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Rat hippocampus structures after experimental traumatic brain injury and GABA-benzodiazepine receptor complex modulators administration

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Abstract: *the global incidence of traumatic brain injury remains high and long-lasting consequences of such injury brings both medical and social burden on public health. Such comorbidity among others includes early and late post-traumatic epilepsy, post-traumatic stress disorder and depression and anxiety, cognitive impairments often require prolonged treatment. The incidence of such negative outcomes of traumatic brain injury is extremely high among deployed military personnel and war-affected civilians. All of the facts that mentioned above require the development of new strategies, including novel drug designing to provide complex treatment and overcome existing difficulties in rehabilitation after traumatic brain injury. The aim of the study was to evaluate the effect of GABA-benzodiazepine receptor complex modulators on rat hippocampus zones after traumatic brain injury. The experiment was carried out on male Wistar rats. To obtain traumatic brain injury we used a modified weight drop model. Rats were divided to intact, placebo (received placebo treatment), MPTD-01 (treated with 4-(4'-Methoxyphenyl)-2,3,4,5-tetrahydro-1H-2,3-benzodiazepin-1-one; intraperitoneally, 5 mg/kg once a day) and BS 34-20 (treated with 6-(4-Methoxyphenyl)-7H-[1,2,4]triazolo[3,4-a][2,3]benzodiazepine; intraperitoneally, 5 mg/kg once a day) groups. The light microscopy with hematoxylin and eosin straining was performed on 3, 7, 14, 21 days after injury. Hippocampus CA1, CA3 zones and dentate gyrus were studied. Unlike the placebo group, the MPTD-01 and BS 34-20 experimental groups demonstrated a smoothing of the swelling phase and its reduction, a shift in the time phase of cellular reactions to a later time. In the BS 34-20 group, minimal edema and more complete restoration of the structure of the hippocampus was observed on the 21st day of the experiment. Even though the studied structures of the hippocampus were not directly traumatized during the performance of the TBI model, they also undergo remodeling according to the revealed patterns. Such remodeling consists in the sequential change of the swelling phase by the phase of cellular reactions followed by the recovery phase. As it was shown in current study, the use of MPTD-01 and BS 34-20 in rats after a moderate traumatic brain injury leads to a time shift and smoothing of the edema phase. The possible explanation of the studied drugs action is excitotoxicity reduction what results in less pronounced secondary alteration. Tested novel hydrogenated benzodiazepines can be considered as promising drugs for neurodegeneration prevention after mild traumatic brain injuries, but their effect on glia activation and other phases of inflammatory cascade both with neuron and gliocyte ultrastructure still need to be investigated.*

Keywords: Brain Injuries, Traumatic; Benzodiazepines; Drug Design; CA1 Region, Hippocampal; Dentate Gyrus

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Introduction

Following epidemiological studies published between 2015 and 2020, the age-adjusted incidence of all severities of TBI was between 476 per 100000 individuals in South Korea (Kim et al., 2020) to 787 per 100 000 individuals in the USA. (Taylor, Bell, Breiding & Xu, 2017).

In 2016 Institute for Health Metrics and Evaluation (global Disease burden metrics) reported that that TBI was a cause of 8·1 million YLDs (Years of healthy life lost due to disability). As for war-related TBI, the U.S. Armed Forces Health Monitoring Centre reported that 375,230 U.S. servicemen suffered from TBI from 2000 to 2016 (Wojcik, Stein, Bagg, Humphrey & Orosco, 2010).

According to epidemiological studies, in general, the incidence of TBI among soldiers who participated in Operation Iraqi Freedom (OIF), Operation Enduring Freedom (OEF), and Operation New Dawn (OND) deployment reached 15.2–22.8% (Wojcik et al., 2010)

Ukrainian national statistics on TBI epidemiology among military personnel are still unalienable or incomplete due to continued russian aggression.

The most common short and long-term TBI-associated complications include immediate seizures, early and late post-traumatic epilepsy, cerebrospinal fluid leakage, hydrocephalus, cognitive and memory impairment, post-traumatic stress disorder (PTSD), and injury-related depression (Kaptalan, Andreeva, Stoyanov, Ostapenko & Oliinyk, 2021).

