



Progesterone and vitamin D: Improvement after traumatic brain injury in middle-aged rats



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ABSTRACT

Progesterone (PROG) and vitamin D hormone (VDH) have both shown promise in treating traumatic brain injury (TBI). Both modulate apoptosis, inflammation, oxidative stress, and excitotoxicity. We investigated whether 21 days of VDH deficiency would alter cognitive behavior after TBI and whether combined PROG and VDH would improve behavioral and morphological outcomes more than either hormone alone in VDH-deficient middle-aged rats given bilateral contusions of the medial frontal cortex. PROG (16 mg/kg) and VDH (5 µg/kg) were injected intraperitoneally 1 h post-injury. Eight additional doses of PROG were injected subcutaneously over 7 days post-injury. VDH deficiency itself did not significantly reduce baseline behavioral functions or aggravate impaired cognitive outcomes. Combination therapy showed moderate improvement in preserving spatial and reference memory but was not significantly better than PROG monotherapy. However, combination therapy significantly reduced neuronal loss and the proliferation of reactive astrocytes, and showed better efficacy compared to VDH or PROG alone in preventing MAP-2 degradation. VDH + PROG combination therapy may attenuate some of the potential long-term, subtle, pathophysiological consequences of brain injury in older subjects.

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Introduction

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in the US and worldwide. In addition to the complexity of the injury mechanisms, factors such as age, gender, alcohol and drug use, metabolic state, comorbidities, combined trauma, and genetics can also influence the effects of an intervention following TBI (Maas et al., 2007). The complexity of TBI provides a compelling reason for the investigation of combination therapies (Margulies and Hicks, 2009). Because treatment with a single agent designed to target one pathological event often does not address the varied mechanisms and associated systemic pathologies of TBI, we asked whether combination treatment might be a more successful strategy.

Progesterone (PROG) and vitamin D (VDH) both function as potent hormones, but have been shown to exert their neuroprotective effects through a number of different pathways (Stein and Cechic, 2011). It is well documented that PROG provides neuroprotection in a variety of experimental brain injury models and neurodegenerative conditions such as TBI (Sarkaki et al., 2013), stroke (Liu et al., 2012), Alzheimer's disease (Carroll et al., 2010), and epilepsy (Velíšková and Desantis, 2013).

Although PROG has been shown in many experiments to be an effective therapeutic for TBI, we hypothesize that VDH, with its own

neuroprotective effects in a number of injury models, might enhance neuroprotection and repair when combined with PROG through multiple mechanisms of action, and more so in older, VDH-deficient subjects with brain injuries (Briones and Darwish, 2012; Buell and Dawson-Hughes, 2010; Cechic et al., 2011; Lin et al., 2005). Recently we showed that *in vitro* VDH + PROG was more effective at protecting primary cortical neurons from cytotoxic insult than either compound alone, and that the infarct size of middle cerebral artery occlusion *in vivo* was reduced (Atif et al., 2009, 2013). We also demonstrated that combination treatment (with a low dose of VDH) was more effective than PROG alone in reducing cognitive impairment in young adult (~2.5 months old) VDH-sufficient rats after TBI (Hua et al., 2012). Another experiment tested the combination in VDH-deficient senescent rats (22 months old) (Cechic et al., 2011) and revealed that VDH deficiency reduces the benefits of PROG treatment in the acute phase after brain injury in these animals.

In addition to playing a critical role in calcium homeostasis and bone health, VDH exerts multiple effects on the cardiovascular and central nervous systems (Deluca et al., 2013; Valtueña et al., 2013). VDH deficiency has been associated with neurological disorders such as Alzheimer's disease (Luckhaus et al., 2009), Parkinson's disease (Evatt et al., 2011); stroke (Kojima et al., 2012; Sun et al., 2012), and some neuropsychiatric diseases (Eyles et al., 2013). It was recently suggested that VDH deficiency can exacerbate injury and its outcome (Balden et al., 2012; Solomon et al., 2011), as well as reduce the beneficial effects of other treatments for TBI (Cechic et al., 2011; Chatterjee, 2001; McCann and Ames, 2008). Given these findings, it is especially important to

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address whether such a deficiency and its treatment could affect functional outcomes in pre-senescent brain-injured subjects.

