

Attenuation of Reactive Astrogliosis Accompanies Neurological Recovery Induced by Novel Poly-Target 2,3-Benzodiazepines After Experimental Traumatic Brain Injury

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Abstract

Introduction.

Traumatic brain injury (TBI) generates multifactorial secondary pathophysiology, which monotherapies have consistently failed to address. We evaluated MPTD-01 and BS34-20, novel 2,3-benzodiazepine derivatives with a computationally predicted multi-target profile, in a moderate TBI rat model.

Methods.

Male Wistar rats (n = 120) were allocated to five groups: Intact (n = 5), Sham (n = 10), Placebo (n = 35), MPTD-01 (n = 35), and BS34-20 (n = 35; 5 mg/kg/day i.p., Days 3–21), followed for 45 days. Primary outcome: 100-point Neurological Deficit Scale (NDS). Secondary outcomes included OFT behaviour, survival, and GFAP immunohistochemistry. Molecular docking (GNINA) against 7 CNS targets provided a hypothesis-generating in silico component.

Results.

Both compounds produced significant NDS improvements from Day 14 to Day 45 ($p < 0.001$; Hedges' $g = 3.5–5.6$). Landmark analysis demonstrated absence of post-acute deaths in treated groups compared with continued mortality in Placebo animals ($\chi^2=6.543$, $p = 0.038$). Total locomotor distance did not differ between groups; BS34-20 animals resumed exploratory behaviour earlier ($p = 0.048$), and both treatment groups exhibited numerically > 2 -fold lower chronic freezing than Placebo at Day 45, representing a clinically meaningful trend ($p = 0.059$). GFAP immunohistochemistry demonstrated attenuation of progressive reactive astrogliosis across the cortex, dentate gyrus, and CA1, with a shift toward a self-limiting astroglial response. Computational profiling supported a seven-target mechanistic hypothesis (GABA-A, GABA-B, AMPA, NMDA, Nav1.2, TSPO, OX1R).

Conclusions.

MPTD-01 and BS34-20 demonstrate neuroprotective efficacy, astroglial response modulation, and behavioural recovery without detectable impairment of gross locomotor output. The proposed multi-target mechanism warrants experimental validation. The 2,3-benzodiazepine scaffold warrants further investigation as a platform for polypharmacological neuroprotection in TBI.

Introduction

Traumatic brain injury (TBI) remains one of the leading causes of mortality and long-term disability worldwide. Data from recent iterations of the Global Burden of Disease study (GBD 2017, 2019, 2021) indicate that more than 20 million new TBI cases occur annually, while the number of individuals living

with persistent sequelae reaches hundreds of millions [1, 2]. The growing incidence of mild-to-moderate TBI is particularly concerning: although it is often not associated with immediate mortality, it may be accompanied by enduring cognitive, emotional-affective, and somatic deficits, resulting in a substantial socio-economic burden for healthcare systems, patients, and their families [3, 4]. In Eastern Europe, including Ukraine, TBI incidence and related costs remain consistently high compared with other regions, reflecting a combination of road-traffic trauma, occupational injuries, and military-related harm [5, 6].

From a pathophysiological perspective, TBI is not a single, instantaneous event but a dynamic process that comprises primary (mechanical) and secondary brain injury. The primary insult causes immediate deformation of neural tissue, axonal disruption, vascular injury, and the formation of contusion foci. However, secondary cascades, ischemia, blood–brain barrier (BBB) dysfunction, glutamatergic excitotoxicity, calcium overload, mitochondrial impairment, oxidative stress, and a blunted yet prolonged neuroinflammatory response largely determine the final extent of injury and the functional prognosis [7, 8]. The multifactorial nature of these processes may partly explain why single-target neuroprotective agents that showed promise in experimental settings have repeatedly failed to demonstrate convincing efficacy in clinical trials [9, 10].

A key component of secondary injury after TBI is glutamatergic excitotoxicity. Trauma triggers massive glutamate release, excessive activation of ionotropic NMDA (N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors, and pathological Ca^{2+} influx into neurons, followed by caspase activation, reactive oxygen species generation, and initiation of apoptosis [7, 8, 11, 12]. Experimental TBI models report sustained, locus-specific alterations in glutamatergic transmission in the cortex and hippocampus that correlate with memory impairment, affective disturbances, and susceptibility to post-traumatic epilepsy [7, 13]. Accordingly, the glutamatergic axis is a central mediator of both acute and chronic brain dysfunction after TBI.

The GABA(gamma-aminobutyric acid)ergic system is likewise critical, as together with glutamate it maintains a fine balance between excitation and inhibition. After TBI, changes have been described in the expression and function of GABA-A and GABA-B receptors, a reduction in phasic inhibition with a relative enhancement of tonic inhibition, and alterations of parvalbumin-positive interneurons in the cortex and hippocampus [14–17]. These alterations promote network hyperexcitability, which contributes to post-traumatic epileptogenesis, cognitive impairment, and emotional lability. Thus, excitotoxicity is not merely a consequence of excessive glutamatergic signalling; it reflects a broader imbalance between excitatory and inhibitory mechanisms across neural circuits.

A logical strategy to correct these disturbances is the use of GABAergic agents, primarily benzodiazepines (BZDs), which enhance inhibitory signalling through allosteric modulation of GABA-A receptors. In clinical practice, BZDs are widely used for sedation in patients with TBI, seizure control, and the management of anxiety and aggression [18]. However, their use is limited by well-recognised adverse effects, including respiratory depression, haemodynamic instability, tolerance and withdrawal, impaired cognitive recovery, and interference with neuromonitoring due to deep and prolonged sedation [19, 20].

Modern recommendations for the management of severe TBI emphasise individualised, "light" sedation aimed at minimising secondary brain injury while avoiding excessive CNS suppression [21, 22]. This has stimulated the search for more selective modulators capable of dissociating anxiolytic and anticonvulsant effects from profound sedation [23].

The pharmacological rationale for this approach is rooted in the concept of subunit-specific GABA-A receptor modulation: sedative-hypnotic effects are predominantly mediated by α_1 -containing receptors, while anxiolytic and anticonvulsant properties are associated with α_2/α_3 -containing receptors, motivating the concept of "anxioselective" modulators that dissociate these profiles [24–28].

In parallel, novel benzodiazepine scaffolds, particularly 2,3-benzodiazepines, have been actively investigated. For this class, a combination of interactions has been proposed, with the main one being non-competitive blockade of AMPA receptors, which are directly linked to glutamatergic excitotoxicity [29, 30]. Several new 2,3-benzodiazepine derivatives have demonstrated pronounced anxiolytic and antinociceptive activity with a relatively favourable safety profile in rodent models of anxiety, depression, and seizures [31–33]. This mechanistically evident poly-target activity makes 2,3-benzodiazepines promising candidates for pharmacological correction of TBI sequelae, where neurotransmission imbalance, neuroinflammation, and impaired neuroplasticity co-exist [34].

Beyond glutamatergic and GABAergic dysregulation, secondary TBI pathophysiology involves sustained neuroinflammation driven by activated microglia and astrocytes, in which the 18-kDa translocator protein (TSPO) plays an established modulatory role, with TSPO ligands demonstrating neuroprotective effects in experimental TBI models [35–43], and axonal ionic dysregulation arising from disrupted voltage-gated sodium channel distribution at sites of diffuse axonal injury [44–47]. These pathological axes represent additional pharmacological targets whose theoretical relevance to the 2,3-benzodiazepine scaffold is explored in the computational profiling component of the present study.

Molecular biomarkers of neuronal and glial injury are essential for assessing TBI consequences. Glial fibrillary acidic protein (GFAP) is the principal intermediate filament of astrocytes and a canonical marker of reactive astrogliosis, reflecting the degree of astrocytic activation and remodelling within the injured brain [48]. Importantly, GFAP immunoreactivity is used here as a quantitative index of astrocytic activation and reactive processes; it does not directly mark mature glial scar architecture, extracellular matrix remodelling, or specific astrocyte subtype composition, and its interpretation throughout this study is confined to the dynamics of reactive astrogliosis. Increased GFAP expression in brain tissue, as well as elevated GFAP concentrations in blood and cerebrospinal fluid after TBI, are associated with parenchymal injury severity, intracranial lesion burden, the need for neuroimaging, and unfavourable functional outcomes, thus broadly reflecting injury severity and survival [49–51]. Accordingly, GFAP can be viewed as an integral surrogate biomarker of neuroglial injury, indirectly capturing broader molecular perturbations in the injured brain. In experimental models, GFAP remains the most extensively validated tissue immunohistochemical marker of reactive gliosis; analysing its regional and temporal dynamics

enables tracking the transition from acute to chronic neuroinflammation and network remodelling, and allows quantitative appraisal of the neuroprotective potential of novel interventions [52].

Taken together, current evidence indicates that effective pharmacological correction of TBI sequelae requires a paradigm shift from single-target interventions to polypharmacological strategies capable of simultaneously limiting glutamatergic excitotoxicity, restoring the excitation-inhibition balance, stabilizing axonal ion homeostasis, and modulating neuroinflammation [9, 53]. In this context, 2,3-benzodiazepine derivatives present a privileged chemical scaffold. While historically characterized primarily as AMPA receptor antagonists, emerging data suggest their potential to engage a broader target spectrum, encompassing those mentioned earlier.

Despite this theoretical promise, evidence supporting the use of multi-target 2,3-benzodiazepines specifically in TBI remains fragmented. There is currently no coherent understanding of how the simultaneous modulation of these distinct molecular checkpoints translates into clinically relevant outcomes, such as survival, neurological recovery, and structural preservation, following traumatic injury [54]. Furthermore, the capacity of these agents to modify the trajectory of reactive astrogliosis, a hallmark of secondary injury, has not been established.

The aim of this study was to evaluate the neuroprotective and restorative potential of two novel 2,3-benzodiazepine derivatives, MPTD-01 and BS34-20, in an experimental model of moderate traumatic brain injury. By integrating *in silico* molecular profiling with *in vivo* assessments of survival, sensorimotor function, cognitive and emotional-affective status, and astroglial remodelling, we aimed to: (i) evaluate *in vivo* efficacy across functional, behavioural, and histological endpoints; and (ii) generate a computational mechanistic hypothesis for subsequent experimental target-engagement validation. The *in silico* analyses are explicitly hypothesis-generating and do not constitute functional evidence of target engagement.

Methods

Animals and Ethics Statement

The study was conducted on 120 male albino Wistar rats, obtained from the Vivarium of Bogomolets National Medical University (Kyiv, Ukraine), aged 3 months and weighing 180–200 g. Two weeks prior to the experiment, animals were acclimatized to a climate-controlled room (18–22°C, relative humidity 40–60%) with a standard 12-hour light/dark cycle (illumination 250 lux). Rats were housed in standard cages (50 × 40 × 25 cm) with *ad libitum* access to standard rodent chow and water. During the habituation period, animals underwent daily handling (8:00–10:00) and training sessions (minimum ten placements) to familiarize them with the experimental environment and assessment procedures.

All animal procedures were conducted in accordance with the "General Ethical Principles of Experiments on Animals" (Ukraine, 2001), consistent with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg,

1986), Law of Ukraine No. 3447IV dated 21.02.2006 "On Protection of Animals from Cruelty," Order of the Ministry of Education and Science, Youth and Sports of Ukraine No. 249 dated 01.03.2012 "Procedure for Conducting Experiments on Animals by Scientific Institutions," and Law of Ukraine "On Protection of Animals from Cruelty" No. 440IX dated 14.01.2020. Approval for the study was obtained from the Commission on Bioethics and Academic Integrity of Bogomolets National Medical University (Protocol No. 200, dated 24 October 2025).

