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Postconditioning Effectively Prevents Trimethyltin Induced Neuronal Damage in the Rat Brain*

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Trimethyltin (TMT) is a toxic substance formerly used as a catalyst in the production of organic substances, as well as in industry and agriculture. TMT poisoning has caused death or severe injury in many dozens of people. The toxicity of TMT is mediated by dose dependent selective damage to the limbic system in humans and other animals, specifically the degeneration of CA1 neurons in the hippocampus. The typical symptoms include memory loss and decreased learning ability. Using knowledge gained in previous studies of global ischaemia, we used delayed postconditioning after TMT intoxication (8 mg/kg i.p.), consisting of applying a stressor (BR, bradykinin 150 µg/kg i.p.) 24 or 48 hours after the injection of TMT. We found that BR had preventive effects on neurodegenerative changes as well as learning and memory deficits induced by TMT intoxication.

Key words: Trimethyltin, postconditioning, hippocampus, bradykinin, morris water maze.

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Trimethyltin (TMT) is a potent neurotoxin that selectively induces neuronal death in the limbic system of humans and other animals, in particular in the hippocampal formation. Animals exposed to TMT develop dose dependent behavioural alterations (hyperactivity and aggression), cognitive impairment (memory loss and learning impairment) and spontaneous seizures. In rats, TMT administration is characterised by a subacute pattern of action, which replicates some peculiar features of human neurodegenerative diseases (GELOSO *et al*. 2011), resulting in progressive neuronal death of CA1 and CA3/hilus pyramidal neurons.

The distribution and time course of TMT-induced neuronal and glial responses to neuronal damage have been examined both morphologically and biochemically (BALABAN *et al*., 1988; GELOSO *et al*. 2004). TMT-induced neurodegeneration is a complex event resulting from different pathogenic mechanisms that include calcium overload, excitotoxicity, neuroinflammation, oxidative stress, and mitochondrial dysfunction (CECCARIGLIA *et al*. 2011; PIACENTINI *et al*. 2008).

Since the localisation of damage and the typical delayed death of neurons is very similar to certain types of acute neurodegeneration, TMT is used as an experimental model to simulate global ischaemia of the brain. The results obtained in the study of ischemic tolerance inspired us to compare options and use this model in the study of mechanisms of ischemic/reperfusion damage and therapy in the CNS.

The phenomenon of ischemic tolerance is currently the most effective mechanism to protect the selectively vulnerable neurons of the brain before ischemia, hypoxia, as well as some forms of intoxication (RIKSEN *et al*. 2004). If an organism, or part thereof, is subjected to an appropriate stress that does not destroy it, then it is able to build up defences, which for some time protects against the same or a similar stress.

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Full tolerance occurs as the synergy of two stresses, for example sublethal followed by lethal or vice versa, which has been shown by the efficacy of postconditioning in preventing apoptosis by the use of various sublethal stressors. Eight minutes of ischemia is lethal for 50% of hippocampal CA1 neurons; it is simultaneously sublethal for the other 50% of CA1 neurons, but if an i.p. injection of norepinephrine or bradykinin is applied two days after ischaemia, less than 10% of these cells will die. Postconditioning after kainic intoxication is equally effective (BURDA *et al.* 2009; BURDA *et al.* 2006; BURDA 2005).

The aim of this study was to observe the survival of neurons in the CA1 region of the hippocampus after TMT intoxication and subsequent postconditioning. As a postconditioner, we used a single dose of bradykinin (BK) 24 or 48 h after TMT intoxication, or two doses of BK, the first 24 h after and the second 48 h after TMT intoxication. We hypothesized that an adequate dose of BR used at an appropriate time after TMT intoxication will be an effective stressor finalizing acquisition of ischemic tolerance, and able to turn thr proapoptotic pathway of delayed neuronal death to antiapoptotic.