In some of our previous work with short-term survival, [Cekic et al. \(2011\)](#), we examined only a limited range of behavioral activities for several days after the injury. Here we extend the findings of our previous study of PROG efficacy in “middle-aged” (~13–14-month-old) brain-injured rats fed a pre-injury, VDH-deficient diet and tested over a longer period of time post-injury on cognitive and sensory tasks. We sought to determine whether a similar combination therapy would have the same benefits in older subjects as in our young adult rats (~2.5 months) ([Hua et al., 2012](#)). We looked at: (1) whether a 3-week period of VDH deficiency exacerbates sub-acute to chronic behavioral impairment compared to a normal diet; (2) whether PROG + VDH combination therapy has better efficacy than either compound alone in improving longer-term cognitive and sensory behavioral and histological outcomes in older, but not yet senescent rats with frontal cortex injury; and (3) whether combination therapy would show different functional and morphological efficacy in VDH-sufficient and deficient rats.

Materials and methods

Animals

One hundred twenty-eight 13-month-old male Sprague-Dawley rats (Harlan Laboratories, Tampa, FL) weighing 500–600 g at the time of injury were obtained and housed individually with unlimited access to food and water. Rats were placed under a 12:12-h reverse light-dark cycle (0800–2000 h) so that behavioral testing could occur during their active phase ([Paulson and Robinson, 1994](#)). This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), Protocol #2001032.

Diet and serum VDH levels

After a 3-day acclimation to the colony, the rats were randomly assigned to two diet cohorts: VDH-sufficient (VitD-S) and VDH-deficient (VitD-D). The VitD-S cohort was given standard rat chow used in our animal care facility (Rodent Diet 5001, LabDiet®, St. Louis, MO). The VitD-D cohort was fed a VDH-null version of the same diet (Diet5A4Y, modified 5001 with no D3, TestDiet®, Richmond, IN) and maintained on the diet for at least 21 days prior to surgery. Although 8 days has been shown to be sufficient to induce a circulating 25OHD₃ level consistent with deficiency ([Narayanan et al., 2004](#)), we extended this interval to allow any sequelae of the VitD-D to become salient and to provide a better model for the human population. For this same reason, our null diet was not altered in any other way, and the VitD-D cohorts were maintained on it until euthanized.

Serum 25OHD₃ is a standard marker for determining VDH status ([Heaney, 2004](#); [Holick, 2005](#)). Five rats from each diet cohort were used to detect serum VDH levels after 21 days on the null diet. Blood (1–1.5 mL) was drawn directly from the right ventricle of the heart with a 21G needle after the rats were rendered unconscious with isoflurane anesthesia. Whole blood was left to coagulate for 30 min at RT and was then centrifuged for 5 min at 1000 ×g. The resulting serum was collected and stored at –80 °C. VDH levels were determined with a 25OHD₃ RIA prepared kit (DiaSorin, Stillwater, MN; catalog no. 68100E).

Surgery and treatment

Rats in each diet cohort were randomly assigned to the following treatment groups: sham-vehicle (S-Sham, D-Sham; n = 12/group), lesion-vehicle (S-Veh, D-Veh; n = 12/group), lesion with 16 mg/kg PROG treatment (S-PROG, D-PROG; n = 11/group), lesion with 5 µg/kg

VDH treatment (S-VDH, D-VDH; n = 12/group), and lesion with PROG and VDH combination treatment (S-Comb, D-Comb; n = 12/group).

The animals were anesthetized with isoflurane (5.0% induction, 2.0–2.5% maintenance) and surgery was performed using aseptic techniques as previously described ([Cutler et al., 2007](#)). A 6-mm diameter mid-sagittal bilateral craniotomy was performed 3 mm anterior to bregma and a cortical contusion injury (CCI) was produced in the medial frontal cortex (MFC) by an electromagnetic cortical contusion device (5-mm diameter impactor tip) with an impact velocity of 2.25 m/s, dwelling time of 500 ms, to a depth of 3.5 mm ventral to bregma. The incision was sutured closed after all bleeding had stopped. In the sham group, there was no impact and the incisions were sutured closed after comparable time under anesthesia.

A dose-response study has shown that, compared to 32 mg/kg, 8 and 16 mg/kg PROG led to better behavioral performance following cortical injury in rats ([Goss et al., 2003](#)). In other studies ([Cekic et al., 2011](#); [Cutler et al., 2007](#); [Hua et al., 2012](#); [Wali et al., 2011](#)), we found that 16 mg/kg PROG is effective in the acute phase after TBI, sparing spatial learning and memory in both adult and aged rats.