Experimental Design

A randomised, placebo-controlled study design was employed. Animals were assigned to groups by computer-generated randomisation stratified by body weight tertile (Table 1). The Intact group (n = 5) served exclusively as a naive histological reference and was not included in statistical comparisons between TBI groups. The Sham group (n = 10) underwent anaesthesia and fixation without craniocerebral impact. TBI groups comprised n = 35 per arm. A CONSORT-style flow diagram detailing animal allocation, mortality, and scheduled withdrawals is provided in Supplementary Figure S1. To ensure methodological rigour: (i) group allocations were held in a master file by SVZ (not involved in data collection), with coded cage labels throughout; (ii) NDS and OFT assessments were performed by a single blinded investigator (DIY); (iii) histological slides were evaluated blind to treatment (VVL); (iv) ICC > 0.85 confirmed inter-rater reliability for NDS; (v) animals designated for scheduled histological withdrawal were pre-selected by random sequence prior to study start, independent of clinical status, ensuring non-informative censoring.

Table 1
Experimental group allocation.

Series	Intervention	Group Name	Number of animals
-	-	Intact	5
-	-	Sham	10
I	Saline administration	Placebo control	35
II	MPTD-01 administration	MPTD-01	35
III	BS34-20 administration	BS34-20	35
Total		120	

TBI was induced using a standard weight-drop model modified for moderate injury severity [30]. Animals were briefly anaesthetised by inhalation of 3% isoflurane in oxygen via a nose cone until loss of righting reflex (~ 60–90 s); no maintenance anaesthesia was required. The head was fixed in a stereotaxic frame, and the weight-drop apparatus, consisting of a 50 g cylindrical metal rod moving freely within a 65 cm vertical tube, was positioned such that the tube opening aligned with the sagittal suture, 5 mm anterior to the interaural line. A single impact was delivered to the cranial vault. Post-injury examination

confirmed closed TBI characterized by subcutaneous haematoma without gross brain displacement, cortical contusion of the temporal and parietal lobes, and diffuse petechial haemorrhages. Following the procedure, animals were returned to their cages with free access to food and water.

The investigational 2,3-benzodiazepine derivatives, MPTD-01 (Fig. 1, A) and BS34-20 (Fig. 1, B), were developed in the Department of Chemistry of Biologically Active Compounds, L.M. Litvinenko Institute of Physical-Organic Chemistry and Coal Chemistry of the National Academy of Sciences of Ukraine (Kyiv, Ukraine) under the leadership of Dr S.L. Bogza [55]. Compounds were administered at a dose of 5 mg/kg body weight intraperitoneally (in solution with normal saline) once daily from Day 3 to Day 21 after TBI. Control animals received equivalent volumes of the vehicle.

(A) MPTD-01: 4-(4"-methoxyphenyl)-2,3,4,5-tetrahydro-1H-2,3-benzodiazepin-1-one. (B) BS34-20: 6-(4-methoxyphenyl)-7H-(1,2,4)triazolo(3,4-a)(2,3)benzodiazepine.

Neurological Assessment

Neurological deficit was assessed using a 100-point Todd-based neurological score (Neurological Deficit Scale, NDS) adapted in accordance with ARRIVE 2.0 reporting principles [56, 57]. The scale quantified level of consciousness (20 points), motor function (30), cranial nerve function (20), sensorimotor integration (20), and autonomic signs (10). Each component included predefined operational criteria. A blinded investigator performed assessments at the same time each day. Inter-rater reliability was tested in a subset of animals (ICC > 0.85). All animals were randomised using a computer-generated sequence.

In the Sham control group, induction of inhalation anaesthesia was accompanied by clouding of consciousness, pupillary constriction, reduced reflexes and pain sensitivity threshold, absence of motor reactions, and slowed breathing. This reaction lasted up to 10–15 minutes, after which animals fully recovered within 20–30 minutes, demonstrating normal responses to light and sound and active grooming. No respiratory, motor, or behavioural disturbances were observed (the 100-point score was 4.1 ± 1.1 throughout the observation period without dynamics). Animals in this group were isolated until removal from the experiment under their usual conditions with free access to food and water.

In the control and treatment groups, injured animals exhibited varying behavioural, motor, and reflex disturbances. Immediately after injury, seizures were observed, rectal temperature decreased to 35–36°C, and heart and respiratory rates decreased. After anaesthesia wore off, transient loss of consciousness or mild obtundation was noted, consistent with moderate TBI. Pupils were constricted; light responses and corneal reflexes were normal or slightly reduced. Auditory responses were normal or weakened. Trunk and limb muscle tone was spastic in most animals, and responses to pain were reduced. Breathing was normal. Animals retained the ability to move independently, although with difficulty. Behavioural responses to the experimenter, ability to drink and eat, and self-care behaviours were reduced.

Open Field Test (OFT)

The OFT was employed as a standard and widely used paradigm in preclinical neuroscience to assess behavioural outcomes after TBI. The value of the OFT lies in its ability to simultaneously evaluate two distinct behavioural domains: general locomotor activity and anxiety-like behaviour. This dual assessment is clinically relevant, as TBI often induces long-term neuropsychiatric consequences, including anxiety disorders, making the assessment of thigmotaxis (anxiety) as important as locomotion.

Each animal was individually placed in a circular opaque arena (diameter 90 cm) with clearly visible boundaries. The arena was located in a quiet, uniformly illuminated room. Animals were habituated to the testing environment for 30 minutes prior to the experiment to minimize novelty-induced stress. During the test, the experimenters left the room, and the behaviour of each animal was recorded for 3 minutes using a top-mounted video camera positioned directly above the arena, providing an unobstructed view of the entire field. All recordings were saved in .mp4 format. After each animal, the arena was thoroughly cleaned with 70% ethanol and water to remove olfactory cues.

Prior to analysis, a pre-specified exclusion criterion was applied: video sessions were excluded if the automated tracker failed to maintain consistent centroid detection for > 10% of frames (due to lighting artefacts or arena contamination). Across all time points and groups, 9 video sessions were excluded (Placebo: 4; MPTD-01: 3; BS34-20: 2). Videos were analyzed offline in Jupyter Notebook using the ezTrack open-source behavioural analysis toolkit [58]. Each video was preprocessed by trimming to the exact 3-minute interval and calibrating the arena boundaries to define the circular region of interest (ROI). Background subtraction was performed by generating a reference frame through temporal averaging of multiple frames without the animal. The software then automatically detected the animal's centroid in each frame based on pixel intensity differences relative to the background. From these frame-by-frame coordinates, ezTrack computed the total distance travelled and produced trajectory plots. 9 behavioural metrics were computed per session: total distance (cm; primary locomotor measure), mean mobile velocity (cm/s), freezing duration (s; displacement < 0.7 cm for ≥ 1.0 s), freezing %, time in centre zone (s; 50% arena radius), time in periphery (s), thigmotaxis index, centre zone entries (n), and latency to first centre entry (s). Identical thresholding, smoothing, and ROI parameters were applied.

Morphological and Immunohistochemical Studies

Morphological and immunohistochemical examinations were performed after decapitation under deep thiopental anaesthesia (50 mg/kg) on days 3, 7, 14, and 21 after TBI. The brain was fixed in neutral buffered formaldehyde solution (pH 7.4) for 24 hours. After dehydration, brain tissue fragments were paraffin-embedded according to a standard protocol. Using rotary microtomes (MPS2 and Microm HM 335 E), sections 3–4 μm thick were prepared and stained with hematoxylin and eosin. Light microscopy was performed using the following systems: Olympus BX40 with Olympus C3030ADU and C2000 ZOOM cameras; Olympus BX43 with an Olympus SC100 camera and Olympus DPSoft software; and an Axio

Imager.A2 microscope (Carl Zeiss, Germany) with Axiovision data processing system, using objective magnifications $\times 5$, $\times 10$, $\times 20$, $\times 40$, binocular head $\times 1.5$, and ocular $\times 10$.

For immunohistochemistry, brain tissue sections were mounted on adhesive Super Frost Plus slides (Menzel, Germany). For antigen retrieval, rehydrated sections were heat-treated in Target Retrieval Solution (DAKO, Denmark) using a microwave oven. Sections were then treated enzymatically with proteinase K (DAKO) for 5 minutes. Endogenous peroxidase activity was blocked using a peroxidase block, and nonspecific binding was blocked using a protein block (DAKO). Primary antibodies against glial fibrillary acidic protein (GFAP; code IS 524) were applied. Visualisation of primary antibodies was performed using the high-sensitivity DAKO Poly Vue HRP/DAB polymer detection system. For cell density quantification, three non-overlapping high-power fields (objective $\times 20$, field area 0.196 mm^2) were examined per brain region (cerebral cortex, dentate gyrus, hippocampal CA1) per time point per animal, in sections separated by $\geq 50 \mu\text{m}$. GFAP-positive astrocytes were counted manually by a blinded investigator (VVL); density expressed as $\times 10^3 \text{ cells/mm}^2$. $n = 5$ animals per group per time point (15 fields per group/region/timepoint).

Statistical Analysis

Data analysis was performed using SPSS 11.0, MedCalc, and Python 3.12 (scipy.stats, numpy). Continuous variables are expressed as mean $\pm$ SD or median [IQR 25–75] for non-normally distributed data (normality: Shapiro-Wilk). Between-group comparisons at each time point used the Kruskal-Wallis test; where significant ($p < 0.05$), pairwise Mann-Whitney U tests with Bonferroni correction were applied (adjusted alpha = 0.017 for three pairs). Within-group longitudinal change: Friedman test with Bonferroni-corrected Wilcoxon signed-rank post-hoc tests. Supplementary analysis: linear mixed-effects model (fixed effects: Group, Time, Group $\times$ Time; random intercept: Animal). Effect sizes: rank-biserial r (non-parametric pairwise) and Hedges' g with 95% CI (between-group at primary endpoints). Survival: Kaplan-Meier (log-rank), Cox proportional hazards regression (Placebo as reference), and pre-specified sensitivity analyses (acute-phase restriction Days 0–7; post-acute landmark analysis conditioned on Day 7 survival). A p -value < 0.05 was considered significant for primary comparisons; the study is exploratory.

Sensitivity analyses further characterised the temporal structure of the survival effect. An analysis restricted to the acute phase (Days 0–7), encompassing the period of shared mortality risk prior to repeated scheduled withdrawals, yielded no between-group difference ($\chi^2 = 0.175$, $p = 0.916$), confirming that the three groups entered the study with equivalent injury severity. In contrast, a post-acute landmark analysis conditioned on survival to Day 7 revealed a significant overall difference ($\chi^2 = 6.543$, $p = 0.038$): Placebo animals sustained 3 additional deaths (at Days 14 and 28), whereas both MPTD-01 and BS34-20 groups recorded zero post-acute lethal events (pairwise: Placebo vs MPTD-01, $p = 0.066$; Placebo vs BS34-20, $p = 0.073$). This temporal dissociation supports the interpretation that treatment did not alter the initial traumatic insult but rather interrupted the secondary injury cascades responsible for delayed mortality.

In Silico Target Profiling and Molecular Docking

To generate mechanistic hypotheses regarding the polypharmacological profile of MPTD-01 and BS34-20, a computational workflow integrating ligand-based target prediction and structure-based molecular docking was employed.

Three-dimensional structures of the investigational compounds and reference ligands (e.g., diazepam, perampanel) were generated from canonical SMILES strings and energy-minimized using the MMFF94 force field. Protonation states at physiological pH (7.4) were standardized. High-resolution crystal structures of the target proteins were retrieved from the Protein Data Bank (PDB): GABA-A receptor (PDB ID: 6HUO) [59], GABA-B receptor (4MS4) [60], AMPA receptor (5L1F) [61], NMDA receptor (7EU7) [93], Nav1.2 (6J8E) [62], and orexin receptor OX1R (6TOT) [94]. For the TSPO, due to the absence of a suitable high-resolution experimental holo-structure, a predicted model was retrieved from the AlphaFold Protein Structure Database [63]. All protein structures were prepared in UCSF ChimeraX by removing solvent molecules, adding polar hydrogens, and assigning Gasteiger partial charges.

