGA to respective 111%, 163% and 252% in the 1st, 2nd and 3rd week compared to unaffected rats. In the *stratum radiatum*, reduction of the slope of excitatory postsynaptic potential was not time-dependent but almost constantly reduced from the 1st to 3rd week after TMT administration (55–60%) compared to control rats. Specific activity of lysosomal enzyme Cat D was significantly increased in the 3rd week after TMT administration. **CONCLUSION:** This work demonstrates a time-dependent reduction of somatic response in the hippocampus of TMT affected rats during the first three weeks. This reduction of neuronal firing was later accompanied with increase of specific activity of NAGA and Cat D, supporting evidence that lysosomal dysfunction may be one of the primary contributors to TMT-induced neurodegeneration.

ORIGINAL ARTICLE

INTRODUCTION

Neurodegenerative disorders, such as dementia, represent a serious health problem associated with physical, psychic and social disability of patients suffering from decreased cognitive functions concerning memory, speech and perception, emotional unbalance, deterioration, etc. The most frequent type of dementia is dementia of the Alzheimer type, characterized by atrophy of the frontal brain cortex, neuronal loss in the hippocampus, presence of β-amyloid peptide-containing senile neuritic plaques and neuronal fibrilary tangles, expressed by cognitive and behavioral failure. Besides the characretistic progressive clinical features of the Alzheimer`s disease (AD) patient only a few biochemical variables have been detected, as e.g. decrease of choline acetyltransferase activity in the cortex and hippocampus, reduced content of corticotropin releasing factor and somatostatin in degenerative neuritis, increased levels of low density lipoprotein cholesterol and increased levels of pro-inflammatory agents in blood serum (Bird *et al.* 1983, Burgos-Ramos *et al.* 2008; Rubio-Perez and Morillas-Ruiz, 2012; Sjögren and Blennow, 2005). Extensive effort has been applied to provide new knowledge on slowing down progressive negative changes. Several models of AD have been developed in experimental conditions (Saraceno *et al.* 2013). One of the important research tools for the study of brain dysfunction could be the model of trimethyltin (TMT)-induced neurodegeneration where the pathology elicited by this neurotoxin is common to most neurodegenerative disorders, *i.e.* neuronal cell death and cognitive impairment. Changes occurring during TMT intoxication are apparent in functional, biochemical and morphological parameters, such as neuronal loss, proliferation of glial cells, alteration of molecular marker levels such as neural cell adhesion molecule, nuclear factor kappa B, c-fos, *etc*., which together lead to behavioral manifestation of the intoxication. TMT elicits neuronal death in the limbic system and causes damage particularly in the hippocampus, thus it has been found a useful experimental model, especially in the investigation of Alzheimer-like diseases (Gasparova *et al.* 2012; Geloso *et al.* 2011; Ishikawa *et al.* 1997; Koczyk 1996; Nilsberth *et al.* 2002). The main target of TMT toxicity is the central nervous system, resulting in pathological damage characterized by neuronal destruction, predominantly in the hippocampus and the cerebral cortex (Aschner and Aschner, 1992). A wide range of experimental approaches from biochemical and histological to magnetic resonance spectroscopy and imaging were used to elucidate the mechanism of TMT action, yet the primary basis for TMT neurotoxicity still remains to be clarified.