In the present study, a single bolus dose of VDH (5 µg/kg) was given 1 h after CCI injury either alone or in combination with PROG. In our previous papers ([Cekic et al., 2011](#); [Cutler et al., 2007](#)), 5 µg/kg VDH was found to be very effective in the acute phase of TBI in aged rats. This choice of a single higher VDH dose was also based on evidence showing that a single megadose can alter VDH status for an extended period ([Diamond et al., 2005](#)).

PROG was administered in 2-hydroxypropyl-β-cyclodextrin solution (HBC, 22.5% w/v solution in dH₂O) as the solvent as well as the vehicle. VDH (1, 25(OH)₂ D₃) was dissolved in 95% ethanol and stored at –80 °C. On the day of surgery, the stock VDH was diluted in HBC and sterile water, resulting in a 22.5% HBC with 2% ethanol solution. All sham and vehicle groups received a volume of vehicle equal to the volume of the PROG and VDH dose. PROG was first administered intraperitoneally 1 h post-injury ([Cutler et al., 2007](#)). This was followed by subcutaneous injections at 6, 24, 48, 72, 96, 120, 144 and 168 h post-contusion. Tapering was introduced as halved dosages over the last 2 days of treatment to avoid PROG withdrawal symptoms ([Cutler et al., 2006](#)). A single dose of VDH was administered intraperitoneally 1 h post-injury to the animals in the VDH and combined therapy groups.

Morris water maze (MWM)

Testing methods from our previous work were used with minor modification ([Wali et al., 2011](#)). Briefly, water maze testing began 10 days after TBI or sham surgery. Rats received 2 trials per day over 7 consecutive days of testing. The platform remained in the same position relative to both the maze and the room throughout testing. Two different starting locations were allocated to the two trials. On each trial the rats swam individually in the maze until they found and climbed onto the platform, where they were permitted to remain for 20 s. Rats that left the platform before 20 s had elapsed were returned to it promptly by the experimenter. If a rat did not locate the platform after 90 s, it was guided to the platform, where it remained for the 20-s period. Rats removed from the platform were then placed in a holding cage near a heater for 5 min before the second trial began. For each trial, a computer connected to a video tracking system detected the contrast of the marked, dark head of each rat against the white opaque water, and latency to reach the platform was recorded. The first trial of each day represents long-term memory because animals have 24 h respite after the previous training session ([Baldi et al., 2005](#)). We used the second trial on each training day as a measure of short-term memory because of the short time period (5 min) between trials.

On the 18th day after surgery the platform was removed and a probe trial was conducted to assess how well the rats remembered the platform location. This trial has dual functionality: it tests long-term

memory and reduces the frequency of finding the platform by accident (Baldi et al., 2005). Each rat was placed in the water in the same location as in the first trial and allowed to swim for 60 s. The percent of time each rat spent in the platform quadrant was recorded.

Somatosensory neglect of the forepaws

Testing was conducted under red light in a quiet environment 3 days before surgery (baseline) and at 3, 8, 15 and 21 days post-surgery. Circular adhesive labels (1.3-cm diameter) were placed on the ventral left forepaw and the rat was placed in a clear Plexiglas testing box. The rat's latency to contact the sticker and then remove it with its mouth was recorded, with a test duration of 4 min. The testing box was cleaned with 70% ethanol and dried between trials.

Forelimb grip strength test

Testing was conducted under red light in a quiet environment 3 days before surgery (baseline) and at 3, 8, 15 and 21 days post-surgery with a grip-strength meter (Columbus Instruments, Columbus, OH). We recorded scores (in Newtons) of 3 successive trials for each animal and took the average for analysis.

Locomotor activity testing

Locomotor activity testing was done under red light in a quiet environment 3 days (baseline) before surgery and at 3, 8, 15 and 21 days post-surgery. Two animals were tested simultaneously in individual boxes using the Digiscan Activity Monitoring System (AccuScan Instruments Inc., Columbus, OH). Rats were placed in the center of the activity box and the recording apparatus was turned on. After 5 min the computer stopped recording movements and animals were returned to their home cages. The activity boxes were cleaned with 70% ethanol and dried between trials.

Tissue preparation

Twenty-two days after surgery, rats were exposed to 5% isoflurane for 5 min. Once they were completely anesthetized, blood (1–1.5 mL) was drawn directly from the right ventricle of the heart and the animals were then transcardially perfused with 0.05 M phosphate-buffered saline (PBS, 200 ml) and fixed with 10% formalin buffer (pH = 7.4, 250 ml). Brains were then extracted from the skull, post-fixed for 24 h at 4 °C in the same fixative, and placed in increasing amounts of 0.1 M phosphate-buffered sucrose (10%, 20%, 30%) each day. Finally, brains were covered with cryoprotectant, frozen using 2-methyl-butane chilled on dry ice, placed in a cryostat, and cut into 20- μ m coronal sections. Sections were stored in cryoprotectant at –80 °C.