Potential targets were initially screened using the SwissTargetPrediction server [64] and cross-referenced with the ChEMBL and BindingDB databases. Subsequent molecular docking was performed using GNINA [65]. For each target, a search space (25 Å cubic grid) was centred on the orthosteric or allosteric active site as defined by the location of co-crystallized native ligands. GNINA integrates the Vina scoring function with a convolutional neural network (CNN) re-scoring model, providing both classical docking scores and CNN-predicted pKd estimates. An exhaustiveness setting of 8 (GNINA default) was employed with the docking grid expanded by a 4 Å margin in all directions from the native ligand. Protocol validation: redocking co-crystallised reference ligands into all six crystal structures confirmed RMSD < 2.0 Å, validating binding site geometry reproduction. Binding poses were ranked by docking score (kcal/mol) and inspected for plausibility. Docking scores are computational estimates and do not represent validated affinities; cross-target comparisons are not valid.

Pharmacokinetic properties and toxicity risks were evaluated using the SwissADME [66] and ADMET-AI platforms. Analysis focused on "drug-likeness" (Lipinski's Rule of Five), predicted blood-brain barrier permeability, and P-glycoprotein substrate status. Toxicity endpoints were assessed using eToxPred [67].

Results

Survival

The distribution of animals across experimental series, accounting for survival and scheduled withdrawal from the experiment, is presented in Table 2. At each time point for morphological assessment (Days 3, 7, 14 and 21), an additional 5 animals from each series were removed from the experiment, recorded as "Withdrawn from experiment", and treated as censored observations in survival analyses.

Table 2

Animal survival analysis and distribution throughout the study. Data represent the number of animals at risk (n), mortality events, and scheduled withdrawals for histological analysis. Survival probabilities are presented as mean $\pm$ SD. There were no statistically significant differences in overall survival between groups ($\chi^2 = 1.747$; $p = 0.417$, Log-rank test).

Series	Day	Number of animals			Survival (cumulative fraction surviving)
		At end of previous period (n)	Died	Withdrawn from experiment	
Placebo	3	35	3	5	0.914 $\pm$ 0.047
	7	27	2	5	0.847 $\pm$ 0.064
	14	20	2	5	0.762 $\pm$ 0.081
	28	13	1	5	0.703 $\pm$ 0.093
	45	7	0	7	-
MPTD-01	3	35	2	5	0.943 $\pm$ 0.039
	7	28	2	5	0.876 $\pm$ 0.059
	14	21	0	5	-
	28	16	0	5	-
	45	11	0	11	-
BS34-20	3	35	3	5	0.914 $\pm$ 0.047
	7	27	2	5	0.847 $\pm$ 0.064
	14	20	0	5	-
	28	15	0	5	-
	45	10	0	10	-

In the Placebo series (I), the cumulative fraction surviving (Kaplan-Meier procedure) was 0.914 $\pm$ 0.047 on Day 3 and decreased to 0.847 $\pm$ 0.064 on Day 7, 0.762 $\pm$ 0.081 on Day 14, and 0.703 $\pm$ 0.093 on Day 28. In series II (MPTD-01) and III (BS34-20), cumulative survival on Day 3 after TBI was 0.943 $\pm$ 0.039 and 0.914 $\pm$ 0.047, respectively, and on Day 7 it was 0.876 $\pm$ 0.059 and 0.847 $\pm$ 0.064. Thus, animals treated with MPTD-01 or BS34-20 demonstrated slightly higher cumulative survival in the acute period compared with the control series. Notably, in both treatment series, lethal events were observed only during the first week after injury, whereas in the control group, the decline in survival continued up to Day 28.

Figure 2 Kaplan-Meier survival analysis across experimental groups.

Survival probability functions for Placebo (I, n = 35), MPTD-01 (II, n = 35), and BS34-20 (III, n = 35) groups over the 45-day post-injury period. No statistically significant difference was observed between groups

(Log-rank test, $p = 0.417$), although treated animals displayed a trend toward stabilization of mortality after the acute phase compared to controls.

Across the entire 45-day observation period, overall survival (accounting for all lethal events) was 77.1% (27/35) in the control series, 88.6% (31/35) in the MPTD-01 group, and 85.7% (30/35) in the BS34-20 group. A Kaplan-Meier plot comparing survival functions across series is shown in Fig. 2; comparison of survival curves using the log-rank test did not identify statistically significant between-group differences ($\chi^2=1.747$; $p = 0.417$). Accordingly, both MPTD-01 and BS34-20 were associated with a trend toward increased overall survival of injured animals (by 11.5% and 8.6%, respectively, versus the control series); however, these differences did not reach statistical significance in this study. Cox proportional hazards regression (Placebo as reference) yielded: MPTD-01 HR = 0.48 [95% CI: 0.13–1.76], $p = 0.269$; BS34-20 HR = 0.62 [95% CI: 0.18–2.07], $p = 0.434$. Both point estimates favour treatment, though neither reached statistical significance; the wide confidence intervals indicate that this study was not powered to detect small-to-moderate survival differences and preclude firm conclusions regarding the direction or magnitude of the survival effect. Numbers at risk at each observation timepoint are displayed beneath Fig. 2.

Neurological Assessment

The Neurological Deficit Scale (NDS) constitutes the primary efficacy outcome of this study, providing the most comprehensive multi-domain assessment of sensorimotor recovery and yielding the clearest and most consistent between-group differentiation across the observation period. After TBI induction, neurological deficit was pronounced in all series, followed by a trend toward gradual recovery (Table 3). Prior to TBI, 100-point scale values were minimal and did not differ between groups (0.2–0.4 points; $p = 0.723$), indicating no baseline intergroup differences. As early as Day 3 after injury, all series showed a marked increase in neurological deficit severity to approximately 45–47 points, with no statistically significant differences between series I (Placebo), II (MPTD-01), and III (BS34-20) ($p = 0.724$). On Day 7, a moderate reduction in neurological impairment severity was observed in all groups (to ~ 39–40 points), while between-group differences also remained nonsignificant ($p = 0.826$), reflecting a similar acute-phase course regardless of pharmacological correction.

Table 3

Dynamics of neurological deficit severity scores. Data are presented as mean $\pm$ SD (points). Effective sample sizes at each time point differ due to mortality and scheduled histological withdrawals; Placebo: Pre-TBI n = 35, Day 3 n = 32, Day 7 n = 25, Day 14 n = 18, Day 28 n = 12, Day 45 n = 7; MPTD-01: Pre-TBI n = 35, Day 3 n = 33, Day 7 n = 26, Day 14 n = 21, Day 28 n = 16, Day 45 n = 11; BS34-20: Pre-TBI n = 35, Day 3 n = 32, Day 7 n = 25, Day 14 n = 20, Day 28 n = 15, Day 45 n = 10. Superscripts indicates significant differences ($p < 0.05$): Roman numerals (I, II, III) denote significant differences vs. the respective group (the Mann–Whitney U test with Bonferroni correction); Arabic numerals (0, 3, 7, etc.) denote significant differences vs. the respective time point within the same group (the Wilcoxon signed-rank test with Bonferroni correction).

Time point (days post-TBI)	Series			p (Kruskal–Wallis)
	Placebo	MPTD-01	BS34-20	
Pre-TBI	0.4 $\pm$ 0.24 ^{3–45}	0.2 $\pm$ 0.2 ^{3–45}	0.4 $\pm$ 0.24 ^{3–45}	0.723
3	47 $\pm$ 3.46 ^{0,7–45}	46.4 $\pm$ 4.1 ^{0,7–45}	45.2 $\pm$ 3.03 ^{0,7–45}	0.724
7	39.2 $\pm$ 2.28 ^{0,3,14–45}	40.4 $\pm$ 2.97 ^{0,3,14–45}	39.6 $\pm$ 3.85 ^{0,3,14–45}	0.826
14	30 $\pm$ 3.16 ^{0,3,7,28,45}	17.4 $\pm$ 3.85 ^{0,3,7,28,45}	17.6 $\pm$ 5.55 ^{0,3,7,28,45}	0.0005
28	24.4 $\pm$ 5.18 ^{0,3,7,14,45, II,III}	5.6 $\pm$ 1.14 ^{0–14, I}	6 $\pm$ 1 ^{0–14, I}	< 0.0001
45	16.8 $\pm$ 2.28 ^{0–28, II, III}	6.4 $\pm$ 1.67 ^{0–14, I}	5.8 $\pm$ 1.1 ^{0–14, I}	< 0.0001

From Day 14 onwards, administration of MPTD-01 and BS34-20 was associated with a more pronounced reduction in neurological deficit compared with the control series. Specifically, in series I the mean neurological deficit score was 30.0 $\pm$ 3.16, whereas in series II and III it was 17.4 $\pm$ 3.85 and 17.6 $\pm$ 5.55, respectively ($p = 0.0005$), which may have indicated accelerated functional recovery under the investigational 2,3-benzodiazepine derivatives. By Day 28, the difference between the control and treatment groups further increased: in series I neurological deficit remained relatively high (24.4 $\pm$ 5.18), whereas in series II and III, it decreased to 5.6 $\pm$ 1.14 and 6.0 $\pm$ 1.0, respectively; between-group differences were highly significant ($p < 0.0001$). A similar pattern persisted on Day 45: the control group mean was 16.8 $\pm$ 2.28, while the MPTD-01 and BS34-20 groups were 6.4 $\pm$ 1.67 and 5.8 $\pm$ 1.1, respectively ($p < 0.0001$). Effect size analysis confirmed very large treatment effects at all late-phase time points: Day 14 (Hedges' $g = 3.49$ [95% CI: 1.52–5.46] for MPTD-01; $g = 2.48$ [0.83–4.13] for BS34-20), Day 28 ($g = 4.53$ and 4.46), and Day 45 ($g = 4.70$ and 5.55; all 95% CI lower bounds > 2.0). A supplementary linear mixed-effects model ($R^2 = 0.78$) corroborated steeper daily recovery in both treatment arms vs Placebo (MPTD-01: $\beta = -0.30$ pts/day, $p = 0.064$; BS34-20: $\beta = -0.30$ pts/day, $p = 0.073$).

Within-group analysis showed that, in all series, neurological deficit peaked on Day 3 and then decreased stepwise to the end of follow-up; differences between sequential time points were statistically significant (as indicated by the indices in Table 3). At the same time, the control group retained a moderate residual

neurological deficit by Day 45, whereas under MPTD-01 and BS34-20 therapy, deficit severity approached minimal values. No material differences between series II and III were observed at any time point, allowing both compounds to be considered similar in effectiveness for correcting neurological sequelae of TBI.

OFT

A detailed quantitative analysis of total distance travelled is presented in Table 4. The primary between-group analysis (Kruskal-Wallis) revealed no significant differences in total locomotor distance at any post-TBI time point (all $p > 0.05$). As the OFT was not designed as a dedicated sedation assay, this finding indicates only that no gross between-group difference in locomotor output was present; sedation cannot be formally excluded or confirmed on this basis alone. Analysis of eight additional OFT metrics yielded the following quantitative findings. At Day 10 (subacute phase), a significant between-group difference in latency to first centre zone entry was detected (Kruskal-Wallis $H = 6.065$, $p = 0.048$); post-hoc Mann-Whitney with Bonferroni correction identified a significant pairwise difference between BS34-20 (median 3.0 [0.8–17.4] s) and MPTD-01 (96.9 [79.7–127.4] s; $U = 20.0$, Bonferroni-corrected $p = 0.048$, rank-biserial $r = -1.00$), indicating earlier reinstatement of exploratory approach behaviour under BS34-20. At Day 45 (chronic phase), Placebo animals exhibited markedly elevated freezing duration (median 151.4 [107.6–162.7] s; 86% of session), substantially exceeding Intact controls (77.0 s; 59%), MPTD-01 (72.1 [65.7–91.4] s; 40%), and BS34-20 (81.1 [72.2–126.3] s; 45%; Kruskal-Wallis $H = 7.442$, $p = 0.059$). The > 2 -fold elevation in Placebo freezing relative to treated groups at Day 45 represents a clinically meaningful signal that approached but did not reach significance at the available sample size.

Table 4
Analysis of general locomotor activity (Open Field Test).