Recently, evidence has been increasing that lysosomes play an important role in neurodegenerative diseases (Zhang *et al.* 2009). Actually, up-regulation of the lysosomal system has been found in experimental models of neuronal injury in rat hippocampal neurons in culture (Adamec *et al.* 2000). Lysosomes contain dozens of hydrolytic enzymes that can break down virtually all kinds of biomolecules. In addition, lysosomes contain cathepsins, a group of proteases which have been associated to degenerative mechanisms in the nervous system (Stoka *et al.* 2016). Both in AD and in experimental models of neuronal injury, lysosomal system activation was found to be an important event associated with early stages of neurodegeneration (Adamec *et al.* 2000). Cathepsin D (Cat D) is a lysosomal aspartic endopeptidase involved in cell apoptosis (Benes *et al.* 2008). Elevated levels were already reported in kainate-induced neurodegeneration (Hetman *et al.* 1995), in patients with mild cognitive impairment (Perez *et al.* 2015), as well as in the aging brain (Kenessey *et al.* 1989). Lysosomal enzyme N-acetyl-β-glucosaminidase (NAGA) was found in most animal cells including neurons. This enzyme is a glycosidase which splits the chemical bonds of glycosides, amino-saccharides, forming structural components of cells (Garvey *et al.* 1995). Activity of both mentioned lysosomal enzymes, NAGA and Cat D, was lowered after administration of antioxidant melatonin to mice (Witek *et al.* 2001). In our previous work, we observed increased specific activity of NAGA in blood serum and brain cortex in TMT-affected rats (8 mg/kg, *i.p.*), determined 31 days after TMT administration, compared to controls obtaining saline *i.p.* (Gasparova *et al.* 2014).

The aim of the present work was to study an association between neuronal function reduction in the hippocampus, the tissue with the main role in learning and memory, in light of the activity of lysosomal enzymes NAGA and Cat D during the first three weeks of TMT-induced neurodegeneration. We focused on the TMT effect during this time period with the aim to recover early marks which could be important in a succesful treatment strategy.

MATERIALS AND METHODS

Animals and TMT administration

Male Wistar rats (*N*=54, weighing 242±3 g, 12 weeks old) were from the breeding station Dobra Voda (Slovak Republic, reg. No. SK CH 24011). The rats had free access to water and certified food pellets (KKZ-P/M) and were kept on a 12h/12 h light/dark cycle. All procedures involving the animals were performed in compliance with the Principles of Laboratory Animal Care issued by the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences and by the State Veterinary and Food Administration of Slovakia. TMT chloride (Sigma-Aldrich) dissolved in 0.1% dimethyl sulfoxide (DMSO) and in sterile saline just prior to application, was administered in a single dose of 7.5 mg/kg of body weight *intraperitoneally* in the volume of 0.2 ml/100 g of body weight (*N*=35). Control rats received an equal

volume of sterile saline *i.p.* with 0.1% DMSO (*N*=19). Both experimental groups of animals, controls and TMT-intoxicated, were terminated under short ether anesthesia during days 1–3 (1st week), 8–10 (2nd week), and 15–17 (3rd week) after TMT /or saline administration. As there is evidence that both hippocampi are equally affected by TMT (Robertson *et al.* 1987), the right hippocampus was used for biochemical analysis and the left for electrophysiological recordings.

Preparation of rat hippocampal slices and extracellular recording

The rats were shortly anesthetized by ether, decapitated and both hippocampi were quickly removed from the brain and the left cut into transversal 400 μm thick slices with a McIllwain Tissue Chopper (Stoelting, USA). The slices were kept in the holding chamber for a recovery period of at least 90 min before the electrophysiological measurement started. During the measurement, one slice was kept in the recording chamber and continuously perfused with artificial cerebrospinal fluid saturated with 95% O_2 and 5% CO_2 at a constant rate monitored by aquatic manometer. The temperature of recording and incubation chambers was kept at 33–34 °C. Electrically evoked responses were recorded by DigiData 1322A (Molecular Devices, Axon Instruments, USA) with a sampling rate of 10 kHz and stored on personal computer for off-line analysis by AxoScope software. Postsynaptic potentials were recorded extracellularly in the *stratum pyramidale* or the *stratum radiatum* of the CA1 area in response to electric stimulation of Schäffer collaterals in hippocampal slices of rats exposed to TMT and compared to responses of control unaffected rats. Postsynaptic neuronal firing was evaluated by measuring the maximal amplitude of somatic population spike (PoS) as a parameter for sizing neuronal vitality, recorded in the *stratum pyramidale*. Postsynaptic dendritic response was evaluated by measuring the slope of field excitatory postsynaptic potential (fEPSP) recorded in the *stratum radiatum*. Maximal somatic and dendritic responses were recorded and compared to responses from control unaffected rats. The stimulus intensity was continuously increased to achieve maximal response checked with cursors on a 100 MHz digital storage oscilloscope (Tectronix 2230, USA), subsequently, AxoScope10.3 software was run and maximal response recorded repeatedly five times from the same slice. Supramaximal stimulation had a stimulus duration of 100 μs at stimulus frequency of 0.05 Hz. Maximal PoS amplitude or fEPSP slope were analyzed off-line, where PoS amplitude was calculated as the distance between the most positive and the most negative peaks of the trace of compound action potential, expressed in mV, and fEPSP slope was calculated as the voltage difference *per* 1 ms in the linear part of the initial downward deflection of the fEPSP trace, expressed in mV/ms. Recordings of control rats which obtained saline *i.p.* were collected and pooled during the three weeks to mean value of PoS amplitude and mean value of fEPSP slope ± S.E.M. Responses of TMT exposed rats were calculated from recordings during the 1st, 2nd and 3rd week after TMT administration and expressed as mean ± S.E.M. at each time point.