Evaluation of necrotic cavity

The necrotic cavity was evaluated as previously described with slight modification (Wali et al., 2011). Slides from 6 levels (every 1 mm from 5 mm anterior to bregma to bregma, Fig. 3A) were selected for Nissl staining (Hua et al., 2012; Wali et al., 2011). After air drying, the slides were washed with PBS (PH = 7.4), stained in 0.1% cresyl violet solution for 5–10 min at 37 °C, rinsed quickly in distilled water, differentiated in 95% ethyl alcohol, dehydrated in 100% alcohol 2 \times 5 min, cleared in xylene 2 \times 5 min, and mounted with a permanent mounting medium. The stained slides were scanned into a computer and saved as digital images using PathScan Enabler IV (Meyer Instruments, Houston, TX). The necrotic area (A) in each section was traced using Image-J software from the NIH. The results were presented as volume (mm³) of tissue lost, calculated by the following formula: $1\text{ mm} \times (A1 + A2)/2 + 1\text{ mm} \times (A2 + A3)/2 + 1\text{ mm} \times (A3 + A4)/2 + 1\text{ mm} \times (A4 + A5)/$

$2 + 1\text{ mm} \times (A5 + A6)/2$ (1 mm is the interval between the sections).

Fluoro-jade C (F-Jc) staining

F-Jc was used to assess delayed loss of degenerating neurons in the brain (Ehara and Ueda, 2009). We selected slides at 3 mm anterior to bregma. Procedures were performed as previously described (Ehara and Ueda, 2009). After tissue-mounted slides had been air-dried for 2 h, they were washed 3 times in PBS and then immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min, rinsed for 2 min with 70% ethanol, and then incubated in 0.06% potassium permanganate solution for 10 min. After rinsing with ddH₂O, the slide-mounted tissue samples were stained with 0.0001% of F-Jc solution for 10 min, rinsed in distilled water 3 \times 1 min, and covered with mounting medium with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). The brain tissue was examined under a fluorescence microscope and pictures were taken of the peri-lesion area by Image-Plus software. The percentage of F-Jc positive cells was counted and calculated.

Double-immunofluorescence staining for GFAP and MAP-2

Slides at 3 mm anterior to bregma were labeled for both glial fibrillary acidic protein (GFAP) and microtubule-associated protein-2 (MAP-2). The slides were air-dried for 2 h, washed 3 times in PBS, incubated with blocking buffer (1% bovine serum albumin (BSA) in PBS) at room temperature (RT) for 30 min, and then co-incubated with rabbit anti-rat GFAP primary antibody (AB5804, Millipore, Billerica, MA) and mouse anti-rat MAP-2 primary antibody (SC32791, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT. Slides were washed 3 times in PBS, and incubated with goat anti-rabbit IgG (H + L) labeled with Alexa Fluor 594 F(ab') fragment (A-11071, Molecular Probes, Carlsbad, CA) and goat anti-mouse IgG (H + L) labeled with Alexa Fluor 488 F(ab') fragment (A-11001, Molecular Probes) for 1 h at RT, then rinsed with PBS for 3 \times 5 min and covered with the mounting medium DAPI. The tissue was examined with a fluorescence microscope and both sides of the cerebral cortex were photographed proximal to the lesion site and then in sub-cortex ventral to the injury (Fig. 7A) with Image-Plus software. GFAP-positive and MAP-2-positive cells were measured and calculated as a percentage of the positive area/total area by Image-J software.

Statistics

All results are expressed as the mean \pm SE. Statistical significance was set at $p < 0.05$. Mixed-model and Tukey *post-hoc* comparisons were used to analyze MWM testing, locomotor activity, grip strength and somatosensory neglect of the forepaws to determine whether there were any 1) significant differences in the longitudinal outcomes across treatment and diet groups, or 2) significant outcomes over time. Two-way ANOVA and Tukey *post-hoc* comparisons were used for MWM probe trial analysis, necrotic cavity size, F-Jc positive cells and GFAP/MAP-2 positive area analyses, and serum VDH. SAS 9.3 and Sigmaplot 11.0 were used for data analyses.