Parameter	Placebo	MPTD-01	BS34-20
Pre-TBI	1445.72 [1398.04–1505.31]	1577.06 [1535.03–1762.74]	1630.76 [1493.46–1642.64]
Day 7 post-TBI	677.56 [654.03–813.10]	1008.74 [804.13–1071.82]	434.57 [394.25–825.41]
Day 10 post-TBI	889.69 [720.26–970.77]	685.89 [534.90–883.93]	690.84 [629.19–698.60]
Day 28 post-TBI	520.50 [468.93–870.25]	476.94 [464.90–793.55]	829.78 [637.95–841.27]
Day 45 post-TBI	955.34 [813.48–1435.21]	1131.11 [1089.83–1355.75]	1299.90 [992.12–1343.33]
p (D7 vs D45)	0.2222	0.1508	0.0079
p (D28 vs D45)	0.2222	0.0317	0.0317

Values represent Total Distance Travelled (cm) presented as Median [IQR 25–75]. Inter-group comparisons (Kruskal-Wallis) showed no significant differences at any time point ($p > 0.05$). Intra-group recovery dynamics were assessed using the Mann-Whitney U test; bold p-values indicate significant late-phase recovery.

The absence of a significant between-group difference in gross locomotor activity is an expected finding. Prior studies indicate that "total distance travelled" in the Open Field Test is often an insensitive endpoint in models of moderate TBI, particularly in chronic phases, because general locomotor capacity frequently recovers or is compensated [33]. This result supports that the present injury model induced specific, subtle neurological deficits (captured by the NDS) rather than a global, catastrophic impairment of gross motor function.

However, a more nuanced intra-group analysis (Mann-Whitney U test) of the recovery trajectories within each group revealed significant findings (Table 4): all groups exhibited a "clinical nadir" (lowest point of activity) at Day 28 post-TBI; both treatment groups showed a statistically significant recovery from this nadir between Day 28 and Day 45 (MPTD-01: $p = 0.0317$; BS34-20: $p = 0.0317$); the Control group did not show this significant late-phase recovery ($p = 0.2222$). Furthermore, the BS34-20 group was the only group to show a statistically significant overall recovery from the acute phase (Day 7) to the final endpoint (Day 45) ($p = 0.0079$).

Visualization of movement patterns for representative animals from the Placebo, MPTD-01, and BS34-20 groups (n = 5 animals per group at each time point). Tracks illustrate general locomotor activity and exploratory behaviour within the 90 cm circular arena.

Morphological and Immunohistochemical Studies

Cerebral cortex

Placebo. On Day 3 post-injury, an accumulation of activated astrocytes with intensely stained cytoplasm was evident within the area of direct damage, primarily in the molecular layer and extending partially into the external granular layer (Fig. 4I-A). By Day 7, astrocyte activation became pronounced. Cells in these layers exhibited a greater number of extensively branched processes (Fig. 4I-B). On Day 14, the number of activated astrocytes continued to increase within the trauma zone, characterized by numerous processes and dense localization in the superficial cortical layers (Fig. 4I-C). By Day 21, a substantial population of activated astrocytes persisted, forming clusters of cells with numerous thin and thick branched processes (Fig. 4I-D). Thus, placebo treatment was associated with a progressive intensification of GFAP-defined astrocytic reactivity that persisted through Day 21, consistent with chronic, sustained reactive astrogliosis.

MPTD-01. On Day 3, GFAP-positive astrocytes formed uneven, local clusters predominantly within the molecular layer (Fig. 4II-A). In the direct damage zone, these cells displayed intense staining and numerous processes, located both freely and perivascularly, with increased GFAP expression in cell bodies and the surrounding neuropil. By Day 7, a distinct focal "clearing" with minimal GFAP expression emerged in the molecular layer (Fig. 4II-B). A broad band of reactive astrocytes with numerous branched processes spanning the deep molecular and external granular layers demarcated this focus. By Day 14, while uneven GFAP expression persisted in the injury zone (Fig. 4II-C), the overall intensity was markedly reduced compared to the previous time point, reflected by fewer astrocytes with reduced process complexity and thickness. By Day 21, GFAP expression was low and uniform across cortical regions (Fig. 4II-D), indistinguishable from adjacent tissue; cells were few in number with predominantly unbranched processes. Thus, MPTD-01 administration induced a transient astrocytic activation peaking on Day 7, followed by rapid resolution to near-baseline levels by Day 21.

Figure 4 Spatiotemporal dynamics of reactive astrogliosis in the cerebral cortex.

Representative photomicrographs of the perilesional cortex immunostained for GFAP with haematoxylin counterstaining (n = 5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III):** treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D):** time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). Note the progressive escalation and persistence of GFAP-defined reactive astrogliosis in the Placebo group (I-D), contrasting with the marked reduction in astrocytic GFAP immunoreactivity observed in the MPTD-01 group (II-D). Scale bar = 50 µm.

BS34-20. On Day 3, GFAP expression was uniformly increased, showing signs of moderate activation characterized by numerous short, weakly branched processes located freely and around microvessels (Fig. 4III-A). The activation zone corresponded to the injury site, extending into the molecular and external granular layers. By Day 7, activation persisted but became spatially heterogeneous (Fig. 4III-B); astrocytes formed clusters creating zones of both increased expression and focal clearing, with numerous long, branched processes. On Day 14, GFAP expression declined relative to Day 7, with astrocytes distributed evenly and possessing fewer elongated, unbranched processes (Fig. 4III-C). By Day 21, the overall degree of expression was further reduced but remained uneven; clustered astrocytes with signs of activation (branched processes, intense cytoplasmic staining) persisted in the molecular and external granular layers (Fig. 4III-D). Thus, BS34-20 was associated with peak activation on Day 3 followed by a gradual but incomplete reduction. While both MPTD-01 and BS34-20 significantly attenuated the sustained GFAP-defined astrocytic reactivity observed in the Placebo group, MPTD-01 showed a more pronounced reduction in GFAP immunoreactivity by Day 21, approaching near-baseline levels, compared to the moderate persistence observed with BS34-20.

Dentate gyrus

Placebo. On Day 3 post-injury, a uniform increase in GFAP expression was observed in the hilus, driven by increased astrocytic branching within the neuropil (Fig. 5I-A). By Day 7, marked activation was evident; the number of GFAP-positive cells increased significantly, and they exhibited numerous extensively branched processes (Fig. 5I-B). This state of elevated activation persisted through Day 14 (Fig. 5I-C), with only a slight spontaneous decline observed by Day 21 (Fig. 5I-D). Thus, the placebo group was characterized by a sustained astroglial response that peaked on Day 7 and remained relatively high throughout the subacute period.

MPTD-01. On Day 3, intense GFAP expression was evident across the hilus and molecular layer, where astrocytes displayed intensely stained cell bodies and numerous long, branched processes (Fig. 5II-A). A distinct subpopulation in the molecular layer exhibited processes penetrating the granule cell layer, in some cases spanning its entire thickness. By Day 7, although overall expression decreased, astrocytes retained activated morphologies with long branched processes uniformly distributed across layers (Fig. 5II-B); notably, an increased number of radially oriented processes within the granule cell layer was observed. High expression persisted through Day 14 (Fig. 5II-C). However, by Day 21, a marked reduction occurred: the density of GFAP-positive cells and the complexity of their processes decreased significantly, returning to near-baseline levels (Fig. 5II-D). Thus, MPTD-01 induced an activation peak on Days 3–7 that progressively resolved, with GFAP immunoreactivity returning toward near-baseline levels by Day 21.

BS34-20. On Day 3, high GFAP expression was observed, though the distribution of activated astrocytes was uneven (Fig. 5III-A). The neuropil exhibited a "foamy" appearance with local clearings, centrally marked by linear accumulations of activated astrocytes extending radially from the granule cell layer to the hilus. By Day 7, activation peaked; cells were uniformly distributed in the hilus and molecular layer with numerous branched processes (Fig. 5III-B), though few processes penetrated the granule cell layer.

High expression persisted through Day 14 (Fig. 5III-C). By Day 21, expression declined relative to the acute phase, but moderate activation remained, characterized by astrocytes with thinner, shorter processes (Fig. 5III-D). Thus, BS34-20 was associated with pronounced activation peaking on Day 7 and remaining elevated through Day 14. In contrast to the MPTD-01 group, where astrogliosis effectively resolved by Day 21, the reduction in the BS34-20 group was less complete, with signs of activation persisting into the chronic phase.

Figure 5 Reactive astrogliosis in the hippocampal Dentate Gyrus.

Representative photomicrographs of GFAP-positive astrocytes in the hilar and molecular layers of the dentate gyrus (n = 5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III):** treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D):** time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). Scale bar = 50 μ m.

Hippocampal CA-1

Placebo. On Days 3 and 7 post-injury, activated astrocytes were evident across all layers of the CA1 region (Fig. 6I-A, 6I-B). By Day 14, signs of marked activation persisted, characterized by a large population of branched cells with intensely stained cytoplasm (Fig. 6I-C). On Day 21, a substantial number of astrocytes with elongated, branched processes remained, located predominantly in close proximity to neurons. Thus, placebo treatment was associated with pronounced astrocyte activation at all time points, showing no tendency toward resolution by Day 21, indicative of chronic, unresolved gliosis.

MPTD-01. On Day 3, activated astrocytes showing a strongly positive GFAP reaction were distributed uniformly throughout all hippocampal layers, featuring long, thick processes with numerous branches (Fig. 6II-A). By Day 7, while astrocytes retained activated morphologies, the intensity of the immunohistochemical reaction began to decline relative to the acute phase (Fig. 6II-B). By Day 14, both GFAP expression and the degree of activation were markedly reduced, reflected by decreased staining intensity and the thinning of astrocytic processes, particularly in the molecular and polymorphic cell layers (Fig. 6II-C). By Day 21, expression returned to near-baseline levels (Fig. 6II-D); GFAP-positive cells were few, predominantly located in the polymorphic layer, and exhibited thin, short processes with minimal branching. Thus, MPTD-01 administration was characterized by an acute activation phase (Days 3–7) followed by a progressive reduction in GFAP immunoreactivity that reached near-baseline levels by Day 21.

BS34-20. On Day 3, intense GFAP expression was observed in all layers, with astrocytes displaying long, branched processes, often localized perivascularly (Fig. 6III-A). By Day 7, this pronounced expression persisted (Fig. 6III-B). By Day 14, a reduction in astrocyte numbers was noted compared to the previous time point (Fig. 6III-C); remaining cells were distributed uniformly with numerous but shorter processes. By Day 21, a moderate number of GFAP-positive cells remained (Fig. 6III-D); unlike the placebo group, these did not form clusters, and their processes were thin and weakly branched. Thus, BS34-20 was

associated with activation that peaked on Days 7–14 and decreased moderately by Day 21. Comparative analysis indicates that while BS34-20 significantly attenuated the sustained GFAP-defined reactive astrogliosis observed in the placebo group, it resulted in a more prolonged GFAP immunoreactivity profile compared to MPTD-01, in which astrocytic reactivity showed a more complete reduction by Day 21.

Figure 6 Reactive astrogliosis in the hippocampal CA1 region.

Representative photomicrographs of GFAP-positive astrocytes in the CA1 field (n = 5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III)**: treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D)**: time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). The Placebo group exhibits persistent hypertrophy and clustering of astrocytes, which is attenuated in both treatment arms. Scale bar = 50 μ m.

A comparison of the number of GFAP-positive astrocytes across the experimental groups is shown in Fig. 7. In the cerebral cortex of the placebo control group, the density of GFAP-positive astrocytes showed a clear tendency to increase on Days 14 and 21, indicating a progression of astrocytic gliosis and potentially reflecting ongoing damage to nervous tissue. Administration of MPTD-01 enhanced astrogliosis on Days 7 and 14, with a decrease by Day 21. Administration of BS34-20 was associated with significant attenuation of the progressive accumulation of GFAP-positive astrocytes. Both compounds significantly reduced GFAP-defined reactive astrogliosis relative to placebo in multiple brain regions; the cortical reduction was more pronounced and temporally advanced under BS34-20, while GFAP immunoreactivity alone does not allow definitive conclusions regarding scar prevention or astrocyte phenotype determination.