Material and Methods

Adult male *albino Wistar* rats weighing 250-300 g were group-housed and maintained on a 12 h light/dark cycle, with ad libitum access to water and rodent chow. The experiments were carried out in accordance with the protocol for animal care approved by both the Slovak Health Committee (1998) and the European Communities Council Directive (86/609/EEC). The rats were randomised into five groups containing seven animals in each group: the control group comprising sham control animals and four groups with trimethyltin intoxication (TMT 8 mg/kg i.p.). Some TMT groups were given bradykinin (BR) as a postconditioner: the first group was not treated with BR, the second group was given BR 24 hours after TMT intoxication, the third group was given BR after 48 hours and the fourth group was given BR after 24 and 48 hours after TMT intoxication. The animals were killed 7 days after TMT intoxication. Transcardiac perfusion via the left ventricle was performed under chloral hydrate anaesthesia (300 mg/kg, i.p.). Perfusion started with a washout of blood vessels with 200 ml of 0.9% NaCl. Brains were perfused fixed with 4% paraformaldehyde solution in phosphate buffered saline, removed and postfixed overnight in the same fixative prior to vibratome sectioning.

Fluoro Jade B staining

33 μm thick coronal sections of the brain from each animal were prepared at the level of bregma -3.3 ± 0.2 mm for the hippocampus and randomly selected for Fluoro Jade B staining, which was used to label all degenerating neurons present in the CA1 region 7 days after TMT intoxication with or without BR postconditioning, regardless of the mechanism of cell death. The sections were mounted on 2% gelatine-coated slides and then dried on a slide warmer at 50°C for 30 min. The slides were then immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate for 10 min, and subsequently rinsed in distilled water for 2 min. After 20 min in the staining solution containing 0.0004% Fluoro Jade B dye (Histo-Chem Inc., Jefferson, AR, USA), the slides were rinsed three times for 1 min in distilled water (SCHMUED & HOPKINS 2000). Excess water was removed by briefly draining the slides (about 15 s) vertically on a paper towel. The slides were then placed on a slide warmer, set at approximately 50°C until they were fully dry (5-10 min). The dry slides were cleared by immersion in xylene for at least a minute before cover slipping with DPX (Sigma-Aldrich). The slides were examined with a Leica DM2500 fluorescence microscope with a Leica camera and LAS V3.6 software (Leica Microsystems, Wetzlar, Germany).

Immunocytochemistry

Immunocytochemistry was performed on the prepared coronal free-floating 33 μm thick vibratome sections. Sections containing the hippocampus were immunostained for NeuN, a neuronal marker. Briefly, the sections were incubated overnight at 4°C with a monoclonal mouse NeuN antibody (Chemicon Int., Temecula, CA, USA; 1:500) in 0.1 mol/l PBS (pH 7.4) with 0.2% Triton-X. After washing with 0.1 mol/l PBS (pH 7.4) containing 0.2% Triton-X, a secondary anti-mouse IgG antibody raised in horse (Vector Laboratories, Burlingame, CA, USA) was applied for 90 min at room temperature. After further washing, the avidin/biotin complex formulation kit (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA, USA) was applied for 90 min, then the slides were rinsed with PBS followed by Tris buffer (pH 7.6), and reacted with DAB (0.1 mol/l Tris, 0.04% DAB, 0.033% H_2O_2); the reaction was stopped with phosphate buffer. The slides were dehydrated, cleared, and coverslipped for analysis.

Cell-counting procedure

Positive cell count was performed by an investigator who was unaware of the treatment conditions, using Image Tool software (UTHSCSA, San Antonio, USA). Quantification of NeuN and Fluoro Jade positive cells were conducted in the middle of the linear part of the CA1 (-3.3 ± 0.2 mm posterior of the bregma). Cells were counted in the ten hippocampal region slices of each animal and expressed as the average of positive pyramidal neurons per 1 mm of CA1 length.