Studies on the epidemiology of late posttraumatic epilepsy demonstrated that patients with mild TBI have an increased probability of late epilepsy onset nearly three times more during the first year after injury and nearly two times more during the next three years. For such patients, standardized incidence ratios are 3,1 (1,0-7,2 95 CI) and 2,1 (1,1-3,8 95% CI). (Annegers & Coan, 2000)

Another wide cohort study showed that patients with mild TBI had significantly (up to 37%) increased risk of affective disorders, especially during the second year (Delmonico et al., 2024)

The last scoping review also stated that long-lasting cognitive impairment including episodic memory impairment can be observed in nearly 55% of the patients after mild TBI during the first year after injury. (McInnes, Friesen, MacKenzie, Westwood & Boe, 2017; Mavroudis et al., 2024)

The hippocampus is considered as a part of the brain that responds to affect, cognitive, and memory functions. Both with this, hippocampus transformation such as hippocampal sclerosis with neuronal cell death and excessive gliosis or mossy proliferation are keystones of epileptogenesis. Prevention of neurodegeneration and epileptogenic brain transformation by decreasing excitotoxic events is a key way of late TBI complications prophylactics (Ostapenko, I. 2022; Ziablitsev, & Khudoley 2020).

Design of novel drugs that can decrease excitotoxicity by increasing or modulation of GABA-ergic transmission can be a perspective way of treatment.

Aim

The current study aimed to investigate of GABA-benzodiazepine receptor complex modulators (hydrogenated 2,3-benzodiazepines MPTD-01 and BS 34-20) effect on rat hippocampus structures after experimental mild traumatic brain injury. The question of the study was are the hydrogenated 2,3-benzodiazepines MPTD-01 and BS 34-20 prevent neurodegeneration in hippocampus after mild traumatic brain injury?

Materials and methods

The experiment was carried out on 125 male Wistar rats (180-240 g) that were housed in standard conditions, natural light cycle, water and food ad libitum.

We used the modified Marmarou weight drop model to perform mild traumatic brain injury by applying a 66,7 g weight drop from 65 cm on interauricalar point at sagital line. (Nasution et al., 2020).

All the rats were divided to four groups:

Rats from I group rats received no injury or treatment (intact)

Rats from II group received placebo treatment

Rats from III group received MPTD-01 (4-(4'-Methoxyphenyl)-2,3,4,5-tetrahydro-1H-2,3-benzodiazepin-1-one; intraperitoneally, 5 mg/kg q.d.)

In IV group rats received BS 34-20 (6-(4-Methoxyphenyl)-7H-[1,2,4]triazolo[3,4-a][2,3]benzodiazepine; intraperitoneally, 5 mg/kg q.d.)

3, 7, 14 and 21 days after injury rats were sacrificed by decapitation under thiopentone overdose. Rat brains were processed for light microscopy. 5-um thick sagital formalin-fixed paraffin-embedded sections were sliced and stained with hema-

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toxyline and eosyne (Mulisch, & Welsch, 2015), observed via MICROmed Evolution ES-4130 light microscope. All obtained photos were processed with ImageJ software (NIH, USA, ver. 1.50), On all slices we defined, studied and described dentate gyrus, CA1 and CA3 hippocampus zones.

All experimental procedures were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council on protection of animals used for scientific purposes. We also consider national regulatory act (The law of Ukraine «On protection of animals from cruelty»).

Results

By the 3^{d} day after injury rats from all experimental groups demonstrated preserved layer structure of hippocampus including dentate gyrus. By this term, we reveal the edematous changes in subgranular layer of dentate gyrus in hippocampus of all experimental groups. Such changes were observed in all experimental groups, but they were more prominent in II (TBI+placebo) group. Such findings were also seen in III experimental group (TBI+MPTD-01) but the degree of their severity was less than in II group. Rats from IV group (TBI+BS 34-20) demonstrated similar in nature but minimal in expression changes.

On the 7th day after injury rats from II (TBI+-placebo) group demonstrated signs of oedema in subgranular layer of dentate gyrus. Part of CA3 hippocampus zone that directly directly contain focus with increased number of small round-shaped cells that have basophilic cytoplasm. The CA1 hippocampus zone contains focus, where typical granular cells are absent, the vast majority of cells have polymorph, polygonal or corrugated shape and basophilic cytoplasm. Such cells are placed in 2-3 rows.