In the dentate gyrus and the CA1 field of the hippocampus in the placebo control group, astrocyte density increased on Days 7 and 14, with recovery by Day 21. Evidently, in deeper brain structures, traumatic injury and astrogliosis were less pronounced and showed a spontaneous tendency to recover by this time point. Comparison of the groups receiving benzodiazepines showed that astrogliosis was less pronounced in the dentate gyrus with MPTD-01 and differed little in the CA1 field of the hippocampus (Fig. 7). The small number of observations did not allow a conclusive comparison between groups; a consistent, quantitatively supported reduction in GFAP-defined astrocytic reactivity was observed across both hippocampal subregions under both investigational compounds.

Figure 7 Quantitative analysis of astrocyte density.

Bar charts representing the density of GFAP-positive astrocytes ($\times 10^3$ cells/mm²) in the (A) Cerebral Cortex, (B) Dentate Gyrus, and (C) Hippocampal CA1 region across four time points (n = 5 animals per group per time point; 3 fields per animal, 15 fields per group per region per time point). In the cortex, attenuation appeared earlier under BS34-20, whereas hippocampal regions (particularly the dentate gyrus) showed a greater reduction under MPTD-01. Data are presented as mean $\pm$ standard deviation.

Note: y-axis values are scaled $\times 10^3$ (a bar reading 6 = 6000 cells/mm²). * $p < 0.05$ vs Day 3 within group; # $p < 0.05$ vs Placebo at the corresponding timepoint (Mann-Whitney U, Bonferroni-corrected).

In Silico

Table 5
Docking scores of 2,3-benzodiazepines and reference ligands.

Target	Protein Data Bank ID	Compound	Docking score (kcal/mol)
Type-A γ -aminobutyric (GABA-A) receptor ($\alpha 1\beta 3\gamma 2$)	6HU0	MPTD-01	-7.235
		BS34-20	-8.379
		Alprazolam (Ref.)	-10.663
		Diazepam (Ref.)	-8.253
Type-B γ -aminobutyric (GABA-B) receptor (GBR1, GBR2)	4MS4	MPTD-01	-5.937
		BS34-20	-6.640
		Baclofen (Ref.)	-7.602
Translocator protein (TSPO)	N/A	MPTD-01	-6.722
		BS34-20	-7.065
		Diazepam (Ref.)	-6.804
AMPA-subtype GluA2 receptor	5L1F	MPTD-01	-6.721
		BS34-20	-7.904
		Perampanel (Ref.)	-9.069
NMDA receptor (GluN1/GluN2B)	7EU7	MPTD-01	-6.767
		BS34-20	-7.216
		Memantine (Ref.)	-4.906
The voltage-gated sodium channel Nav1.2	6J8E	MPTD-01	-6.361
		BS34-20	-7.356
		Lamotrigine (Ref.)	-6.841
Orexin receptor OX1R	6TOT	MPTD-01	-7.817
		BS34-20	-8.369
		SB-334867 (Ref.)	-8.230

Docking scores were computed using GNINA (Vina scoring function + CNN re-scoring). Reference compounds (Ref.) represent established ligands for each target. Values are Vina docking scores (kcal/mol; more negative = more favourable predicted binding); cross-target comparisons are not valid.

The compounds additionally showed predicted interactions with further targets. At Nav1.2 (6J8E): BS34-20 -7.356, MPTD-01 -6.361 kcal/mol. For TSPO (AlphaFold model), docking scores were - 7.065 kcal/mol for BS34-20 and - 6.722 kcal/mol for MPTD-01, compared with - 6.804 kcal/mol for diazepam. Docking against the NMDA receptor (PDB 7EU7) yielded scores of -7.216 (BS34-20) and - 6.767 (MPTD-01) kcal/mol, both exceeding the reference channel blocker memantine (-4.906 kcal/mol), generating a hypothesis for NMDA channel blockade as an additional excitotoxicity-limiting mechanism. Docking against the orexin OX1 receptor (PDB 6TOT) yielded - 8.369 (BS34-20) and - 7.817 (MPTD-01) kcal/mol, comparable to the selective OX1R antagonist SB-334867 (-8.230 kcal/mol). OX1R modulation may represent a novel contribution: orexin signalling is implicated in post-traumatic arousal dysregulation and neuroinflammation, warranting dedicated experimental investigation. Collectively, these results generate a seven-target polypharmacological hypothesis spanning glutamatergic, GABAergic, neuroinflammatory, ionic, and arousal axes.

The predicted pharmacokinetic and ADMET profile (Table 6) indicated favourable drug-like properties for both compounds. All ADMET outputs are computational predictions requiring experimental validation. Both MPTD-01 and BS34-20 were fully compliant with Lipinski's "rule of five" and showed high predicted gastrointestinal absorption. Critically for a CNS-acting drug, both compounds were predicted to be BBB-permeable. A key predicted difference was found in P-glycoprotein substrate status: MPTD-01 was predicted not to be a substrate, while BS34-20 was predicted to be a P-gp substrate. Both compounds were predicted to have a low risk of mutagenicity (AMES), cardiotoxicity (hERG), or hepatotoxicity.

Table 6
Predicted ADMET and physicochemical properties.

Parameter	MPTD-01	BS34-20	Description
Physicochemical properties			
Molecular weight (g/mol)	268.32	290.33	
LogP	2.23	2.76	Lipophilicity coefficient
Lipinski rule violations	0	0	Oral bioavailability marker
SA score	0.19	0.21	Synthetic accessibility index
Pharmacokinetics			
GI absorption	High	High	Likelihood of absorption after oral administration
BBB permeability	Yes	Yes	-
P-glycoprotein substrate	No	Yes	Possibility of active efflux
Toxicity			
AMES (mutagenicity)	No	No	DNA damage risk
hERG blockade	No	No	Cardiotoxicity risk
Hepatotoxicity (DILI)	No	No	Liver injury risk
Tox-score	0.60	0.60	Overall toxicity index (lower = better)

ADMET: Absorption, Distribution, Metabolism, Excretion, and Toxicity. GI: Gastrointestinal; BBB: Blood-Brain Barrier; DILI: Drug-Induced Liver Injury.

Thus, the 2,3-benzodiazepine derivatives MPTD-01 and BS34-20 may achieve an "anxiolytic" profile, presumably due to a more focused dual modulation of the GABAergic and glutamatergic systems (GABA-A and AMPA receptors, respectively). This balanced mechanism may help avoid the global CNS suppression that is typical of less selective agents.

Discussion

Principal Findings and Translational Significance

The primary finding of this investigation is that the novel 2,3-benzodiazepine derivatives, MPTD-01 and BS34-20, significantly enhance long-term functional recovery following moderate TBI. This robust outcome, evidenced by highly statistically significant improvements on the comprehensive Neurological Deficit Scale from Day 14 onwards ($p < 0.0001$), suggests that these compounds effectively mitigate the

subacute and chronic sequelae of brain injury. Crucially, while the difference in overall survival did not reach statistical significance ($p = 0.417$), the temporal profile of lethal events revealed a clinically relevant stabilization: mortality in treated groups was confined strictly to the acute phase, whereas placebo-treated animals continued to succumb to injury complications through Day 28. This temporal dissociation, confirmed by a statistically significant post-acute landmark analysis ($p = 0.038$), combined with profound functional recovery, indicates that MPTD-01 and BS34-20 may interrupt the persistent secondary injury cascades, such as excitotoxicity and neuroinflammation, that typically drive late-stage decompensation [68, 69]. Quantification of effect sizes revealed Hedges' $g = 3.5\text{--}5.6$ at Days 14–45, with all 95% CI lower bounds above $g = 2.0$, effects of exceptional magnitude unlikely to reflect sampling variability. A supplementary mixed-effects model confirmed a steeper daily rate of NDS improvement in treated animals (Group x Time interaction beta ~ -0.30 pts/day, $p = 0.064\text{--}0.073$), consistent with active pharmacological acceleration of the recovery trajectory.

From a translational perspective, these findings address a critical gap identified by recent consensus statements, including the *Lancet Neurology* Commission, which attribute the failure of historical TBI trials to a reliance on single-target agents [4, 70]. The observed efficacy of MPTD-01 and BS34-20 supports the emerging paradigm of "rational polypharmacology," where agents are selected for their ability to simultaneously modulate multiple pathological nodes [53, 71]. By targeting a broader neuroprotective network rather than a single receptor, these findings provide preclinical rationale for investigating the 2,3-benzodiazepine scaffold as a platform for polypharmacological neuroprotection; translation to clinical settings will require replication across diverse TBI models, female animals, and injury severities.

Astroglial Remodelling as a Structural Basis for Recovery

The profound functional recovery observed in treated animals was mirrored by a fundamental reorganization of the astroglial response. In placebo-treated animals, the trajectory of GFAP-defined reactive astrogliosis followed a maladaptive pattern: immunoreactivity progressively intensified, culminating in dense cellular conglomerates consistent with chronic reactive astrogliosis by Day 21 [72]. This chronic persistence of reactive astrocytes is known to create a physical and chemical barrier to axonal regeneration and synaptic reintegration, thereby locking the injured brain into a state of permanent deficit [73–75]. This finding is discussed in the context of the proposed astrocyte reactive state dichotomy: the early, transient GFAP response observed under treatment has been hypothetically linked in the literature to neuroprotective reactive states (associated with lesion isolation and trophic support), whereas the sustained, progressive GFAP elevation in Placebo animals is consistent with chronic reactive astrogliosis associated with impediment of axonal regeneration. We emphasise that GFAP immunoreactivity alone cannot determine astrocyte subtypes, reactive state identity, or phenotype switching; this interpretation is speculative and requires dedicated transcriptomic or proteomic characterisation in future work.

In contrast, administration of MPTD-01 and BS34-20 did not simply abolish the glial response but rather temporally reshaped it. Both compounds induced an early, transient peak in astrocyte activation (Days 3–7), which rapidly resolved, with GFAP immunoreactivity returning toward baseline in both treatment

arms by the chronic phase. This temporal pattern is conceptually consistent with a “boundary-forming” rather than “scar-forming” trajectory of reactive astrogliosis as described in the literature [76–78]. The early phase likely preserves the beneficial function of astrocytes, isolating the lesion and limiting the spread of excitotoxic damage, thereby preventing progression to the chronic scarring phase that inhibits plasticity [79]. By retuning the glial program toward this adaptive phenotype, the investigational compounds apparently preserved the structural integrity of the perilesional cortex and hippocampal networks (Dentate Gyrus and CA1), providing a preserved neuroanatomical substrate for the reintegration of complex sensorimotor functions [80].

Behavioral Specificity and Restoration of Adaptive Strategies

The dissociation between the pronounced improvement on the NDS and the absence of between-group differences in gross locomotor activity (OFT) is fundamental to interpreting the pharmacological profile of MPTD-01 and BS34-20. In experimental neurotrauma, improved scores on functional scales can sometimes be confounded by non-specific psychomotor effects; for instance, classical 1,4-benzodiazepines often induce dose-dependent sedation that masks sensorimotor deficits, or conversely, paradoxical hyperactivity [81–83]. In this study, the total distance travelled did not differ between treated and placebo animals. While the OFT does not constitute a dedicated sedation assay, the absence of between-group differences in gross locomotor output makes pharmacological sedation or stimulation an unlikely confound for the large NDS effect sizes, supporting genuine sensorimotor reintegration as the most parsimonious interpretation [23, 32].

However, the quantitative distance metric requires interpretation through the lens of qualitative trajectory analysis. As reported in the Results (Fig. 3), trajectory inspection revealed that quantitative “equivalence” in total distance masked qualitatively distinct behavioural states. During the acute phase, non-locomotor microsaccades associated with freezing episodes inflated distance estimates in Placebo animals, masking true suppression of exploratory drive. At later time points, the apparently normalised distance in Placebo animals coincided with sporadic, unstructured centre entries absent of preceding peripheral exploration, a pattern suggestive of disruption to risk-assessment circuits (potentially reflecting prefrontal-hippocampal axis dysfunction) resulting in disinhibited rather than genuinely exploratory behaviour.