Morris water maze test

Cognitive and memory functions of the rats which underwent experimental procedures were tested with the Morris water maze (MORRIS 1984) on the sixth and seventh reperfusion days. A washing tank (150 cm in diameter and 58 cm deep) was filled to 22 cm with 26 ± 1°C water. Approximately 500 ml of milk was added to the water, making it opaque. A submerged escape platform (20 cm tall and 15 cm diameter) was located in the southeast quadrant of the maze. A variety of extra-maze visual cues were visible from within the maze. The experimenter was unfamiliar with the treatment received by the subject and remained at a fixed location approximately 0.5 m away from the outside edge of the tank for each trial. The water maze training procedure lasted two days. On the sixth day after TMT intoxication, each rat underwent two trials. For each trial, a rat was placed in the water facing the same place at the edge of the pool. The rat was allowed 60 s to locate the platform. If after 60 s it did not find the escape platform, it was guided by the experimenter and allowed to remain on the platform for 10 s. The inter-trial interval for each subject was 5 min, during which the rat was dried and returned to the home cage. On the second day, the decisive probe trial was performed: all rats started from the same starting position opposite from the quadrant where the submerged escape platform had been positioned during the tests. The escape latency (the time each subject required to locate the hidden platform after being released) of each subject were measured. The maximum value from each probe taken for statistical analysis was 60 sincerely.

Statistical analysis

Data were analysed with one-way ANOVA followed by Tukey-Kramer's test using GraphPad InStat 3.1 software (Graph-Pad Software Inc., La Jolla, CA, USA). The results were represented as mean ± SD. A value of P less than 0.05 was considered to be statistically significant.

Results

On average, 296 neurons were detected in 1 mm of the line pyramidal neurons CA1 of the hippocampus of control animals if 30 μm thin slices were used. TMT was used at a dose able to destroy approximately one half of these cells. Seven days after TMT intoxication (8 mg/kg, i.p.), we observed the degeneration of 168.65 ± 8.01 (56.98%) neurons (Fig. 1A,B). Due to our previous results indicating that the death of CA1neurons occurs similarly after ischaemia by so-called delayed neuronal death, we used a procedure of delayed postconditioning with the injection of BR 48 hours after TMT (Fig. 1A). The result of this process was that the number of destroyed neurons decreased to 40.01% (118.42±14.84). This decrease was statistically significant. When BR was applied 24 hours after TMT, the number of damaged neurons was 48.6% (143.84±15.11). However, when the first dose of BR was 24 hours and the second was 48 hours after TMT intoxication, the number of dead neurons decreased to 14.2% (42.03±1.58) (Fig. 1A, 2A).

A massive decrease in the number of NeuN positive cells was observed in the CA1 pyramidal cell layer after TMT intoxication and 7 days of reperfusion, to only 86.55±12.4 neurons/mm (Fig. 1B, 2B). Rats with BR administration 24 hours and 48 hours after TMT intoxication demonstrated a significantly increased number (180.98±15.9 vs. 201.53±15.6) of NeuN positive cells. Photomicrographs of the immunoreaction with NeuN show that neurons in the CA1 region were best preserved if BR postconditioning was administered 24 hours after and repeated 48 hours after TMT intoxication (86.2% NeuN immunoreactivity) in comparison with TMT intoxication without postconditioning (29.23% NeuN immunoreactivity).

The function of CA1 neurons in terms of their ability to participate in learning and memory after TMT intoxication, with or without bradykinin application (Fig. 3), was evaluated using the Morris water maze test. Despite the fact that the chart contains all the data from the test, the probe on the second day of the test was the most important result. We observed significant changes in the time needed to find the hidden platform. Finding the hidden platform took significantly ($P<0.001$) longer, from 13.57 ± 2.56 s in sham control rats to 58.25 ± 4.11 s in the TMT group without bradykinin application. The animals treated with BR 24 or 48 hours after TMT intoxication were able to locate the platform in less than half the time (27.51 ± 3.05 s or 25.84 ± 3.55, $P<0.05$) needed by the TMT group. When the first dose of BR was given 24 hours after and repeated 48 hours after TMT intoxication, the animals were able to locate the platform in 15.85 ± 2.15 s. These data confirm not only