Biochemical determination of N-acetyl-β-D-glucosaminidase and cathepsin D specific activities

The right hippocampi were stored under liquid nitrogen for later biochemical analyses. On the day of biochemical determination, the tissue samples were thawed and put into ice-cold phosphate-buffered saline, pH 7.4, containing Triton X-100 (0.1%) and homogenized with a knife homogenizer at 4 °C. Homogenates were centrifuged at 15 000×g in a cold centrifuge at 4 °C for 20 min and the supernatants were used for enzyme and protein assays. The activity of NAGA and Cat D were assayed according to standard methods (Barret & Heath 1977). Proteins were determined by the method of Lowry *et al.* (1951). All chemicals used were of analytical grade (Sigma-Aldrich, USA).

Statistical analysis

The data are expressed as mean ± S.E.M. Statistical difference was calculated by One-way analysis of variance (ANOVA) with Dunnett multiple comparison test, comparison *vs.* control value and with Tukey-Kramer comparison test, comparison of all columns. A p-value <0.05 was considered significant.

RESULTS

Electrophysiological changes in the hippocampus

Electrophysiological recordings were focused to elicit maximal somatic and dendritic responses recorded extracellularly in the *stratum pyramidale* and in the *stratum radiatum* in the hippocampus of rats monitored one, two and three weeks after TMT administration with regard to responses from control unaffected rats. Time-dependent continuous mark decrease in PoS amplitude was observed during the 1st to 3rd week (Figure 1A). In the 3rd week, maximal PoS amplitude reached only 11% of the response of control rats. Moreover, this fatally reduced response was induced due to significantly increased stimulus intensity needed for induction of maximal response compared to control rats (Figure 1B). Dendritic response expressed as a slope of initial phase of fEPSP was reduced during the three experimental weeks, yet this reduction was not significant compared to controls (Figure 2A). Similarly as in the case of PoS response, stimulus intensity had to be increased to elicit maximal dendritic response during the 3rd week after TMT administration compared to response of control rats, indicating also a fatally damaged dendritic response (Figure 2B). Typical traces of PoS in control and TMT exposed rats are shown in Figure 3A and typical traces of fEPSP in control and TMT exposed rats are shown in Figure 3B.