Conversely, the behavioural trajectory of the MPTD-01 and BS34-20 groups followed a coherent physiological sequence: freezing followed by thigmotaxis (caution), and, finally, exploration. This progression indicates that the compounds did not merely stimulate motor output, but facilitated the reinstatement of adaptive behavioural programs. The treated animals regained the capacity to suppress panic (freezing) and exercise appropriate caution (thigmotaxis) before engaging in complex exploratory tasks. This is further supported by the intra-group dynamics, which revealed a statistically significant late-phase recovery in exploratory activity from Day 28 to Day 45 ($p < 0.05$) in treated animals, a rebound absent in the placebo group. Thus, the therapeutic effect extends beyond simple sensorimotor recovery

to the preservation of higher-order executive and emotional-volitional functions, allowing for a return to natural ethological strategies, likely underpinned by the preserved hippocampal and cortical architecture described earlier [84, 85].

Computational Target-Engagement Hypotheses: Translating In Silico Predictions to Testable Mechanisms

This section articulates testable mechanistic hypotheses derived from the computational target-profiling analysis. These predictions are not offered as explanations of the observed in vivo findings; the in vivo data establish what was observed, while the in silico data generate where to investigate next. All statements in this section represent working hypotheses requiring experimental target-engagement validation (radioligand binding, whole-cell electrophysiology, selective antagonist challenge in vivo). With this framing, the predicted multi-target profile of MPTD-01 and BS34-20 may, if confirmed, engage a synergistic network addressing distinct pathological axes of secondary TBI. Notably, the predicted engagement of OX1R, an interaction not previously attributed to 2,3-benzodiazepines, would represent a novel mechanistic contribution warranting dedicated pharmacological investigation.

The 2,3-benzodiazepine scaffold (prototyped by GYKI 52466) is structurally distinct from classical 1,4-benzodiazepines and is characterized by non-competitive AMPA receptor antagonism [30]. The predicted high affinity of MPTD-01 and BS34-20 for the AMPA receptor aligns with the observed attenuation of early reactive astrogliosis, as AMPA-mediated excitotoxicity is a primary trigger for the calcium-dependent injury cascades that drive astrocyte hypertrophy [86–88]. By dampening this initial excitotoxic surge, the compounds may reduce the sustained astrocytic reactivity observed in placebo controls; whether this prevents a pathological scar-forming glial transition cannot be determined from GFAP immunohistochemistry alone and remains a hypothesis requiring structural validation.

Predicted interaction with TSPO suggests a direct mechanism for modulating neuroinflammation. TSPO ligands are known to shift activated microglia and astrocytes from pro-inflammatory to restorative phenotypes [41, 89, 90]. This putative mechanism offers a biological explanation for the rapid resolution of GFAP expression observed in the treated groups, contrasting with the persistent inflammation in placebo controls.

The dual modulation of the GABA-A receptor (benzodiazepine site) and voltage-gated sodium channels (e.g., Nav1.2) addresses the ionic dysregulation and network hyperexcitability that characterize the subacute post-injury period [91]. This action likely underpins the durable recovery of complex sensorimotor functions (NDS) by stabilizing neuronal networks without inducing the global suppression associated with broad-spectrum sodium channel blockers or sedative hypnotics [47, 92]. Collectively, this computational framework generates a polypharmacological hypothesis in which MPTD-01 and BS34-20 may function as network-level modulators of secondary injury cascades; however, without direct functional target-engagement data (e.g., radioligand binding, patch-clamp electrophysiology, or

target-occupancy studies), all mechanistic attributions remain predictive and require experimental confirmation.

Methodological Considerations, Limitations, and Future Directions

A primary methodological strength of this study is its multi-domain design, which integrates *in silico* prediction of polypharmacological interactions with a rigorous *in vivo* assessment of functional recovery and tissue-level remodelling. This approach aligns with contemporary standards in preclinical neurotrauma research, where translational validity is strengthened by combining mechanistic hypotheses with clinically relevant endpoints like the NDS. Furthermore, the extended follow-up period (45 days) distinguishes this work from acute-phase studies, allowing for the evaluation of long-term recovery trajectories and the durability of the therapeutic effect.

However, several limitations must be acknowledged. First, the study enrolled only male Wistar rats. Sex is an established biological variable modulating post-traumatic neuroimmune responses, astrogliosis, and drug pharmacokinetics; female rodents demonstrate distinct neuroinflammatory and recovery patterns partly attributable to oestrogen- and progesterone-mediated neuroprotection [31]. Generalisability to females or to the full heterogeneity of clinical TBI populations cannot be assumed; future studies must include both sexes. Second, the study was not powered to detect small mortality differences; larger cohorts are required to confirm the observed trend (MPTD-01 HR = 0.48; BS34-20 HR = 0.62). Third, all mechanistic insights regarding receptor engagement remain computational predictions; biophysical validation (radioligand binding, patch-clamp, target-occupancy studies) is required. Fourth, GFAP immunoreactivity captures reactive astrogliosis as an integral marker but cannot distinguish neuroprotective (A2-like) from neurotoxic (A1-like) astrocyte phenotypes, nor capture full neuroinflammatory cellular heterogeneity. Fifth, with $n = 5$ animals in the terminal behavioural cohort, the LMM Group x Time interaction approached but did not reach significance; all findings are exploratory. Finally, OFT metrics were derived from automated centroid tracking; future studies should incorporate multi-point body tracking (e.g., DeepLabCut) for higher-fidelity behavioural phenotyping.

Future investigations will focus on validating these specific molecular targets and expanding the biomarker panel to include indices of microglial activation (e.g., Iba1, CD68) and synaptic integrity. Additionally, evaluating the compounds in diverse TBI models (e.g., blast injury or repetitive mild TBI) will be critical for establishing their broader therapeutic potential as pleiotropic neuroprotective agents.

Conclusions

- The integration of *in silico* predictive modelling with *in vivo* functional and morphological assessments provides a comprehensive evaluation of the novel 2,3-benzodiazepine derivatives, MPTD-01 and BS34-20, as potential neuroprotective agents for traumatic brain injury.

- Computational profiling predicts that the 2,3-benzodiazepine scaffold possesses a multi-target pharmacological profile characterised by predicted interactions with AMPA and NMDA glutamate receptors, the GABA-A benzodiazepine site, GABA-B receptors, TSPO, voltage-gated sodium channels (Nav1.2), and the orexin OX1 receptor, a mechanistic breadth spanning excitotoxicity, GABAergic dysregulation, neuroinflammation, ionic destabilisation, and arousal dysregulation. This computationally predicted multi-target profile generates a mechanistic hypothesis spanning excitotoxicity, GABAergic dysregulation, neuroinflammation, ionic destabilisation, and arousal dysregulation; experimental target-engagement validation is required before mechanistic claims can be substantiated.
- Treatment with MPTD-01 and BS34-20 produced highly significant and large-magnitude improvements in long-term sensorimotor integration (NDS) in a rat model of moderate TBI (all $p < 0.001$; Hedges' $g = 3.5-5.6$ at Days 14–45). Gross locomotor activity did not differ between groups, confirming genuine neurological restoration rather than sedation. Quantitative OFT analysis demonstrated earlier restoration of exploratory behaviour under BS34-20 at Day 10 (Bonferroni-corrected $p = 0.048$) and substantially lower chronic freezing in both treatment groups vs Placebo at Day 45.
- While the increase in overall survival did not reach statistical significance in this study, the therapeutic intervention was associated with a confinement of lethal events to the acute phase, preventing the late-stage lethal events observed in placebo-treated controls.
- Functional recovery was associated with a significant modulation of the GFAP-defined astroglial response. Both compounds attenuated the progressive, sustained phase of reactive astrogliosis in the cortex and hippocampus, reducing GFAP immunoreactivity toward near-baseline levels and preserving the neuroanatomical substrate required for recovery. These histological findings are confined to GFAP-defined astrocytic reactivity and do not constitute direct evidence of glial scar prevention or astrocyte phenotype determination.

Collectively, these data position MPTD-01 and BS34-20 as promising prototype agents for a "network-based" pharmacological strategy against secondary brain injury, warranting further validation through direct biophysical target engagement studies.

Declarations

Ethics approval and consent to participate.

Approval for the study was obtained from the Commission on Bioethics and Academic Integrity of Bogomolets National Medical University (Kyiv, Ukraine). No privately owned animals were used, and therefore informed consent from animal owners was not applicable.

Consent for publication.

Not applicable.

Clinical trial number.

Not applicable.

Competing interests.

The authors declare that they have no competing interests.

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None reported.

Author Contribution

S.Z. – conceptualization, study design, data analysis and interpretation; N.A. – data acquisition and investigation, data analysis and interpretation, manuscript drafting; D.Y. – data acquisition and analysis, study organization and conduct, manuscript drafting; M.B. – data acquisition, study organization and conduct, manuscript drafting; V.L. – morphological data and interpretation. All authors have read and approved the final version of the manuscript.

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Data Availability

The datasets generated and analysed during the current study, including raw NDS scores, OFT tracking data, and immunohistochemical cell density measurements, are available from the corresponding author on reasonable request.

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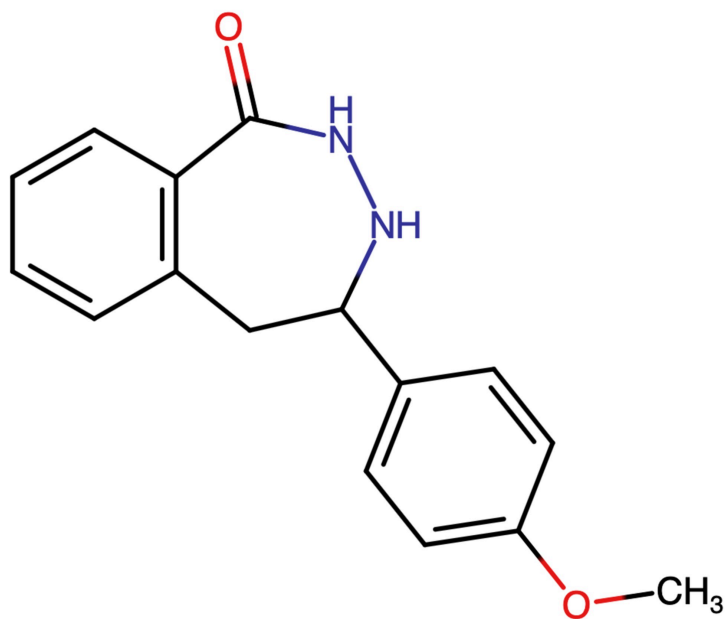
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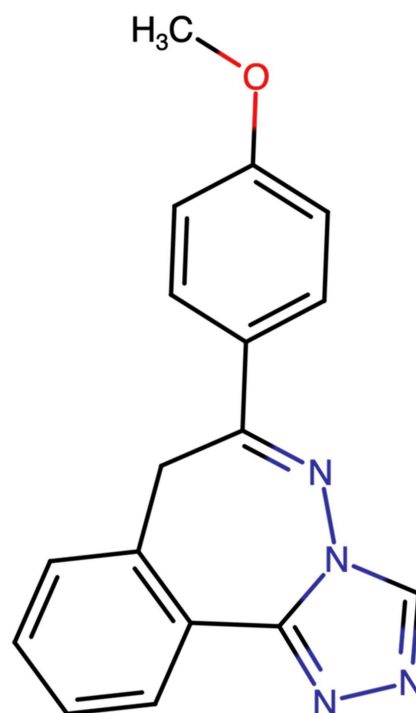
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Figures



A MPTD-01



B BS34-20

Figure 1

Chemical structures of the investigational 2,3-benzodiazepine derivatives.

(A) MPTD-01: 4-(4"-methoxyphenyl)-2,3,4,5-tetrahydro-1H-2,3-benzodiazepin-1-one. (B) BS34-20: 6-(4-methoxyphenyl)-7H-(1,2,4)triazolo(3,4-a)(2,3)benzodiazepine.

