

PKC activator therapeutic for mild traumatic brain injury in mice

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ABSTRACT

Traumatic brain injury (TBI) is a frequent consequence of vehicle, sport and war related injuries. More than 90% of TBI patients suffer mild injury (mTBI). However, the pathologies underlying the disease are poorly understood and treatment modalities are limited. We report here that in mice, the potent PKC activator bryostatin1 protects against mTBI induced learning and memory deficits and reduction in pre-synaptic synaptophysin and post-synaptic spinophylin immunostaining. An effective treatment has to start within the first 8 h after injury, and includes 5× i.p. injections over a period of 14 days. The treatment is dose dependent. Exploring the effects of the repeated bryostatin1 treatment on the processing of the amyloid precursor protein, we found that the treatment induced an increase in the putative α -secretase ADAM10 and a reduction in β -secretase activities. Both these effects could contribute towards a reduction in β -amyloid production. These results suggest that bryostatin1 protects against mTBI cognitive and synaptic sequela by rescuing synapses, which is possibly mediated by an increase in ADAM10 and a decrease in BACE1 activity. Since bryostatin1 has already been extensively used in clinical trials as an anti-cancer drug, its potential as a remedy for the short- and long-term TBI sequelae is quite promising.

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Introduction

Traumatic brain injury (TBI) is a wide spread public health concern, affecting a large percentage of the population, with young men being especially at high risk (Bruns and Hauser, 2003). In the U.S., 1.4 million people suffer from TBI each year. A recent survey of patients at the Walter Reed Brain Injury Center estimated TBI to be about a third of all war related injuries (Hoge et al., 2008).

Victims of TBI suffer from a broad range of short- and long-term physical, cognitive, and emotional impairments resulting from injury to the brain and from diffuse axonal damage. Most TBI patients (over 90%) suffer from mild trauma (mTBI), while only a small portion suffer from moderate to severe TBI (Thornhill et al., 2000; Moppett, 2007). Severe TBI patients usually suffer from either direct damage to the brain tissue, the blood brain barrier (BBB) and/or develop post-injury edema and thus are easy to diagnose. However, mild TBI (mTBI) patients are difficult to diagnose because they fail to show any clear morphological brain damage (Kibby and Long, 1996; Margulies, 2002). Still, mTBI patients frequently suffer from long-lasting cognitive and emotional difficulties termed “post-concussive syndrome” (Thornhill et al., 2000; van der et al., 1999). Despite the severe

impact of TBI on public health, the physiological and morphological pathologies underlying the disease are poorly understood. Since no effective pharmaceutical TBI therapies are available, current treatment is directed towards providing optimal intensive care management to aid the body's own regenerative processes (Johnston and Gupta, 2002; Narayan et al., 2002; Jones et al., 1994).

The initial mechanical impact of TBI triggers a complex set of events that causes secondary damage by activating endogenous auto-destructive biochemical processes which can last many years (Berger et al., 1999; Waxweiler et al., 1995; Assaf et al., 1999; Shapira et al., 1993; Gorrie et al., 2002; Graham et al., 2000; Albeni, 2001; Pierce et al., 1996, 1998; Reilly, 2001). One of TBI's secondary consequences is increased synthesis and expression of the amyloid precursor protein (APP) and its proteolytic product β A, which is the main constituent of plaques observed in brains of Alzheimer's disease (AD) patients (Gorrie et al., 2002; Pierce et al., 1996; Lewen et al., 1995; Murai et al., 1998; Ciallella et al., 2002; Smith et al., 2003; Roberts et al., 1991; Gentleman et al., 1997; Iwata et al., 2002; Smith et al., 1998; Uryu et al., 2002). APP is cleaved at distinct sites by three proteolytic enzymes (α -, β - and γ -secretase) to produce amyloidogenic and non-amyloidogenic pathways. The amyloidogenic pathway produces β A peptides by cleaving the APP by β (BACE1)- and γ -secretases (Nunan and Small, 2000; Esler and Wolfe, 2001). In the non-amyloidogenic pathway, α -secretase cleaves the APP within the sequence of β A, thus precluding its formation and releasing the neuroprotective segment sAPP α (Postina, 2008; Deuss et al., 2008; Nunan and Small, 2000; Kojro and Fahrenholz, 2005). Members of the ADAM (a disintegrin

Abbreviations: ADAM, a disintegrin and metalloproteases; mTBI, mild traumatic brain injury.

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and metalloproteases) family, especially ADAM10 and the less abundant ADAM17, are expressed in the brain and show α -secretase activity (Karkkainen et al., 2000; Asai et al., 2003).

PKC regulates many cell functions, including α -secretase activity, and thus attracts major interest as an anti-tumorigenic therapy (Goekjian and Jirousek, 2001; Basu, 1993; Blobel et al., 1994; Gescher, 1998). In the brain, PKC activates signal transduction pathways involved in many processes such as synaptic transmission and plasticity and activation of pro- and anti-apoptosis processes and is also involved in higher brain functions such as learning and memory (Bank et al., 1988; Alkon and Rasmussen, 1988; Craske et al., 2005; Nogues, 1997; Alkon et al., 2005; Bonini et al., 2007; Farrow et al., 2002; Lan et al., 2001; Majewski et al., 1997; Olds et al., 1989; Van der Zee et al., 1997). The non-tumorigenic PKC modulator bryostatin1 (a macrocyclic lactone derived from a marine bryozoan) binds to the PKC's C1 regulatory domain, as does the endogenous ligand diacylglycerol (Mutter and Wills, 2000; Paterson and Anderson, 2005; Amador et al., 2003; Newton, 1997; Mellor and Parker, 1998; Kortmansky and Schwartz, 2003). Both bryostatin and phorbol esters show temporal biphasic PKC modulation. Short-term exposure to these ligands promotes activation of PKC, whereas their prolonged exposure causes significant PKC down-regulation, presumably through upregulated proteasome proteolytic degradation (Lorenzo et al., 1999, 1997; Kortmansky and Schwartz, 2003). Bryostatin1 has been shown to have cognitive enhancing effects and promising efficacy in animal models of AD and stroke (Sun and Alkon, 2006; Kuzirian et al., 2006; Wang et al., 2008; Sun et al., 2009). These properties of bryostatin1 led us to the hypothesis that it will protect against the cognitive and cellular sequelae of TBI.