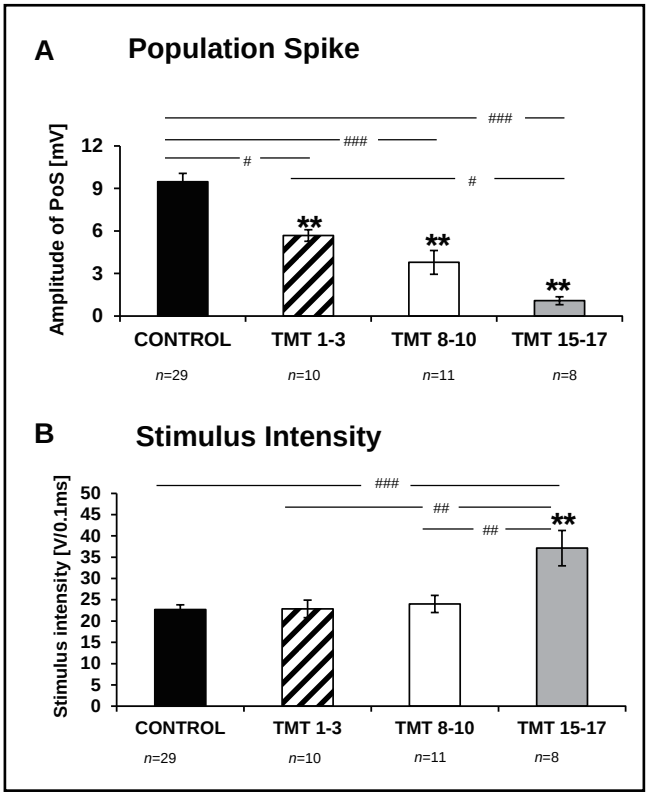

Fig. 1. Effect of trimethyltin (TMT; 7.5 mg/kg of body weight, *i.p.*) on hippocampal population spike amplitude recorded on the CA3–CA1 synapse in the *stratum pyramidale* on days 1–3 (*N*=6 rats); on days 8–10 (*N*=6 rats) and on days 15–17 (*N*=6 rats) after TMT administration compared to population spike of unaffected male Wistar rats (*N*=19) (**A**). Stimulus intensity needed to evoke maximal amplitude of population spike (**B**). Values are expresed as means ± S.E.M. Statistical difference was calculated by ANOVA with Dunnett multiple comparison test, comparison *vs.* control value (**p<0.01) and with Tukey-Kramer comparison test, comparison of all columns ($^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001), *n*=number of hippocampal slices.