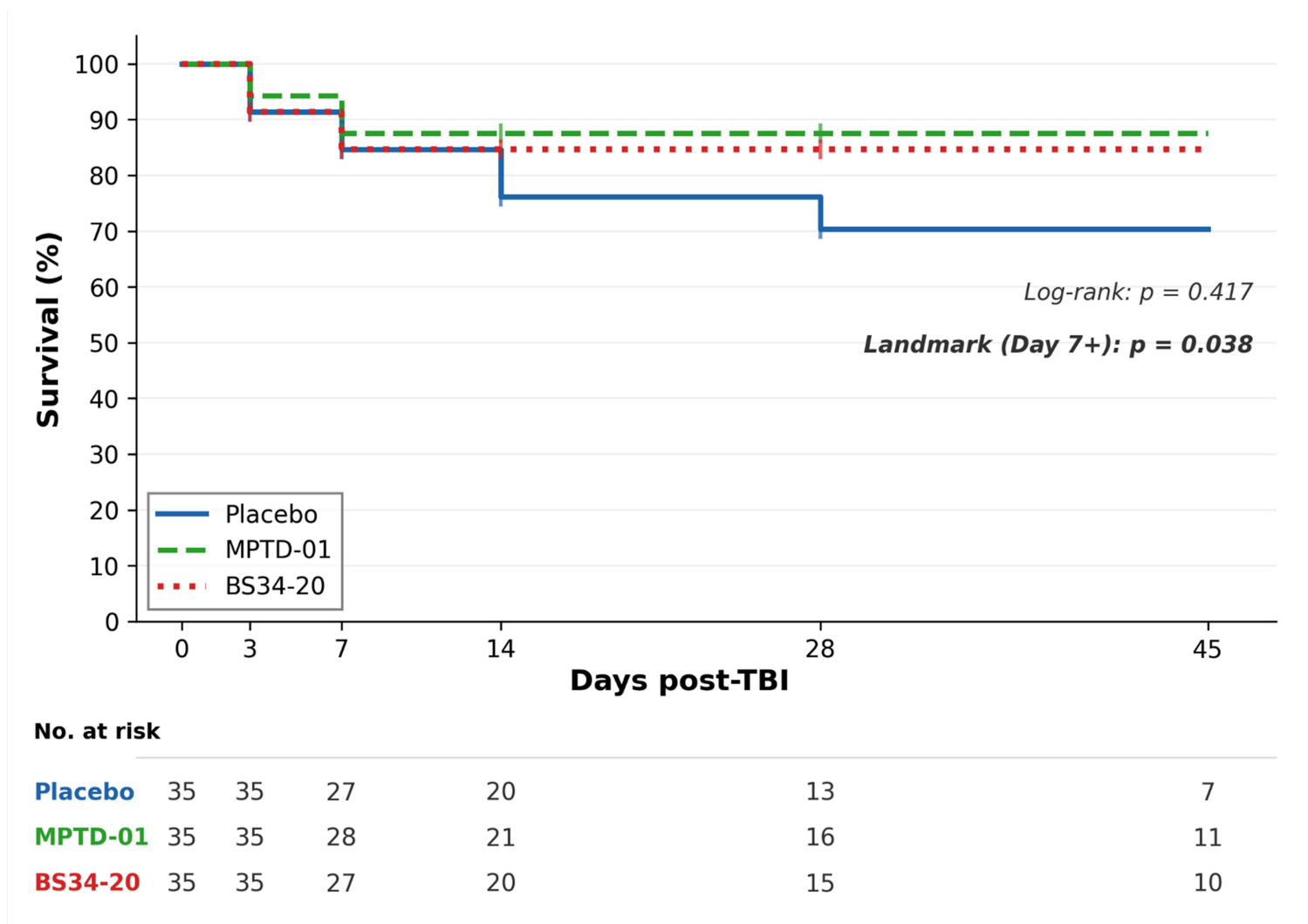


Figure 2

Kaplan-Meier survival analysis across experimental groups.

Survival probability functions for Placebo (I, n=35), MPTD-01 (II, n=35), and BS34-20 (III, n=35) groups over the 45-day post-injury period. No statistically significant difference was observed between groups (Log-rank test, $p=0.417$), although treated animals displayed a trend toward stabilization of mortality after the acute phase compared to controls.

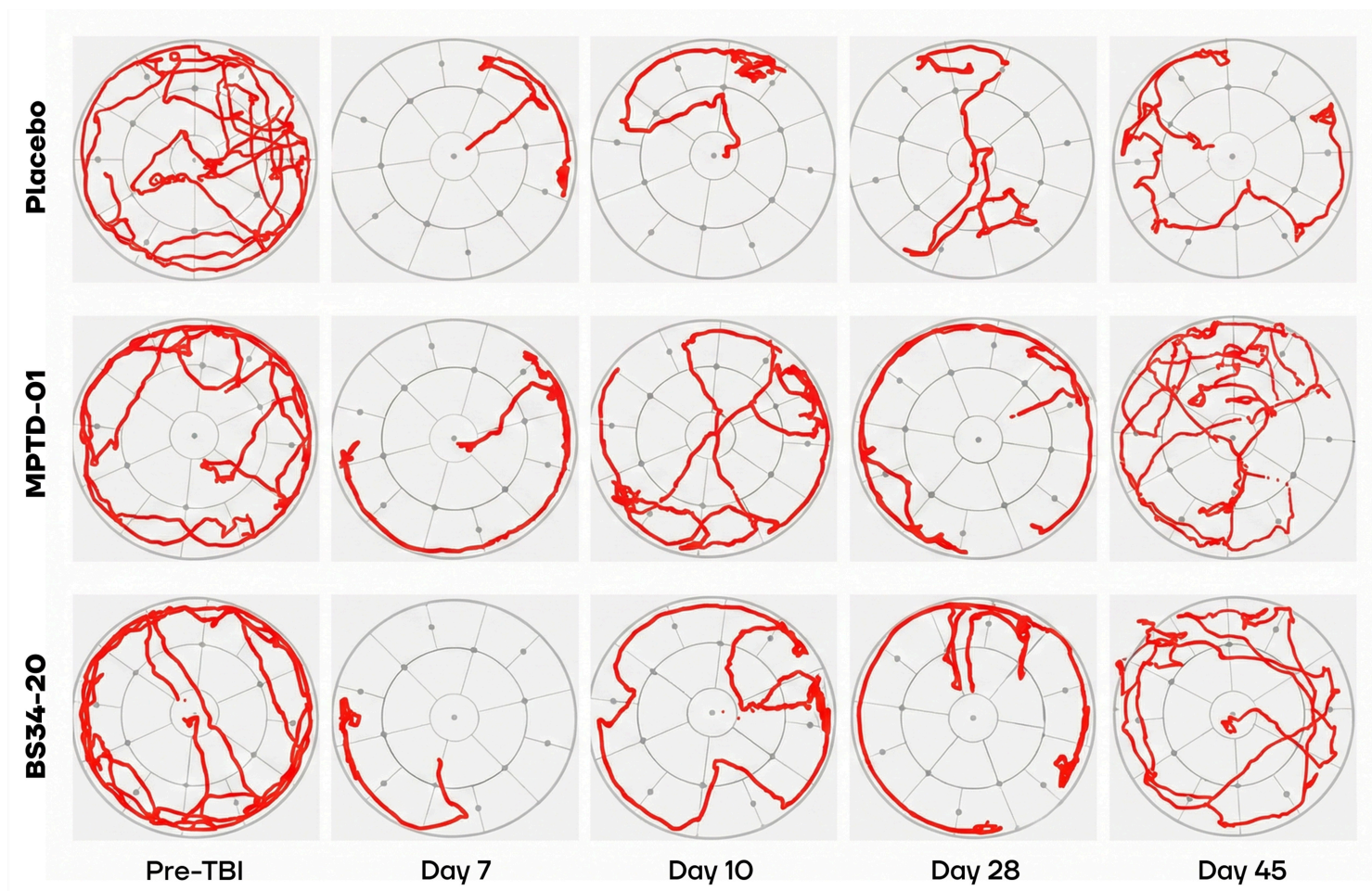


Figure 3

Representative locomotor trajectories in the Open Field Test.

Visualization of movement patterns for representative animals from the Placebo, MPTD-01, and BS34-20 groups (n=5 animals per group at each time point). Tracks illustrate general locomotor activity and exploratory behaviour within the 90 cm circular arena.

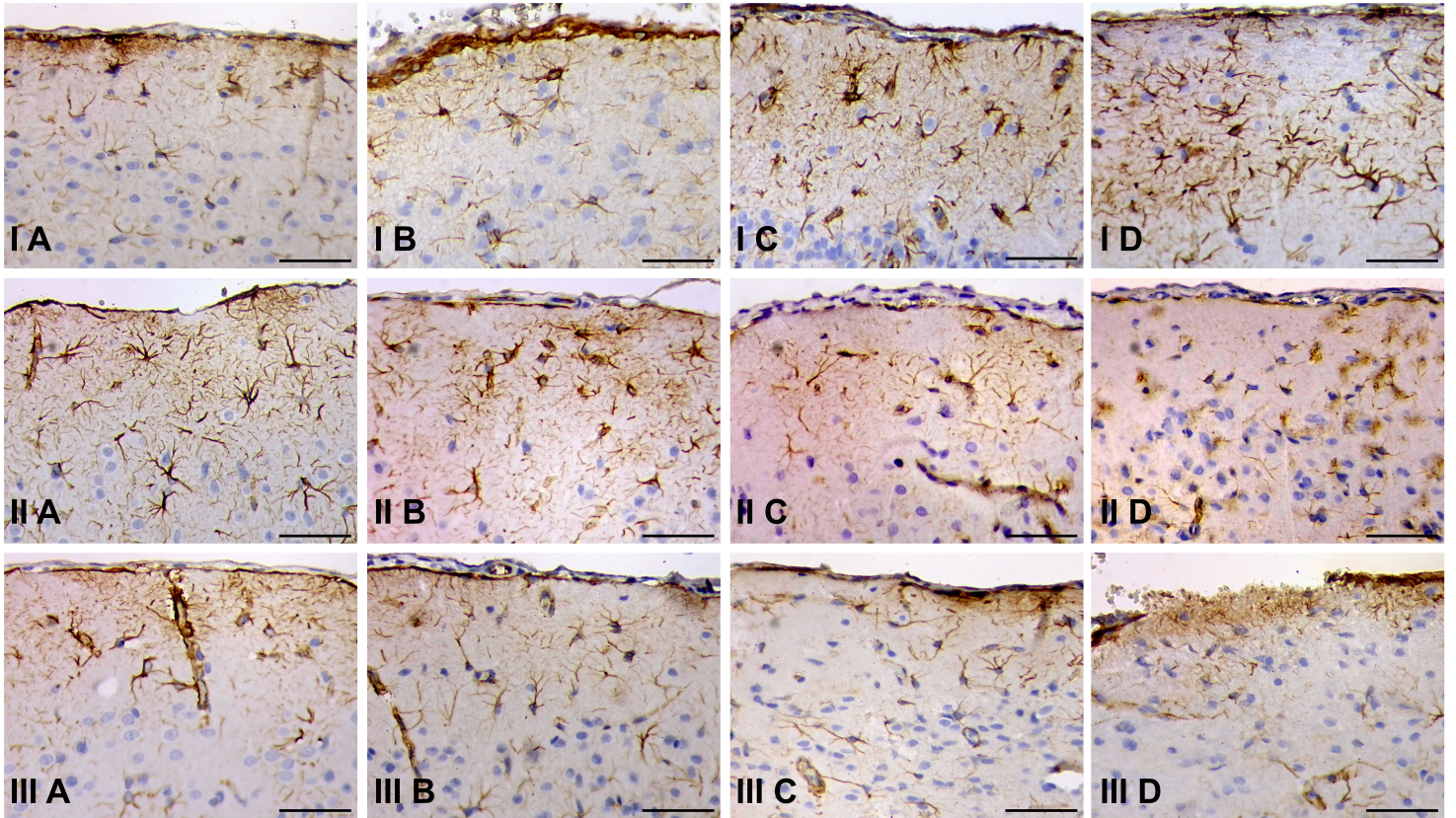


Figure 4

Spatiotemporal dynamics of reactive astrogliosis in the cerebral cortex.