In this study we tested the ability of bryostatin1 to protect against the cognitive and cellular sequelae of mild TBI. The mTBI was delivered utilizing a modified weight drop model that closely mimics symptoms of human "post-concussive syndrome" (Zohar et al., 2003; Milman et al., 2005; Pan et al., 2003) and produces neuronal apoptosis (Tashlykov et al., 2007, 2009). Our results indicate that an extended treatment of 5 i.p. bryostatin1 injections administered over a period of 14 days, effectively protects against the cognitive and synaptic pathologies induced by the mTBI. This protection may be mediated by ADAM10 activation and BACE1 deactivation.

Materials and methods

Mild TBI induction

C57B6/J male mice, 3–4 months old, weighing 25–30 g (Jax Lab) were supplied with *ad libitum* chow and water and kept under 12/12 light cycle at 23 °C. Concussive closed-head mTBI was induced in the mice using the head trauma device previously described (Zohar et al., 2003). In brief, mice were lightly anesthetized by 0.5 ml isoflurane in a closed glass chamber, and then their heads placed under a device consist of an 80 cm long metal tube (inner diameter 13 mm). Each mouse was situated under the tube so that the impact on the skull (closed scalp) was administered anteriolaterally, just anterior to the right ear. During the injury, the head was supported by a sponge immobilization board allowing small head movements during the injury, analogous to those that occur in real life closed-head injury. The injury was induced by dropping a metal weight (30 g) through the metal tube to strike the skull. Immediately after the injury, mice were placed back in their cages for recovery. Injured mice did not exhibit any apnea or any patho-neurological effects that would indicate significant pain after the injury.

Drug treatment regime

Bryostatin1 (Biomol International, USA) was reconstituted using 100% ethanol and the appropriate dose was diluted into 200 μ l of PBS

and i.p. injected to the mice. The bryostatin was injected using two injection regimens; 1) a single 30 μ g/kg injection given one hour after the trauma. 2) Extended treatment of five injections administered over a period of 14 days (on days 3, 7, 10 and 14 after the first injection). The extended treatment was started one, eight or fourteen hours after the mTBI induction. In the treatment that started one hour after trauma two doses were given 20 or 30 μ g/kg. In the two other treatments only one dose of 30 μ g/kg was administered. Sham control mice were subjected to the same treatments but injected with PBS only.

Brain pharmacokinetics of bryostatin1

To test for a possible breach of the BBB and to elucidate the bryostatin1 tissue pharmacodynamics, we i.p. injected [$^{26-3}$ H]-labeled bryostatin1 to mice. [$^{26-3}$ H]-bryostatin1 was synthesized by oxidizing the 26-OH with CrO_3 followed by reduction with [3 H]- NaBH_4 as described (Lewin et al., 1991). The 3 H-bryostatin was purified by TLC (Silica Gel G) using hexane/acetone (70:30 v/v), and the purity and identity of the product was verified by UV spectroscopy. One μ Curie of labeled bryostatin1 (30 μ g/kg) was diluted into 200 μ l of PBS and injected immediately after the mTBI induction or to control mice. Tissue homogenate (25 μ l) was mixed with scintillation fluid and counted (Beckman LS3801 scintillation counter) using quench correction to calculate DPM. Mice blood, brain, and liver tissues were homogenized 1, 4, and 24 h post injection. Counts were normalized for tissue weight. To test for possible breach of the BBB counts from control and mTBI, brains were compared 1 h after the injury using students *t*-test, and the pharmacokinetics groups were compared using one way ANOVA (SigmaStat 3.5) ($N=10$ per time point).

Morris water maze

Effects of bryostatin1 treatment on the cognitive ability of brain injured mice were assessed using the Morris Water Maze (MWM). We tested for the effectiveness of bryostatin1 protection using either a single injection, or the 5 repeated i.p. injection paradigm described earlier in the following groups ($N=9$ in each group).

- 1 mTBI only,
- 2 mTBI followed by a single injection,
- 3 mTBI followed by the 5 repeated i.p. injection treatments in four sub groups: A – a dose of 20 μ g/kg starting 1 h post-trauma, B – a dose of 30 μ g/kg starting 1 h post-trauma, C – a dose of 30 μ g/kg starting 8 h post-trauma and D – a dose of 30 μ g/kg starting 14 h post-trauma.
- 4 Uninjured mice receiving only bryostatin1 injection in three subgroups: A – a single 30 μ g/kg injection, B – a 5 repeated i.p. injection treatment of 20 μ g/kg, and C – a 5 repeated i.p. injection treatment of 30 μ g/kg.
- 5 Sham control for each of the above treatments.

Experiments were performed in a featureless circular pool reduced in size (90 cm diameter and 60 cm deep) to accommodate for the learning deficits of the brain injured mice. A hidden clear circular Plexiglas platform was submerged in opaque water in the center of one of the quadrants. Mice were placed at a predetermined random starting position and allowed to swim for 80 s or until climbing onto the escape platform, where they were allowed to stay for 20 s. The swimming path was tracked by a computerized video system (2100 Plus tracking system, HVS) and mice escape latency and swim speed were recorded. Mice were trained at 6 trials per day, for 4 consecutive days. On day five, the platform was removed and mice were allowed to swim for 80 s, and the number of times mice crossed the missing platform position and time spent swimming in the missing platform quadrant was recorded (both measures are indicative of mice

memory retention abilities). Mouse performance in the acquisition and the memory phases of the MWM were analyzed using repeated measure one way ANOVA (SigmaStat 3.5).