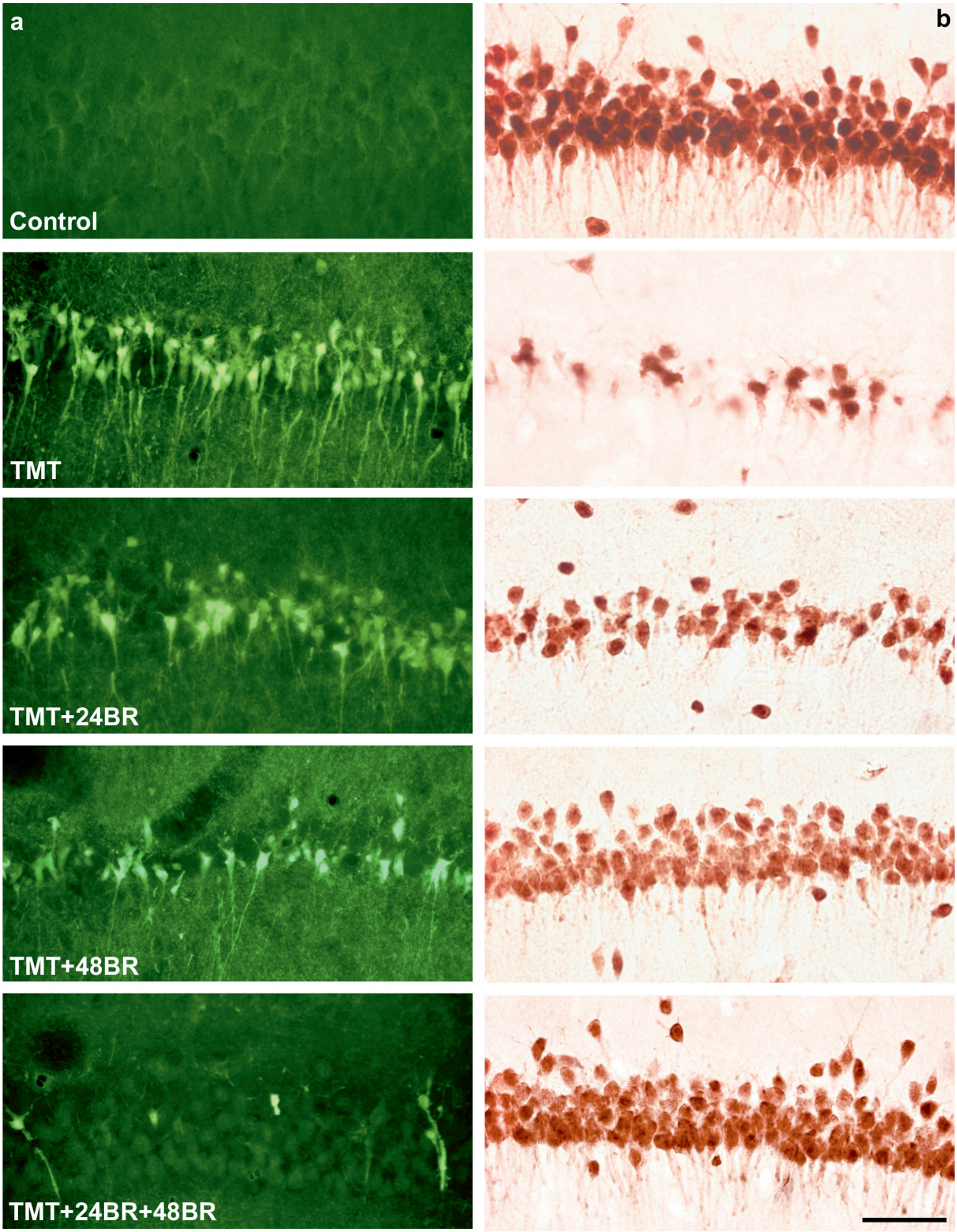

Fig. 1. Representative microphotographs of neurodegeneration visualised by Fluoro Jade B staining (left column) and NeuN immunostaining of surviving neurons (right column) in the hippocampal CA1 region following trimethyltin (TMT) intoxication (8 mg/kg i.p.) and 7 days of reperfusion. Panel a – microphotographs of FJ B staining of the CA1 region of the hippocampus. Bradykinin (BR) 150 μg/kg i.p was administered 24 or 48 hours after TMT intoxication. Scale bar = 50 μm. Panel b – microphotographs of NeuN immunohistochemistry of the CA1 region of the hippocampus. Bradykinin (BR) 150 μg/kg i.p was administered 24 or 48 hours after TMT intoxication. Scale bar = 50 μm. Control – Sham-operated animals, TMT – Trimethyltin + 7 days of reperfusion, TMT+24BR – Trimethyltin + bradykinin administered 24 hours after TMT intoxication + 6 days of reperfusion, TMT+48BR – Trimethyltin + bradykinin administered 48 hours after TMT intoxication + 5 days of reperfusion, TMT+24BR+48BR – Trimethyltin + bradykinin administered 24 and 48 hours after TMT intoxication + 5 days of reperfusion.

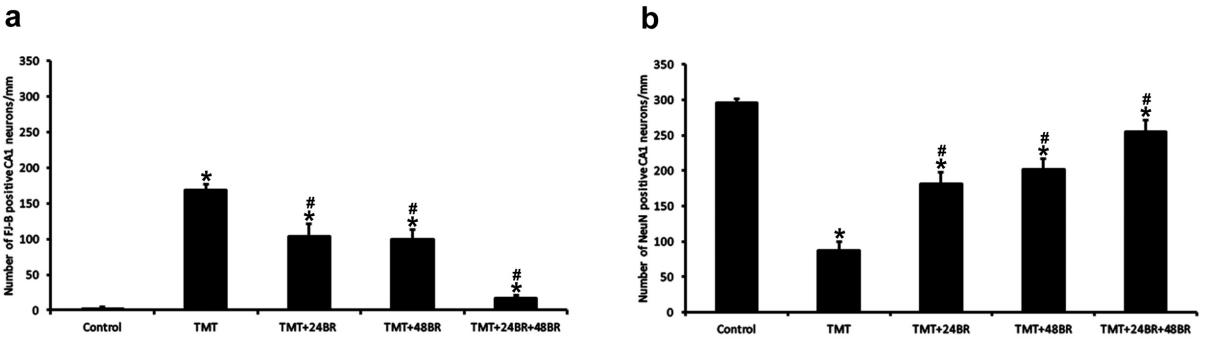