Results

Weight

In the VitD-S cohort, the weights of the rats in all the injury groups decreased significantly in the first three days after surgery (Fig. 1A). The weight of the Comb group increased steadily from the seventh day after surgery, but did not reach pre-injury levels (Fig. 1A). The weight of the Veh and PROG-alone groups stabilized from the seventh day after surgery. The Sham group had significantly lower loss of weight

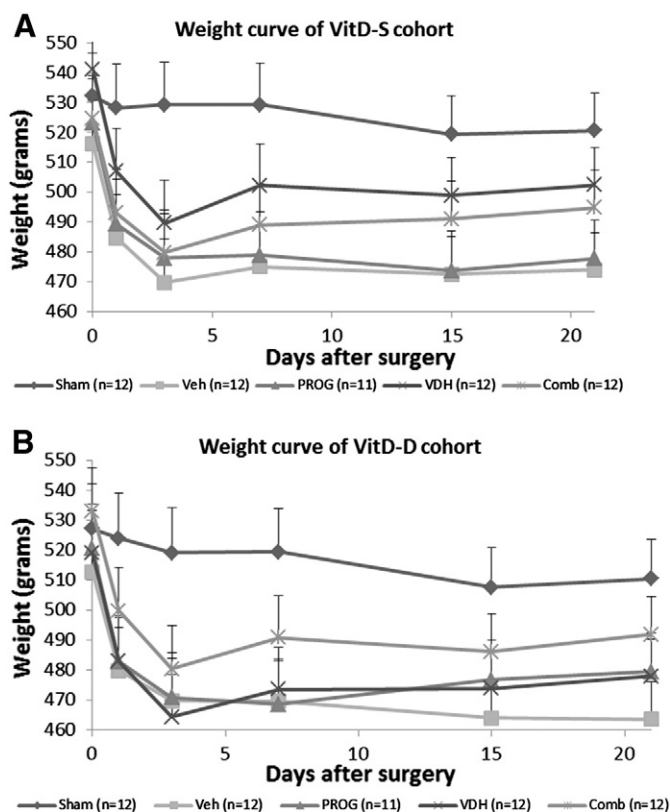


Fig. 1. Changes in body weight with both diets. A. Weight curve with VitD-S diet. B. Weight curve with VitD-D diet. Body weights were measured on the day of surgery (day 0), and 1, 3, 7, 15, and 22 days post-surgery. Body weights decreased significantly after surgery except in Sham with both diets. There was no significant difference across the two diets.

over time compared to the Veh ($p < 0.01$), PROG ($p < 0.01$) and VDH ($p < 0.05$) groups. There were no significant differences in weight loss between the Comb group and Veh. In the VitD-D cohort, the weight-change pattern was almost the same as that of the VitD-S cohort, except that the weight of the PROG group increased steadily from the seventh day after surgery (Fig. 1B). The weights of all the groups with the VitD-D diet did not differ significantly from those from the VitD-S cohort.

Serum VDH levels

Assays showed that rats fed with a VitD-D diet for 21 days had significantly lower average serum VDH levels than those on a VitD-S diet

($p < 0.01$, Fig. 2A). Serum VDH Levels remained low at 22 days post-surgery among the VitD-D diet cohort compared to VitD-S ($p < 0.01$, Fig. 2B). There was no significant difference among treatment groups with either diet.

Locomotor activity

In the VitD-S cohort, the distance traveled for all the treatment groups decreased by the third day after surgery and increased thereafter (Fig. 3A). The changes in distance traveled on different days were significant in every treatment group. The travel distance of the VitD-D cohort followed the same pattern as that of the VitD-S cohort (Fig. 3B). The resting time of the Sham group with VitD-S increased steadily and reached its peak on the fifteenth day after surgery, followed by a gradual decline (Fig. 3C). The resting time of all the other treatment groups with VitD-S increased on the third day after surgery and decreased thereafter (Fig. 3D). The resting time of Sham, Veh, VDH, and Comb groups with VitD-D had the same pattern of change as that of their counterparts on the normal vitamin D diet. The resting time of PROG-treated VitD-D animals increased on the third day after surgery and decreased thereafter. However, the resting time increased again from the seventh day post-surgery. The changes in the two parameters on different days were significant in every treatment group with both diets. However, there was no significant difference in distance traveled or resting time across the treatment groups and diet cohorts (Table 1A). In this study, locomotor activity was not predictive of combination treatment or dietary effectiveness.