Histology

Effects of bryostatin1 treatment on the injured brain synapses were assessed using the following groups; 1 – mTBI only, 2 – mTBI followed by the 5 repeated bryostatin i.p. injection treatments of a 30 µg/kg dose starting 1 h post-trauma, 3 – uninjured mice receiving only bryostatin1 i.p. injection treatment of a 30 µg/kg dose and Sham control for each of the above treatments ($N = 4$ in all groups). At the experimental end point, mice were anesthetized (choral hydrate 35 mg/kg) and their brains were trans-heart fixed by paraformaldehyde (4% in PBS) followed by overnight post-fixation and a 3x PBS wash. The right dorsal hippocampus (the injured side) was sectioned to 400 µm and then re-sectioned to 35 µm thickness using a vibratome. All incubations were performed on free-floating slices at room temperature, gently rocked in a shaker. Nonspecific binding was blocked using a PBS solution containing 15% horse serum and 0.05% Triton X-100 for 1 h. Following the blocking primary antibodies were incubated overnight in the blocking solution in a mixture of anti-synaptophysin monoclonal Ab (1:2000; Chemicon/Millipore) and anti-spinophilin polyclonal Ab (1:100; Upstate/Millipore). After a 3x PBS wash, slices were incubated for 3 h in biotinylated Ab (horse anti-mouse, 1:20; Vector Lab) in PBS and washed 3x in PBS. Alexa Fluor 488-streptavidin (1:100; Invitrogen) and Alexa Fluor 568 horse anti-rabbit IgG (1:200; Invitrogen) were conjugated to the biotinylated antibodies for 3 h. After 2x PBS wash (10 min) and a distilled water wash, sections were mounted in DAPI medium (Vectashield, Vector Lab) on glass slides, dried, and covered with cover glass.

Spinophilin and synaptophysin profiles were imaged utilizing a Zeiss Axiovert 200 M microscope equipped with 510 confocal scanning system (Thornwood, NY), using 63x Plan-APO Chromat oil immersion objective (1.4 NA). Confocal images (512x512 pixels of 63x63 µm² area) were collected in line scan mode (averaged 1:8) with 1.00 Airy unit pinhole from the stratum radiatum of the hippocampal CA1 area. Data was quantified using ImageJ (<http://rsb.info.nih.gov/ij>). The number of spinophilin and synaptophysin puncta (representing post-synaptic dendritic spines and pre-synaptic boutons respectively) was counted after photographic negative and background subtraction ($N = 4$ mice for each group, 4 slices per mice, and 4 random CA1 areas per slice.) Treatment effect on the number of pre-synaptic vesicles was estimated by measuring total Synaptophysin intensity per 63x63 µm² frame. Control data were set at 100%, and all other experimental conditions were defined as percent of controls. Data were analyzed using one way ANOVA followed by Dunnett, *t*-test (SigmaStat 3.5).

Enzymatic activity

Enzymatic activities of ADAM10, ADAM17 and BACE1 were measured following a single or the 5x i.p. repeated bryostatin1 (30 µg/kg) injection treatments in the following four groups ($N = 6$ in each group):

- 1 One hour after mTBI.
- 2 mTBI followed by the repeated injection treatment starting 1 h post-trauma.
- 3 5x i.p. repeated injection treatment of bryostatin1 only.
- 4 Sham control for each of the above groups.

Enzymatic activity was measured in six brain areas; frontal cortex, parietal and temporal cortices together and hippocampus of the injured area and the contra-lateral sides. Enzymatic activity was measured using fluorescence resonance energy transfer assays (FRET) (Jin et al., 2002) using a Spex Fluorolog-2 spectrofluorometer. The

FRET assays rely on two chromophores with excellent spectral overlap and an efficient energy transfer, bound together by a substrate of the selected enzyme. One chromophore serves as a donor and the other as a quencher absorbing all the donor fluorescence. Once the substrate is cleaved by the enzyme, the donor chromophore is released from the quench and can give fluorescence upon excitation. For each enzyme, data were analyzed using two way ANOVA (treatment by brain area) followed by pair-wise multiple comparisons procedures using the Holm-Sidak method (SigmaStat 3.5).

The enzymatic activity was measured using the following protocols:

ADAM 10 – homogenized brain areas were incubated for 60 min at 38 °C in a mix containing: ADAM10 substrate (10 µM) (Mca-PLAQAVDap(Dnp)RSSSR-NH₂, MCA is the donor and NH₂ is the quencher; Calbiochem), Tris-HCl (50 mM; pH 7.4), NaCl (25 mM), and 4% glycerol, final volume 100 µl. The reaction was stopped by cooling on ice, then diluted DDH₂O to 1.4 ml and fluorescence was measured (excitation – 319 nm; emission – 394 nm.)

ADAM17 – brain area homogenate was incubated for 20 min at 38 °C in a mix containing ADAM17 (TACE) substrate IV (10 µM) (Abz-LAQAVRSSSR-DPa, Abz is the donor and DPa is the quencher; Calbiochem), NaCl (25 mM), and 4% glycerol, final volume 100 µl. The reaction was stopped by cooling on ice, then diluted to by DDH₂O to 1.4 ml and fluorescence was measured (290 nm excitation and 415 nm emission).

BACE1 – activity was measured using a kit based on the BACE1 substrate 7-methoxycoumarin-4-acetyl[Asn⁶⁷⁰,Leu⁶⁷¹]-Amyloidβ/A4 protein precursor 770 fragment 667–676-(2,4-dinitrophenyl)-Lys- Lys-Arg amide trifluoroacetate (Sigma). Samples were incubated with 100 µM substrate in a total volume of 100 µl for 1 h at 38 °C, and fluorescence was measured (320 nm excitation and 405 nm emission.)