Fig. 2. Quantification of fluorescence intensity (a) and quantification of the number of NeuN positive cells (b) counted in the middle of the linear part of the CA1 region (-3.3 ± 0.2 mm posterior to bregma) and expressed as the average of 10 measurements of positive hippocampal CA1 pyramidal neurons per 1 millimetre. Values were taken as a mean ± SD of seven animals in each group. Control – Sham-operated animals, TMT – Trimethyltin + 7 days of reperfusion, TMT+24BR – Trimethyltin + bradykinin administered 24 hours after TMT intoxication + 6 days of reperfusion, TMT+48BR – Trimethyltin + bradykinin administered 48 hours after TMT intoxication + 5 days of reperfusion, TMT+24BR+48BR – Trimethyltin + bradykinin administered 24 and 48 hours after TMT intoxication + 5 days of reperfusion, * P 0.05 compared to control group, # P 0.05 compared to the TMT group.

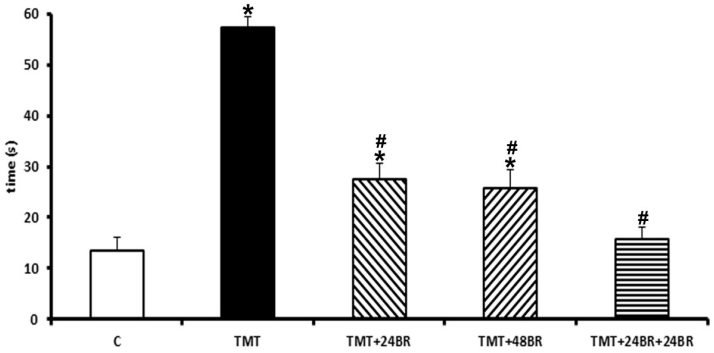

Fig. 3. Results of Morris water maze test performed on day 7 after TMT intoxication with BR administration 24 and 48 hours after TMT intoxication. The time latency to reach the platform in all groups is displayed. Data are presented as mean ±SD (n = 7 in each group). C – sham-operated animals, TMT – Trimethyltin (8 mg/kg, ip) and 7 days of reperfusion. TMT+24BR, TMT+48BR and TMT+24BR+48BR groups were treated with bradykinin (BR, 150 μg/kg i.p) 24 or 48 hours after TMT intoxication.
*Significantly different (p 0.05) versus the sham control group.
#Significantly different (p 0.05) versus the TMT group.