Representative photomicrographs of the perilesional cortex immunostained for GFAP with haematoxylin counterstaining (n=5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III):** treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D):** time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). Note the progressive escalation and persistence of GFAP-defined reactive astrogliosis in the Placebo group (I-D), contrasting with the marked reduction in astrocytic GFAP immunoreactivity observed in the MPTD-01 group (II-D). Scale bar = 50 μ m.

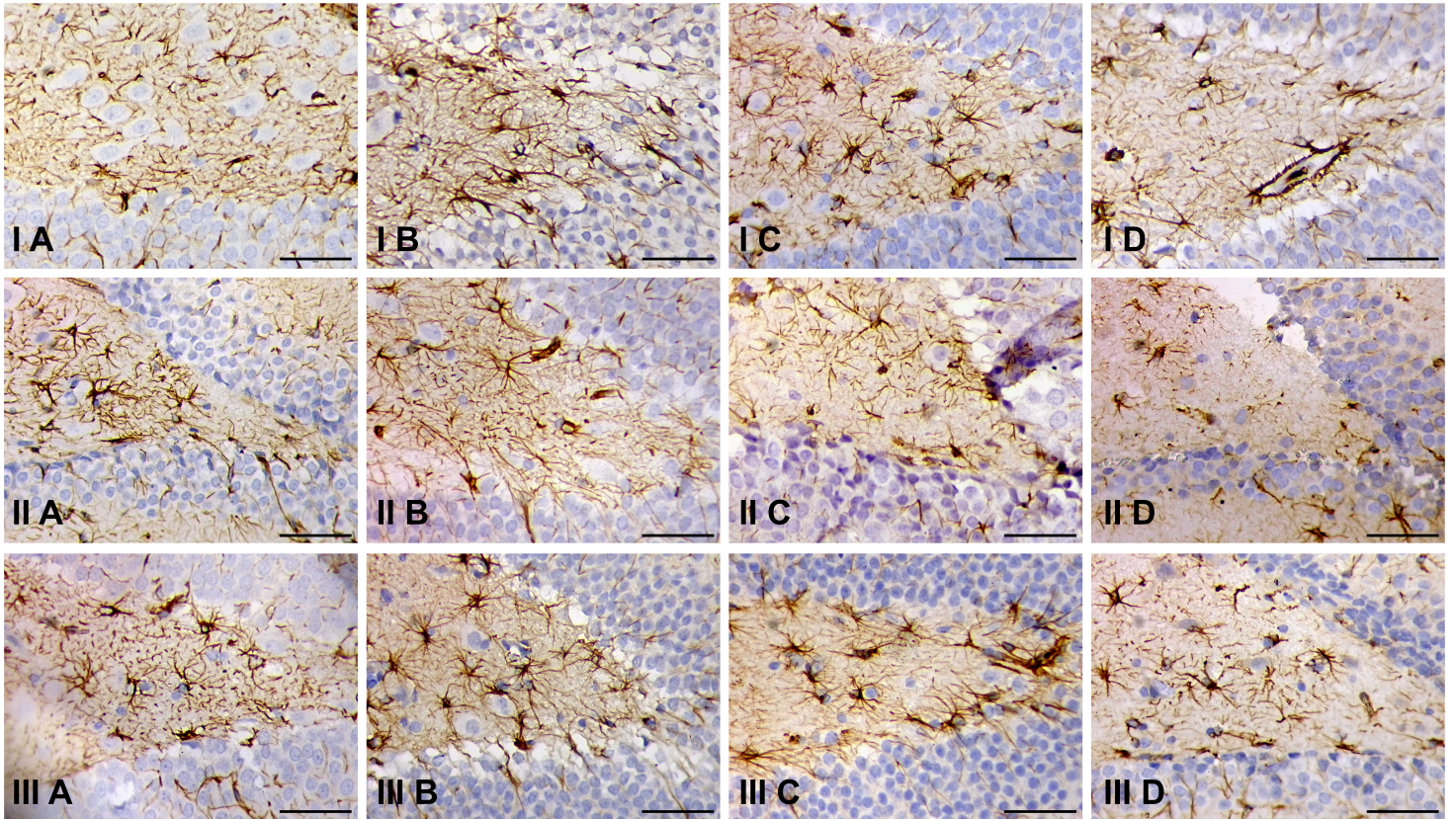


Figure 5

Reactive astrogliosis in the hippocampal Dentate Gyrus.

Representative photomicrographs of GFAP-positive astrocytes in the hilar and molecular layers of the dentate gyrus (n=5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III):** treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D):** time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). Scale bar = 50 μ m.

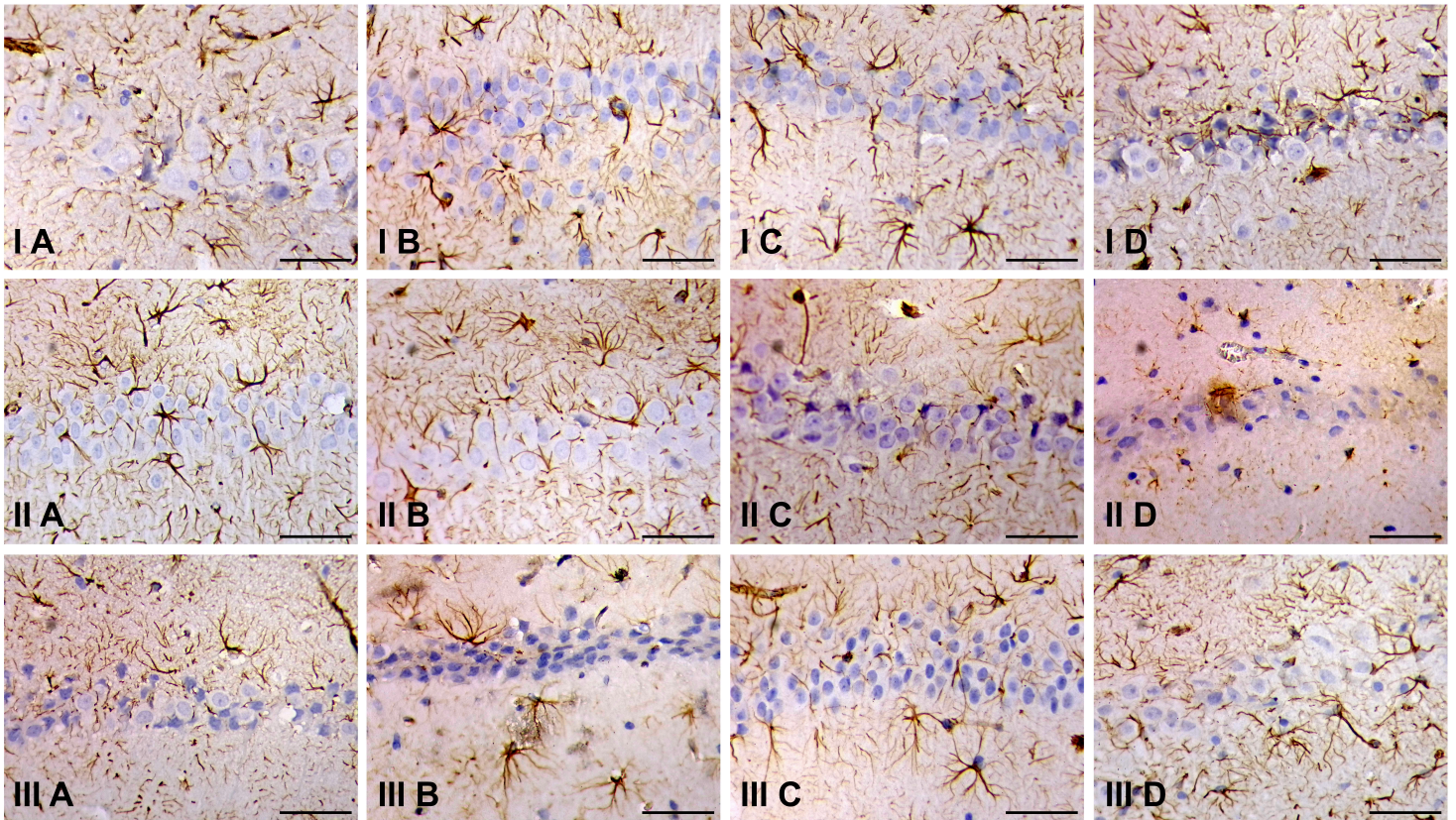


Figure 6

Reactive astrogliosis in the hippocampal CA1 region.

Representative photomicrographs of GFAP-positive astrocytes in the CA1 field (n=5 animals per group per time point; 3 non-overlapping fields per animal). **Rows (I–III):** treatment groups (I: Placebo; II: MPTD-01; III: BS34-20). **Columns (A–D):** time points post-TBI (A: Day 3; B: Day 7; C: Day 14; D: Day 21). The Placebo group exhibits persistent hypertrophy and clustering of astrocytes, which is attenuated in both treatment arms. Scale bar = 50 μ m.

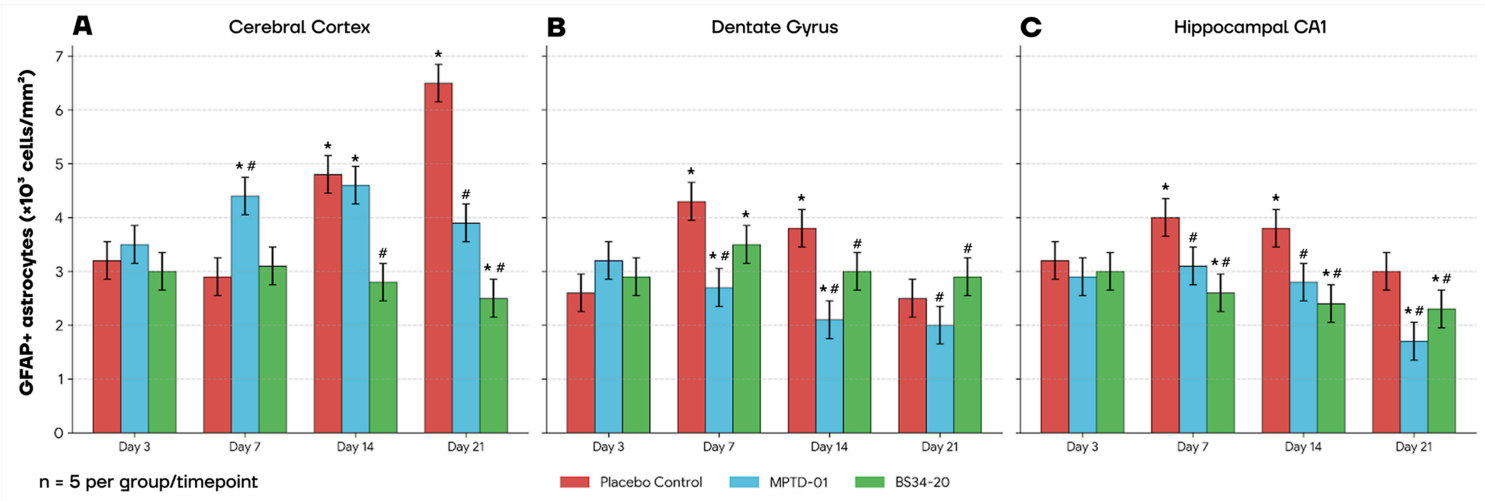


Figure 7

Quantitative analysis of astrocyte density.

Bar charts representing the density of GFAP-positive astrocytes ($\times 10^3$ cells/mm²) in the (A) Cerebral Cortex, (B) Dentate Gyrus, and (C) Hippocampal CA1 region across four time points (n=5 animals per group per time point; 3 fields per animal, 15 fields per group per region per time point). In the cortex, attenuation appeared earlier under BS34-20, whereas hippocampal regions (particularly the dentate gyrus) showed a greater reduction under MPTD-01. Data are presented as mean $\pm$ standard deviation. Note: y-axis values are scaled $\times 10^3$ (a bar reading 6 = 6000 cells/mm²). * p<0.05 vs Day 3 within group; # p<0.05 vs Placebo at the corresponding timepoint (Mann-Whitney U, Bonferroni-corrected).

Supplementary Files

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