Results

The extended bryostatin1 treatment did not affect mouse weight, regardless of whether the mouse suffered mTBI or not (Control 29.8 ± 1.6, $n = 10$; mTBI 29.6 ± 0.6, $n = 10$; mTBI + bryostatin1 29.2 ± 0.6, $n = 16$; and bryostatin1 alone 29.2 ± 0.8, $n = 16$). Visual inspection of the injected mice did not reveal any external (mouth, nose or ear) or internal (around the eye, heart cavity, or intestine membranes) bleeding, and no abnormal cage behavior. The BBB was not breached by the mTBI as normalized counts of radioactive bryostatin injected i.p. to the mice did not differ between mTBI and normal brains measured 1 h after the injury (Fig. 1A). The brain to blood concentration ratio was consistently around 0.5 (1 h 0.5; 4 h 0.49; and 24 h 0.39; $p < 0.006$) and did not vary within the first 24 h of the injections (Fig. 1B).

Morris water maze

Our results indicate that repeated 5x i.p. bryostatin1 injections administered over a 2 week period completely protect against mTBI induced cognitive deficits, whereas one injection has no effect. Repeated injections of both doses used here (20 and 30 µg/kg) protected against the mTBI induced learning deficits ($p < 0.01$ and $p < 0.02$ accordingly; Figs. 2A and C). The higher injections dose (30 µg/kg) had also improved the learning of control uninjured mice (Fig. 2C; $p < 0.02$), while the lower dose had no effect on uninjured mice (Fig. 2A). Surprisingly, the lower 20 µg/kg dose administered to the mTBI group improved their acquisition of the learning task even beyond that of control mice ($p < 0.015$; Fig. 2A). Memory retention

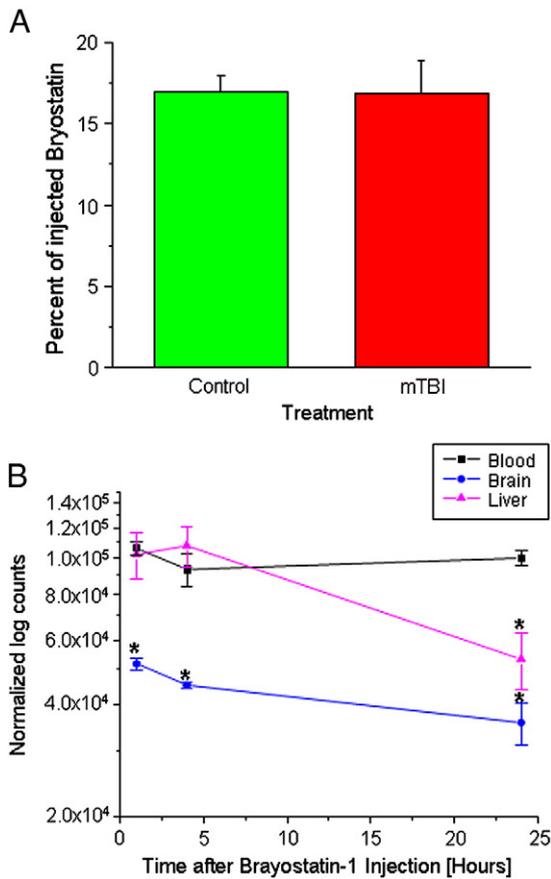


Fig. 1. The distribution of i.p. injected [²⁶⁻³H]-labeled bryostatin1 was used to elucidate its pharmacodynamics and breach of the BBB by the mTBI. Mouse blood, brain, and liver tissues were homogenized 1, 4, and 24 h post injection and their radiation counted ($N=10$ in each time point). Counts were normalized for tissue weight. Data presented as mean \pm S.E.M.A. The induced mTBI did not breach the BBB since the brain concentration of the i.p. injected bryostatin1 was not affected by the injury. B. Following i.p. injection the brain concentration of bryostatin1 was half of the blood's 1, 4 and 24 h after the injection. One way ANOVA, * $p<0.006$ compared to blood concentration.

was improved only in injured mice that received the 30 $\mu\text{g}/\text{kg}$ bryostatin1 treatment ($p<0.01$; Fig. 2D).

Our MWM results defined an effective time window for bryostatin1 to protect against mTBI. When the 5 \times i.p. repeated 30 $\mu\text{g}/\text{kg}$ bryostatin1 injections treatment started 8 h after the mTBI, it prevented both the mTBI induced learning and memory deficits ($p<0.05$ in both; Figs. 3A and B accordingly), while a treatment that started 14 h after the injury had no effect (Fig. 3).

Histology

Microanatomical examination preformed 14 days after the injury, revealed damage to both post- and pre-synaptic structures (Figs. 4A and E accordingly). At this time point, the number of spinophilin stained post-synaptic dendritic spines was reduced by 10% ($p<0.002$; Fig. 4I; for representative images see Figs. 4A and D), and the number of synaptophysin stained pre-synaptic vesicles was reduced by 15% ($p<0.006$; Fig. 4K). The number of synaptophysin puncta which estimates axonal boutons number was not affected by the trauma (Fig. 4J; for representative images see Figs. 4E and H). Repeated bryostatin treatment protected against both the post- and pre-synaptic damages. Following the treatment, both spinophilin and synaptophysin maintained normal distribution in the injured brains (Figs. 4I–K; for representative images see Figs. 4B and F). Repeated bryostatin1 treatment did not affect the distribution of these synaptic

markers in non-injured mice (Figs. 4C, G, and I–K). These data suggest a TBI-induced reduction of synaptic connections that is rescued by the bryostatin treatment.