that postconditioning is able to prevent neuronal death but also that surviving neurons retain a substantial part of their function with the ability to learn and remember.

Discussion

Trimethyltin (TMT) is a potent neurotoxic material which produces a dose-dependent degeneration of neurons in the limbic system (CORVINO *et al.* 2013). It particularly damages the neurons in the hippocampus, amygdala, and cortex (GELOSO *et al.* 2011). TMT-induced neurodegeneration is characterised by massive neuronal death and is accompanied by reactive gliosis, epilepsy, and other neurobehavioral alterations (CORVINO *et al.* 2012). It has been well-documented that TMT impairs hippocampal and non-hippocampal learning and memory processes using passive avoidance, water maze, and radial arm maze tests (EARLEY *et al.* 1992). The Morris water maze is a well-known behavioural method which has been frequently used in rodents to evaluate hippocampal-dependent learning and memory impairment in neurocognitive disorders (D'HOOGE & De DEYN 2001).

The use of trimethyltin is an extremely simple, reliable, and inexpensive model suitable for the study of acute neurodegeneration, similar to what occurs after cardiac arrest. TMT intoxication appears to be a useful tool to damage the brain and study the various responses to damage. In the present study, we demonstrated that a single dose of TMT significantly impaired learning and memory in rats, which was accompanied by the degeneration of more than 50% of the neurons in the CA1 region of the hippocampus. Using BR as a post-

conditioner improved the deleterious effects of TMT on learning and memory and also increased the number of surviving neurons in the CA1 region of the hippocampus.

After TMT intoxication, the initiation of hippocampal neuronal death occured after two days (BROCK & O'CALLAGHAN 1987). Delayed death of neurons, which likely occurs via apoptosis, may be stopped and significant numbers of cells can be rescued when delayed postconditioning is used. We believe that the extent of the therapeutic window is indirectly proportional to the intensity of the lethal insult and lasts from several hours to two days. Our therapeutic approach provides a therapeutic window of around two days for transient global ischaemia lasting up to 10 min as well as intoxication with kainic acid in dose 8 mg/kg (BURDA *et al*. 2009; BURDA *et al*. 2006; BURDA *et al*. 2005; DANIELISOVA *et al*. 2008; DANIELISOVA *et al*. 2009; DANIELISOVA *et al*. 2006).). However, in comparison with the effect of postconditioning on ischaemia or intoxication kainic acid, the effect of postconditioning was significantly lower in the present study. One of the explanations for the lower efficiency was that the death of neurons after TMT intoxication occurs more rapidly than after kainic acid exposure or transient global ischemia.

The optimal procedure used after trimethyltin intoxication is to provide the first dose of the postconditioner 24 hours after insult and to repeat it 48 hours after TMT; this resulted in surprisingly high efficacy.

We expect that the 24 hour interval between TMT administration and postconditioning is enough to prevent early neurodegeneration in CA1 but is too short to build full tolerance as previously demonstrated after preconditioning (BURDA *et al*. 2003), but a single dose of the postconditioner at 48 hour interval is too late because apoptosis begins earlier after TMT than after ischaemia or kainite exposure. We hope that this work may be useful for elucidating the role of putative candidates for translational medical research on neurodegenerative diseases.

Conclusions

In general, it was found that postconditioning significantly ameliorated cognitive impairment and reduced the death of neurons in TMT-treated rats. In the current study, deficits in learning and memory were prevented after two doses of BR. . However, the exact mechanism(s) of postconditioning preventing TMT-induced learning and memory deficits are still under debate and further investigation is required.

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