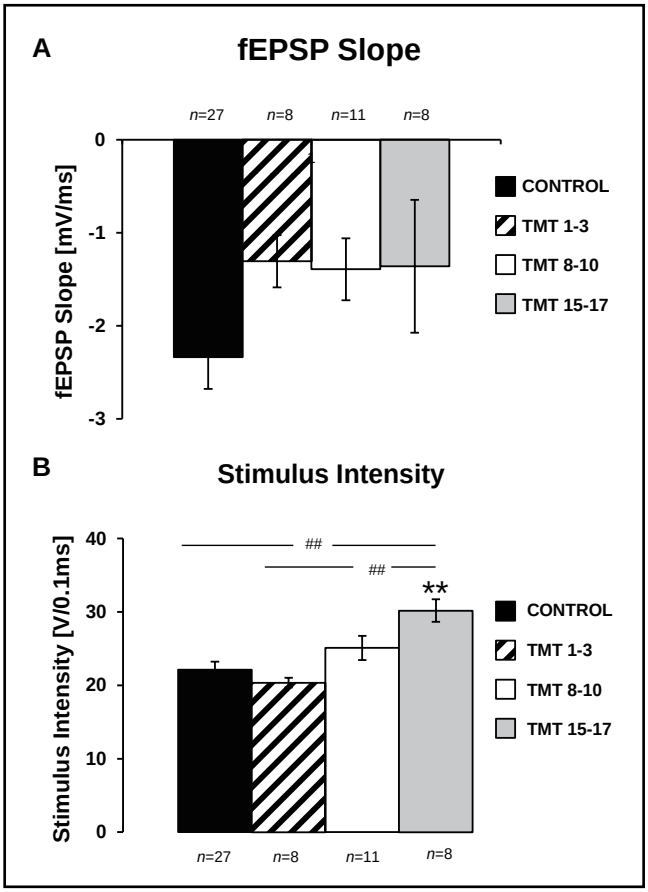

Fig. 2. Effect of trimethyltin (TMT; 7.5 mg/kg of body weight, *i.p.*) on hippocampal dendritic fEPSP slope recorded on the CA3–CA1 synapse in the *stratum radiatum* in control male Wistar rats (*N*=19 rats) and rats affected with TMT on days 1–3 (*N*=6 rats); on days 8–10 (*N*=6 rats)and on days 15–17 after TMT administration (**A**). Stimulus intensity needed to evoke maximal response (**B**). Values are expresed as means ± S.E.M. Statistical difference was calculated by ANOVA with Dunnett multiple comparison test, comparison *vs.* control value (**p<0.01) and with Tukey-Kramer comparison test, comparison of all columns ($^{\#\#}p$<0.01), *n*=number of hippocampal slices.

Changes in lysosomal enzyme activities

NAGA specific activity increased to respective 111%, 164% and 253% in the 1st, 2nd and 3rd week compared to unaffected rats (Figure 4A). Cat D specific activity significantly increased in the 3rd week (up to 161%) compared to unaffected rats (Figure 4B).

DISCUSSION

Regarding functional distortions induced by TMT in brain tissue, behavioral and electrophysiological studies seem to be appropriate. In electrophysiological experiments on hippocampal cultures or slices, *in vitro* bath application of TMT was used (Armstrong *et al.* 1986,1987; Allen and Fonnum, 1984; Harkins and Armstrong, 1992; Janigro and Costa 1987; Krüger *et al.* 2005; Melani *et al.* 2005; Noraberg *et al.* 1998, 1999; van Vliet *et al.* 2007). To date, administration of TMT to experimental animals systemically with the aim to study evoked potentials has been quite rare (Gasparova *et al.* 2012; Hasan *et al.* 1984; Ray, 1981; Segal, 1988). Despite one of the main ideas of using *in vitro* exposure to TMT and thus to assist in replacing animal experimentation *in vivo*, bath application represents direct action of the drug tested on hippocampal tissue when only acute neurotoxicity is detected in the case of TMT. Systemic administration mirrors the development of time-dependent pathological changes and thus offers higher potential as a tool for investigation of progressive neurodegenerative diseases. This guided us to investigate time-dependent electrophysiological changes in the hippocampus of *in vivo* TMT-intoxicated rats and simultaneously to determine changes in lysosomal enzyme activity, known as a hallmark of degeneration (Zhang *et al.* 2009).

In our previous work on hippocampal slices from rats exposed to 7 mg/kg of TMT, the input-output curve