In III experimental group (TBI+MPTD-01) the dentate gyrus composed of light granular cells, with oedema in subranular layer, The CA3 and CA1 hippocampus zones contain mostly light granular cells that form several layers with few of the pyramid cells with bright basophilic cytoplasm in between other cells.

In the IV experimental group (TBI+BS 34-20) the structure and cell presence of dentate gyrus, CA3 and CA1 hippocampus zones are similar to one in I (intact) group.

By the 14th day in II group (TBI+placebo) The dentate gyrus composed of majorly granural cells, nearly half of them have basophilic cytoplasm. Edema in subgranular layer is like such in previous term. CA3 hippocampus zone contains both granular and pyramidal cells with basophilic cytoplasm and nuclei with clearly seen nucleoli. CA1 zone contains focus with decreased number of granular cells. Half of them have normal structure and cytoplasm tinctorial properties. The rest of the cells had polygonal or corrugated shape and basophilic cytoplasm.

In III group (TBI+MPTD-01) The dentate gyrus is majorly composed of granular cells and a few pyramidal cells that preserve their structure. CA3 hippocampus zone contains decreased number of granular cells with preserved structure; the CA1 zone contain oval-shaped granular cells with light cytoplasm and nuclei with clearly seen nucleoli, few pyramidal cells with basophilic cytoplasm.

In IV group (TBI+BS 34-20) the dentate gyrus composed of huge amount of small round-shaped granular cells with basophilic cytoplasm. Signs of edema are less prominent than in previous term. CA3 and CA1 hippocampus zones contain granular cells with slightly basophilic cytoplasm. In CA1 zone below cortical impact place granular cells appear corrugated, have basophilic cytoplasm.

By the 21st day in II group (TBI+placebo) Dentate gyrus contain granular cells with signs of pericellular and intracellular edema that majorly present in molecular layer. CA3 and CA1 hippocampus

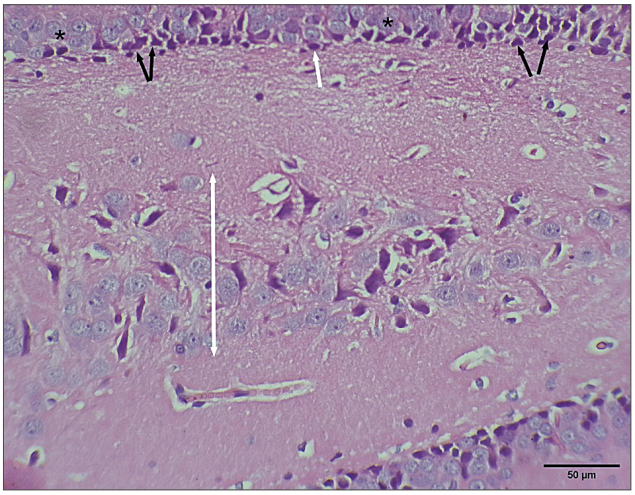

Fig. 1. Dental gyrus and hillus hippocampus zone, BS 34-20 treatment group, 21st day after injury. Scale bars is 50 um. * – granule neuron; black arrows – pyramidal cells in subgranular layer; white arrow – glia cell; double headed arrow – hillus with granular cells and interneurons

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zones contain decreased number of cells, majority of cells are corrugated, wrinkled and have basophilic cytoplasm.

In III group (TBI+MPTD-01) dentate gyrus both with CA3 and CA1 hippocampus zones contain typical cells preserve their quantity, shape and orientation, but nearly all of them have basophilic cytoplasm.

In IV group (TBI+BS 34-20) dentate gyrus, CA3, CA1 hippocampus zones contain both oval-shaped granular cells with narrow light cytoplasm and pyramidal cells with basophilic cytoplasm. In CA1 zone, under the impact zone most cells are pyramidal with basophilic cytoplasm.

Discussion

As we observed, the dentate gyrus structures react to injury as swelling of subgranular layer that was more prominent at 3rd at 7th days after injury and gradually decreased from 14 to 21st day in II (TBI+placebo group). The edema in subgranular zone in both experimental groups are more prominent at 7th and 14th days after injury, with decrease to 21st day. In contrast to II and III groups, rats from IV group demonstrated minimal signs of subgranular layer swelling. The reaction of cells in dentate gyrus to injury manifests in the form of cytoplasm basophilia that was observed at 14th day in II (TBI+placebo) group; together with decrease in number of cells at 21st in this group it can be considered as sign of apoptosis. In contrast to this, in III (TBI+MPTD-01) group such prominent basophilic strain of granular layer cells observed at 7th day with strain features incomplete recovery at day 14. Rats from IV group demonstrate similar changes with cell incomplete recovery till day 21.