Enzymatic activity

We have examined the effects of mTBI and the repeated 5 \times i.p. bryostatin1 treatment on the activity of three key enzymes involved in APP processing; the non-amyloidogenic putative alpha secretases ADAM10 and ADAM17, and the amyloidogenic BACE1 (Fig. 5 and Table 1). Enzymatic activity was a measure of fluorescence intensity in a FRET assay and thus is presented as normalized activity.

Two way ANOVA analyses of ADAM 10 activity showed significant interaction between treatments and brain areas ($F(3,15)=1.76$; $p<0.049$). However, only the treatments contributed significantly to the interaction with about 70% increase in its brain activity ($F(3)=17.98$; $p<0.001$) (Figs. 5A–B, data for P/T cortex not shown). The treatments caused a significant increase in the brain's ADAM10 activity (Holm-Sydk analyses, mTBI + bryostatin1 $p<0.01$; bryostatin1 only $p<0.009$; and mTBI only $p<0.013$), where bryostatin1 only treatment caused higher ADAM10 activity than mTBI only ($p<0.017$). Within the frontal cortex all treatments caused significantly higher activity than control, while within the hippocampus both bryostatin1 only and mTBI + bryostatin1 were different from control (in both $p<0.01$). In the injured side frontal cortex, the injury by itself caused brain activity of ADAM10 to become higher than all other treatments ($p<0.001$), probably due to auto-protective processes in response to the injury induced calcium autotoxicity.

ADAM17 activity was not affected by the bryostatin1 treatment, but its activity was slightly increased by the mTBI induction itself (Table 1). This increase was significant only by analyzing the whole brain data ($F(3)=9.64$; $p<0.003$).

The protective effect of the bryostatin1 treatment against mTBI was further demonstrated by its effect on the activity of the amyloidogenic BACE1 (Figs. 5C and D). Here, only the treatment affected BACE1 activity with no other interactions ($F(3)=16.06$; $p<0.001$). The mTBI caused a 10% increase in brain BACE1 activity ($p<0.025$). The bryostatin1 treatment protected against the injury induced increase in BACE1 activity and reduced it to normal ($P<0.017$). The bryostatin1 only treatment had a similar effect on sham control mice and reduced their BACE1 activity by 20% ($P<0.01$).

Discussion

TBI is a growing public health concern that affects mostly young men. Over 90% of TBI patients are defined as suffering from mild TBI, with symptoms that include short- and long-term cognitive and emotional sequelae. Remedies for TBI are lacking and treatment is restricted to the patients' own healing capabilities. In this study we developed a drug treatment for mild TBI in mice that protects against the cognitive and the synaptic sequela of the injury. Our drug of choice was the PKC activator bryostatin1. Bryostatin1 has been shown to have cognitive enhancing effects in normal and Alzheimer's disease animal models (Sun and Alkon, 2006; Kuzirian et al., 2006; Wang et al., 2008), and was determined to be safe for human use in extensive anti-cancer drug studies (Mutter and Wills, 2000; Kortmanský and Schwartz, 2003; Amador et al., 2003).

We demonstrated the treatment modalities of bryostatin1 by utilizing a closed-head mild TBI mouse model that mimics patients behavioral symptoms (Zohar et al., 2003, 2006; Milman et al., 2005; Pan et al., 2003), paralleled by necrotic and apoptotic neuronal death, this without BBB breach, edema or structural damages (Tashlykov et al., 2007, 2009; Tweedie et al., 2007). Similar to previous studies (Zhang et al., 1996), we found bryostatin1 to cross the BBB and its blood concentration to be higher than the brain's where it is slowly dissipating away. Further supporting previous finding (Pan et al.,