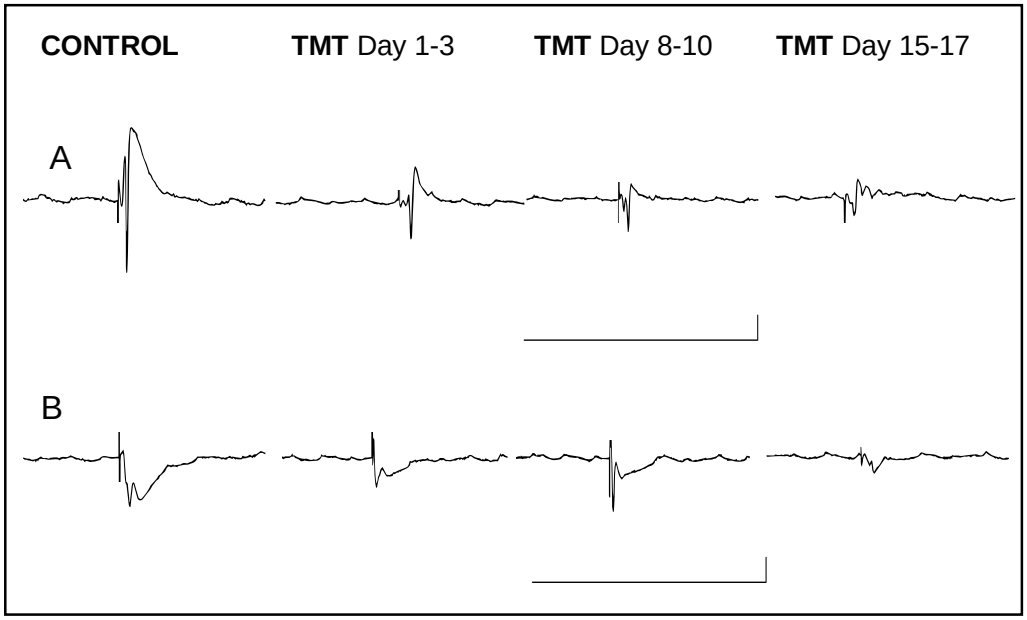

Fig. 3. Typical traces of maximal postsynaptic responses registered in the CA1 somatic (**A**) and dendritic (**B**) layer of hippocampal slices of control (left top and left botom) compared to slices from rats affected with timethyltin (TMT, 7.5 mg/kg of body weight, *i.p.*) registered on days 1–3; 8–10 and 15–17 after TMT administration. Insert: horizontally 100 ms, vertically 2 mV.

of dendritic response was done with increasing stimulus intensity in the range from 10–15 V and we showed a significant of about 50% reduction of the fEPSP slope and amplitude on days 22–24 after TMT administration (Gasparova *et al.* 2012). The present work focused on maximal somatic and dendritic responses during the first three weeks after TMT administration. An apparent time-dependent reduction of PoS amplitude was observed while a slope of EPSP was of almost constant reduction during 1st to 3rd week compared to response of unaffected rats. These results may indicate different vulnerability of somas and dendrites to TMT intoxication in the rat hippocampal CA1 area. The progress of TMT induced weakening of somatic response resembles a time dependent worsening of spatial learning and memory in the Morris water maze, where a behavioral test has to be performed at a minimum of 21 days after administration of TMT to obtain significant worsening of spatial memory (Earley *et al.* 1992; Gasparova *et al.* unpublished observation). Thus time-dependent impairment of spatial memory in the Morris water maze may be a result concerning the time-dependent progress in reduction of the hippocampal PoS amplitude during the first three weeks of TMT action in the rat body and seems to be unrelated to the time-independent and almost constant reduction of dendritic response. This made us incline to the opinion and hypothesis of Kleshcevnikov and Marchbanks (1993) that probably hippocampal population spike, but not dendritic EPSP, is a physiological correlate for spatial memory and cognitive decline. Moreover, our informative study of hematoxylin-eozin stained hippocampal sections of TMT exposed rats tested in this work revealed time-dependent reduction of pyramidal cell number in the CA1 area during the 1st to 3rd weeks with enhanced proliferation of glia cells in 3rd week of TMT