We also observe differences in cell reaction in the CA3 and CA1 hippocampal zone between groups: the peak of reactivity and cell activation in II group (TBI+placebo) is on the 7th and 14th day after injury and appears as hyperstrained basophilic cytoplasm of cells in granular zone. In the III group (TBI+MPTD-01) such neuronal cytoplasm activation was observed only on 21st day after injury, whereas in IV group (TBI+BS 34-20) peak of neuronal cytoplasm hyperactivity was on 14th day and neuronal straining features were restored at day 21. (Tucker, Burke, Fu & McCabe, 2017).

Conclusions

Even though the studied structures of the hippocampus were not directly traumatized during the

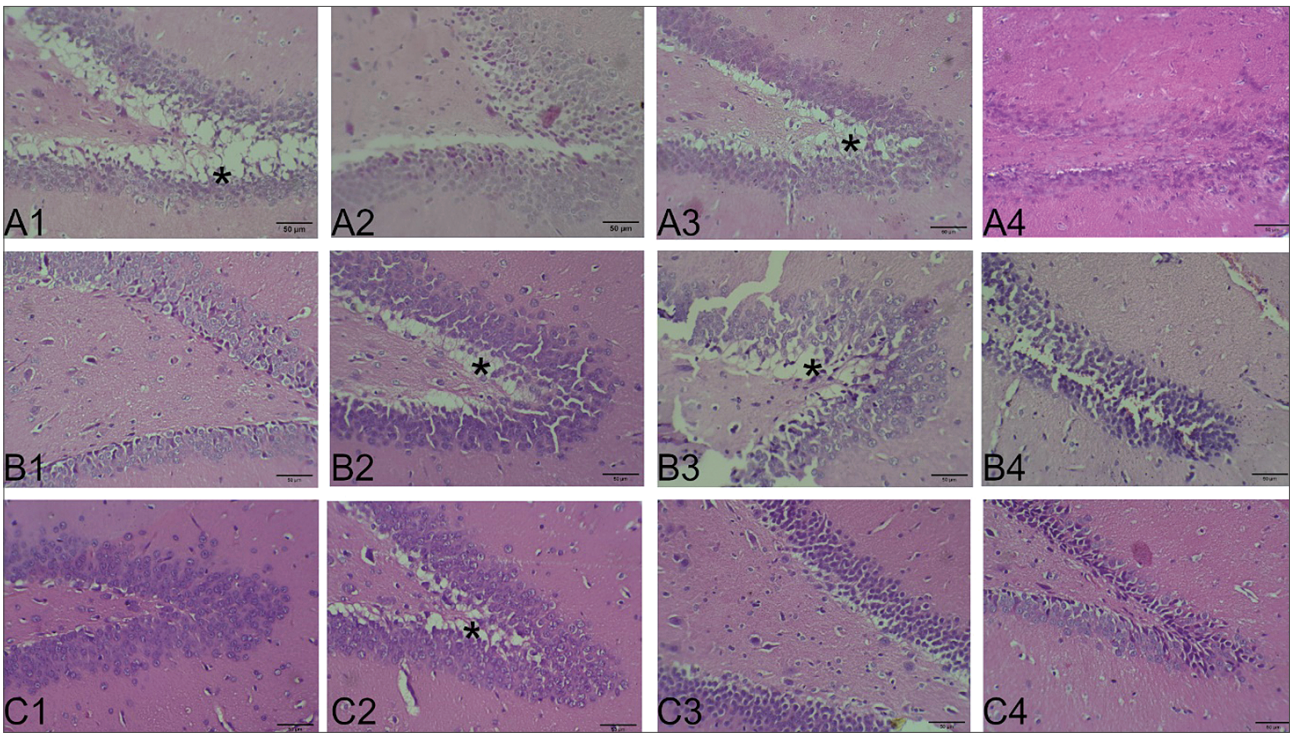

Fig. 2. Dental gyrus hippocampus zone. Prefixes – «A» – placebo treatment group, «B» – MPTD-01 treatment group, «C» – BS 34-20 treatment group; 1, 2, 3, 4 – 3, 7, 14 and 21 days after injury respectively. All scale bars are 50 um. * – pronounced edema in subgranular layer.