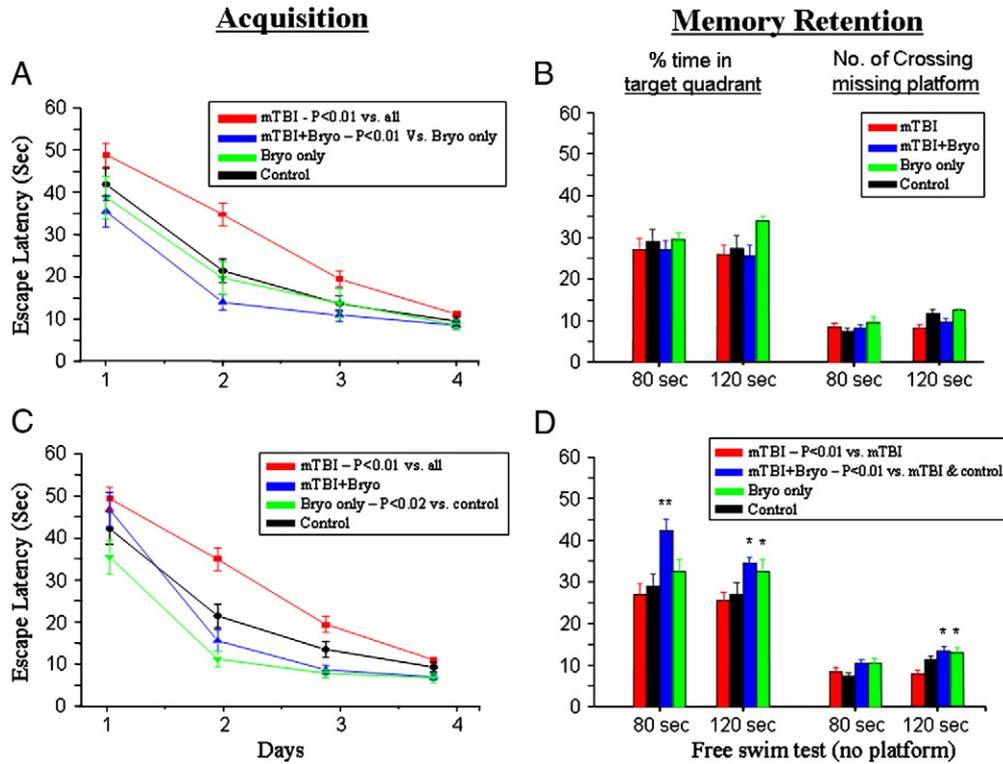


Fig. 2. Dose dependency of the anti-TBI bryostatin1 treatment. One hour after the mTBI was induced the mice received a 5× i.p. bryostatin1 injection treatment over a period of 14 days, in two injection doses 20 and 30 µg/kg (N = 9 in each group). One hour after the last injection of the series, the cognitive ability of the mice was tested in the MWM. Mice were tested for 4 days 6 times a day. On day 5 the platform was removed and the mice memory retention was tested. Both doses completely protects against mTBI induced cognitive deficits. Data was analyzed using repeated measure one way ANOVA and presented as mean ± S.E.M. Both doses protected the learning abilities of the injured mice (p < 0.01). A. Acquisition and B. Memory retention of mice that received a dose of 20 µg/kg bryostatin1 per injections. C. Acquisition and D. Memory retention of mice that received a dose of 30 µg/kg bryostatin1 per injection.

2003), we also found that the mTBI did not breach the BBB as its brain concentration in normal and mTBI mice showed similar bryostatin1 concentrations 1 h post-injury.

For bryostatin1 to protect against the mild TBI sequelae, a repeated injections treatment regime that starts within the first eight hours after the injury needs to be administrated. A treatment that started 14 h post-injury had no effect. Further, our results in-

dicates that bryostatin1 protection is not due to immediate acute effects of the last injection, but rather to the repeated application of the drug. This conclusion is based on the fact that the testing for the spatial learning of the mice always started one hour after the last bryostatin1 injection of the series regardless of dose, number of injections, or time elapsed for the treatment initiation. Nevertheless the dose, number of injections, and time elapsed for the treatment to

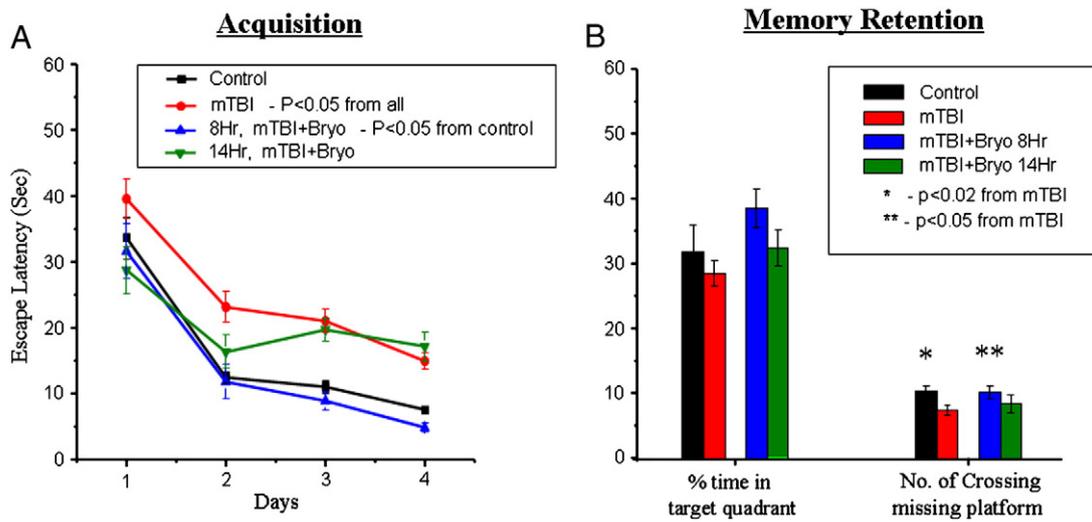


Fig. 3. Time frame for effective bryostatin1 treatment against TBI. To determine the effective time window for the bryostatin1 treatment for mTBI injured mice, the treatment started 8 or 14 h after the injury. (A. Acquisition and B. Memory retention). The cognitive ability of the mice was tested one hour after the last injection (30 µg/kg) of each series in the MWM. Data was analyzed using repeated measure one way ANOVA and presented as mean ± S.E.M (N = 9 in each group). The induced mTBI (red) decreased the acquisition (A, p < 0.05) and memory retention (B, p < 0.05) of the injured mice. Bryostatin1 treatment that started 8 h after the injury (blue) protected from the injury while the treatment that started 14 h after the injury (green) did not.