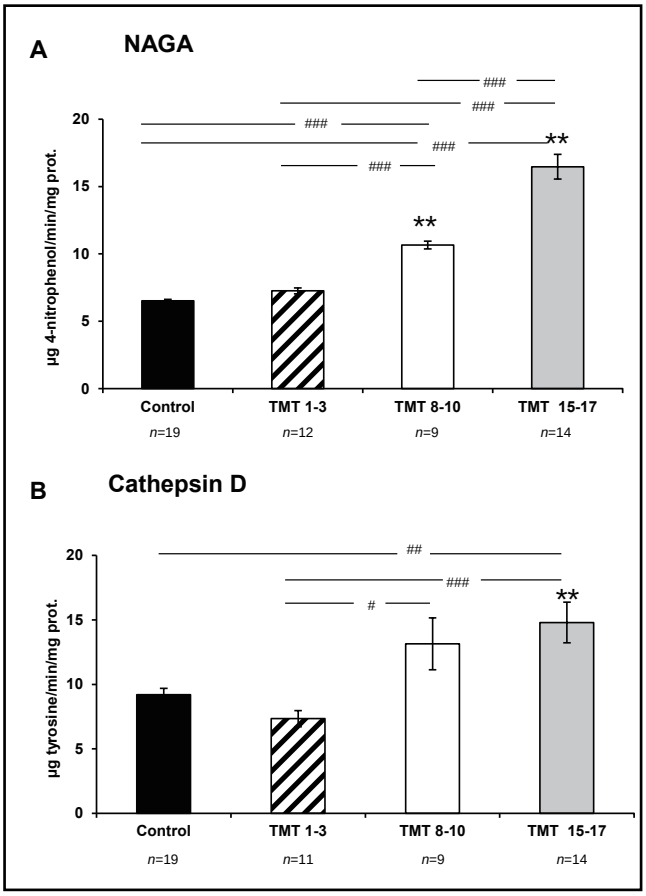

Fig. 4. Effect of trimethyltin (TMT; 7.5 mg/kg of body weight, *i.p.*) on specific activity of lysosomal enzymes NAGA (**A**) and cathepsin D (**B**) in the whole hippocampus of TMT affected rats (*N*=35 rats) compared to control value in the hippocampus of unaffected rats (*N*=19 rats). Values are expressed as means ± S.E.M. Statistical difference was calculated by ANOVA with Dunnett multiple comparison test, comparison *vs.* control value (***p*<0.01) and with Tukey-Kramer comparison test, comparison of all columns ($^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001), *n* = number of hippocampi in each group.

intoxication (not shown) suggesting glial contribution to disease progression (Gómez-Nicola *et al.* 2013).

Changes in the lysosomal degradation have been found during normal brain aging and in age-related neurodegenerative diseases. Cathepsin imbalance during age-related diseases may have a malign effect on neurons in the brain. New knowledge of consequences of lysosomal age-related changes in neurons could contribute to the development of therapeutic tools in progressive neurodegenerative diseases. Cat D, a lysosomal aspartic protease involved in some neurodegenerative processes, seems to play an important role in the regulation of apoptosis (Ceccariglia *et al.* 2011). In response to pathogenic protein accumulation, lysosomal activation was observed. In the present results, functional damage of the hippocampus reflected by the reduced electrophysiological responses was accompanied with increase in NAGA and Cat D specific activities. An enormous increase in specific activity of both lysosomal enzymes was found especially in the 3rd week, thus at time when the amplitude of PoS was fatally reduced (11% of control PoS). As to our knowledge, in the scientific literature there is only above mentioned work concerning lysosomal enzyme activity in the TMT experimental model of Ceccariglia and co-workers (2011). The authors concluded that TMT treatment in the hippocampus induced high levels of Cat D activity both *in vivo* and *in vitro*, in glial cells and in the CA3 hippocampal area neurons, where a marked TMT-induced neuronal loss also occured. Cat D has been sugested to be actively involved in CA3 neuronal death and the protease increase is a calcium-calpain dependent phenomenon (Ceccariglia *et al.* 2011). Regarding NAGA, only data from our previous work are available, where increased activity of this lysosomal enzyme in blood serum and in the cortex were reported contrary to controls in the TMT experimental model (Gasparova *et al.* 2012). In the present work, increasd NAGA specific activity was determined in the hippocampus, the tissue involved in learning and memory.

CONCLUSION

The present work demonstrates time-dependent reduction of somatic population spikes in the hippocampus of TMT affected rats during the first three weeks after its administration and suggests different vulnerability of somas and dendrites to TMT. Reduction of neuronal firing was later accompanied with gradual increase of specific activity of NAGA in the 2nd and 3rd week and with significant increase of specific activity of cathepsin D in the 3rd week in the hippocampus of TMT intoxicated Wistar rats, supporting evidence that lysosomal dysfunction may be one of the primary contributors to TMT-induced neurodegeneration. It is however not excluded that biochemical changes, although not prominent in the first time interval studied, may trigger a mechanism affecting reduction of somatic neuronal firing. Further studies are needed to clarify the causes and consequence in the observed electrophysiological and biochemical events.

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