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performance of the TBI model, they also undergo remodeling according to the revealed patterns. Such remodeling consists in the sequential change of the swelling phase by the phase of cellular reactions followed by the recovery phase.

As it was shown in current study, the use of MPTD-01 and BS 34-20 in rats after a moderate traumatic brain injury leads to a time shift and smoothing of the edema phase. The possible explanation of the studied drugs action is excitotoxicity reduction what results in less pronounced secondary alteration. Tested novel hydrogenated benzodiazepines can be considered as promising drugs for neurodegeneration prevention after mild traumatic brain injuries, but their effect on glia activation and other phases of inflammatory cascade are still needed to be investigate.

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Conflict of interests

The authors declare no present or potential conflict of interests.

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of article.

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Структура гіпокампа щурів після експериментальної травми головного мозку та застосування модуляторів ГАМК-бензодіазепінового рецепторного комплексу

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Анотація: *частота травми головного мозку серед країн усього світу залишається високою і довготривалі наслідки таких травм привносять та збільшують як медичний так і соціальний тягар на сферу громадського здоров'я. Такі наслідки травм серед іншого, включають в себе ранню та пізню посттравматичну епілепсію, посттравматичний стресовий розлад, депресію, когнітивні порушення часто вимагають тривалого лікування. Частота таких негативних наслідків травм головного мозку надзвичайно висока серед військовослужбовців та цивільних громадян під час війни. Викладені вище факти обумовлюють необхідність розробки нових стратегій лікування та профілактики, включаючи розробку нових препаратів для забезпечення комплексного лікування та подолання складнощів, що зустрічаються у процесі реабілітації після травм головного мозку. Експеримент проведено на щурах-самцях лінії Вістар. Для відтворення травми головного мозку використовували модель вільного падіння вантажу. Щури були розділені на інтактну, плацебо та 2 експериментальні групи MPTD-01 (отримували 4-(4'-Methoxyphenyl)-2,3,4,5-tetra-hydro-1H-2,3-benzodiazepin-1-one в дозі 5 мг/кг внутрішньоочеревинно, 1 раз на добу) та BS 34-20 (отримували 6-(4-Methoxyphenyl)-7H-[1,2,4]triazolo[3,4-a][2,3]benzodiazepine в дозі 5 мг/кг внутрішньоочеревинно, 1 раз на добу). Через 3, 7, 14, 21 день після моделювання травми тварин виводили з експерименту, проводили мікроскопічне дослідження зрізів забарвлених гематоксилін-еозином матеріалу із зон CA1, CA3 та зубчастої звивини гіпокампу. На противагу групі плацебо, у групах із застосуванням MPTD-01 та BS 34-20 відзначалося згладженість перебігу фази набряку та її зменшення, зсув часу фази клітинних реакцій на більш пізній час. В групі із застосуванням BS 34-20 відзначався мінімальний набряк та більш повне відновлення структури гіпокампа на 21 добу від початку експерименту. Не зважаючи на той факт, що досліджувані структури гіпокампа не зазнають прямого впливу під час моделювання травми, вони також піддаються ремоделюванню по відомим паттернам. Таке ремоделювання складається із послідовних фази набряку, фази клітинних реакцій та фази відновлення. Наші досліди показали, що застосування MPTD-01 та BS 34-20 у щурів після травми головного мозку середнього ступеня призводить до відстрочення у часі та згладженості фази набряку. Можливим поясненням механізму дії досліджуваних речовин є зменшення ексайтотоксичності нейронів із вірогідним зменшенням вторинної альтерації. Досліджувані нові бензодіазепіни можуть вважатися перспективними препаратами для попередження нейродегенерації після травми головного мозку, проте, їх ефект на активацію нейроглії та інші фази нейрозапалення так само як і ультраструктура нейронів та нейроглії при їх застосуванні потребують подальших досліджень.*

Ключові слова: черепно-мозкові травми; бензодіазепіни; дизайн препаратів; Область CA1, гіпокамп; Зубчаста звивина