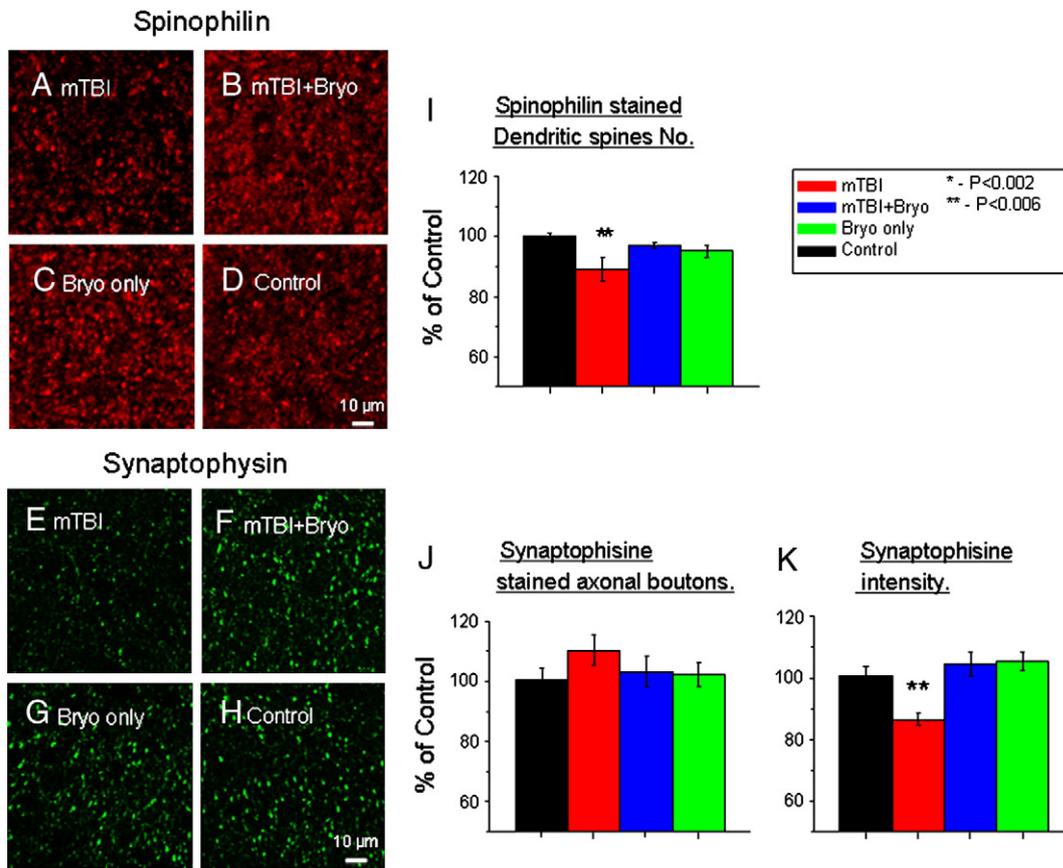


Fig. 4. Microstructural changes of CA1 hippocampal synapses after mTBI and the 14 days 5× i.p. bryostatin1 injection treatment that started 1 h post-trauma (30 µg/kg). Data was compared to control using student's *T* test, and presented S.E.M. ($N=4$ in each group). (A–H) Representative images. A. Spinophilin stain in mTBI mice, B. mTBI followed by bryostatin1 treatment, C. Bryostatin1 only and D. Normal brains. E. Synaptophysin stain in mTBI mice, F. mTBI followed by bryostatin1 treatment, G. Bryostatin1 only and H. Normal brains. I. Number of spinophilin stained post-synaptic structures. J. Number of synaptophysin1 stained pre-synaptic axonal boutons. K. Estimate of synaptophysin1 pre-synaptic vesicles number. * – different from control ($p<0.002$), and ** – different from control ($p<0.006$).

start all affected the outcome of the treatment. The need for repeated bryostatin1 application was further supported by the fact that even when given as close as 20 min after the injury, a single bryostatin1 injection had no effect on the mice. Bryostatin1 was previously reported to enhance the learning in several animal species (Alkon et al., 2005; Sun and Alkon, 2005; Wang et al., 2008). We have found that the chronic application of the higher dose used here enhanced both the learning and memory retention of normal uninjured mice.

To elucidate the mechanisms underlying bryostatin1 protection against brain injury, we have explored the treatments effects on the integrity of the injured synaptic structures and on the activity of the APP secretases. The processes of spatial learning and memory, especially of the MWM task, have long been postulated to depend on hippocampal synapses (Bird and Burgess, 2008; Tashlykov et al., 2009; Hongpaisan and Alkon, 2007). Therefore, we have concentrated our study on the pre- and post-synaptic microstructures of the hippocampal CA1 area. We found that the mTBI reduced the intensity of both the presynaptic synaptophysin and the post-synaptic spinophilin immuno-stains. These results suggest that the injury cause a reduction in the number of pre-synaptic spinophilin and post-synaptic synaptophysin stained structures. The mTBI induced reduction in synaptic microstructures correlated well with our previous demonstration of necrotic and apoptotic neuronal death that was associated with similar mTBI induction procedure in the mouse brain (Tashlykov et al., 2007, 2009). The repeated bryostatin1 treatment regimen rescued the damaged synaptic structures and retained their normal distribution. Taken together with the MWM, these results suggest that bryostatin1 protects against the mild TBI

sequelae by rescuing synapses from the injury induced damage, a process that may contribute to the cognitive recovery of the injured mice.

It is well documented that bryostatin1 activates PKC in a non-tumorigenic pathway (Mutter and Wills, 2000; Kortmansky and Schwartz, 2003; Amador et al., 2003). In a transgenic mouse model for Alzheimer's disease, bryostatin was found to reduce the load of soluble A β in the brain. Bryostatin also enhance the secretion of the α -secretase product sAPP α (Etcheberrigaray et al., 2004), a peptide that has many neuroprotective effects (Postina, 2008; Deuss et al., 2008). We thus have explored the mechanisms underlying the beneficial effects of bryostatin1 for brain trauma, by testing for the treatments effects on the activity of two of the putative α -secretases ADAM10 and ADAM17, and the activity of the amyloidogenic BACE1.

Our results indicate that bryostatin1 has complex effects on the APP secretases in mice brains. The repeated bryostatin1 treatment increased ADAM10 activity especially in the hippocampus, whether or not if they suffered brain injury, but had little effect on the activity of ADAM17. Interestingly, in the frontal cortex the brain injury by itself had also increased the activity of ADAM10, which suggests activation of auto-protective response in the injured brain. One of the sequelae of brain trauma is activation of excitotoxicity process that includes large calcium release. ADAM10 activity was shown to be associated with elevated cytoplasmic calcium levels (Nagano et al., 2004). It is possible that the injury induced increased calcium release, which in turn caused the increase in ADMA10 activity as a tertiary auto-protective event.

The mTBI induced an increase in BACE1 activity that lasted 14 days after injury. Here also, the repeated bryostatin1 treatment had a

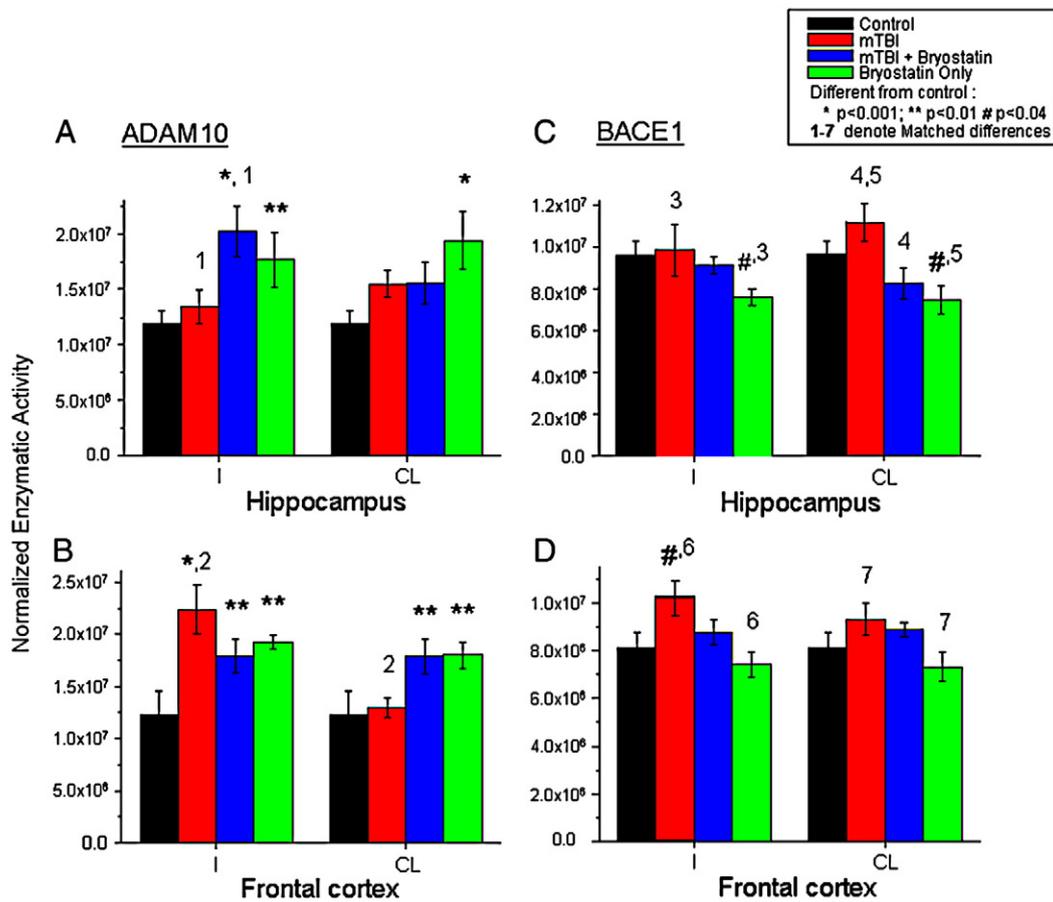


Fig. 5. Enzymatic activity of ADAM10 (A–B) and BACE1 (C–D) in brain areas of mice that suffered mTBI followed by a 14 days 5× i.p. bryostatin1 injections starting 1 h post-trauma. One hour after the last injection brain areas were extracted, dissected, homogenized and their enzymatic activity measured. Enzymatic activity was a measure of fluorescence intensity in a FRET assay and thus is presented as normalized activity. Data was analyzed using two way ANOVA followed by Holms-Sydk analyses, and is presented as mean ± S.E.M. (N=6 in each group). * – different from control (p<0.001); ** – different from control (p<0.01) and # – different from control (p<0.004). Numbers (1–7) denote matched differences: 1 – p<0.01; 2 – p<0.004; 3 – p<0.035; 4 – p<0.005; 5 – p<0.001; 6 – p<0.001; and 7 – p<0.016. Abbreviations: I – injured side, and CL – contra-lateral side.

beneficial effect and lowered BACE1 activity to normal. These effects of bryostatin1 in reducing β-secretase and increasing α-secretase activity may result in a reduction of amyloidogenic APP processing, leading to reduced βA burden in the mTBI brain. This adds to the beneficial effects of bryostatin1 as a possible protectant against one of the long-term devastating sequelae of TBI: the increased risk of developing Alzheimer's disease.

The results of the current study point to bryostatin1 as a promising drug for treating brain injury. Intra-peritoneal repeated bryostatin injection treatment that starts within the first eight hours after mild TBI prevents its induced cognitive deficits and synaptic damage. This protection may be mediated through increased ADAM10 and decreased BACE1 activity. Collectively, these effects may result in in-

creased net production of the neuroprotective sAPPα over the toxic βA.

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Table 1

Enzymatic activity of ADAM17 following mTBI+5× i.p. bryostatin1 treatment administrated over 14 days.

Area	Control	mTBI	mTBI + Bryo	Bryo
H	20.0 ± 0.8	22.9 ± 1.3	18.5 ± 2.9	21.4 ± 2.9
Hc	20.0 ± 0.8	21.0 ± 1.8	18.1 ± 2.4	20.8 ± 4.8
F	18.8 ± 1.5	22.3 ± 1.17	17.1 ± 2.6	18.3 ± 0.8
Fc	18.8 ± 1.5	20.8 ± 1.3	20.4 ± 1.2	22.1 ± 2.3
P/T	19.1 ± 1.1	24.1 ± 1.5	18.2 ± 1.5	20.9 ± 1.7
P/Tc	19.1 ± 1.1	20.6 ± 1.1	19.0 ± 2.3	19.8 ± 1.2

Numbers represent average ± S.E.M. × 10⁶ normalized enzymatic activities. Abbreviations: Bryo – bryostatin1 (30 µg/kg), F – frontal, Fc – contra-lateral frontal cortex, P/T – parietal and temporal cortices, and P/Tc – contra-lateral parietal and temporal cortices.

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