Sticky task

In the intact rats, the latency to notice and remove the sticker under either of the diets decreased slightly after surgery and then leveled off (Figs. 4A, B). The removal latency of other treatment groups with either diet increased, reached a peak at the third day after surgery and then decreased (Figs. 4A, B). There was no significant difference in latency to notice the sticker across diet groups (Table 1A). The changes in latency on different days of testing were significant for every treatment group. The changing patterns in latency to notice the sticker were also significantly different across the 5 treatment groups. Pairwise comparisons revealed that the latency of the Sham group was significantly better compared to the other groups ($p < 0.05$, Table 1B). There were no significant differences among the Comb, PROG, and VDH treatment groups fed either diet (Table 1B). This measure was not sensitive to diet or the effects of combination or monotherapy treatment.

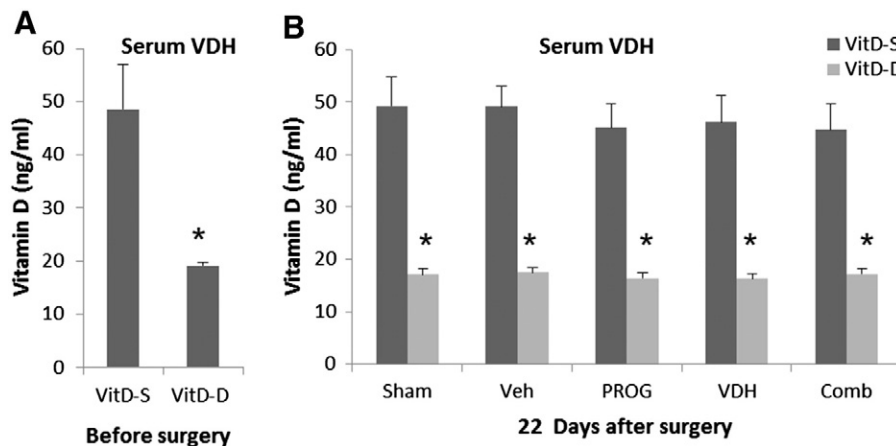


Fig. 2. Serum VDH level. A. Serum VDH level after 21 days' diet conditioning. Rats fed a VitD-D diet had significantly lower serum VDH levels than those fed the VitD-S diet ($p < 0.01$, $n = 5$). B. Serum VDH levels of both diet cohorts at 22 days post-surgery. Serum VDH levels remained low at 22 days post-surgery within the VitD-D diet cohort compared to the VitD-S cohort ($p < 0.01$, $n = 5$). * compared with VitD-S, $p < 0.05$.

Grip strength task

The grip strength of Sham, Veh, VDH, and Comb groups with VitD-S decreased by the third day after surgery and increased thereafter (Fig. 4C). The grip strength of PROG with VitD-S increased over time after injury. The grip strength of Sham with VitD-D did not change significantly from its original level (Fig. 4D). The grip strength of Veh, PROG, and Comb with VitD-D increased steadily after the surgery. The grip strength of VDH with VitD-D decreased by the third day after surgery and increased afterward. Changes in grip strength over time were significant for every treatment group (Table 1A). There were no significant differences in grip strength across the 5 treatment groups or either of the diets. Thus, grip strength in this study was not sensitive to diet or the effects of combination treatment or monotherapy.

Water maze

There were two MWM trials on each testing day, representing long- and short-term memory respectively (Baldi et al., 2005). Mixed-model analysis revealed significant differences in MWM latency across the 5 treatment groups with both VitD-S and VitD-D diets (Figs. 5A, B; Table 2A) in trial 1. The changes in latency over time were also significant (Table 2A). Under either diet, the latency of the Sham group to reach the platform was significantly lower than that of the other 4 treatment groups throughout testing for the first trial on each day ($p < 0.01$, Table 2B). The overall MWM latency of the Comb group decreased significantly faster than that of the Veh group for both diets ($p = 0.01$, Table 2B). The results of trial 2 were consistent with trial 1 in that they demonstrated that combination therapy improved acquisition of the spatial learning task compared to Veh for both long- and short-term memory ($p < 0.01$, Figs. 5C, D, Table 2).

There was no significant difference in probe test results across diet cohorts, but there was a significant difference across treatment groups (Fig. 5E). Pairwise comparison among treatment groups showed that time spent in the platform quadrant for the Veh-treated rats was significantly shorter than that of the PROG ($p < 0.05$), Comb ($p < 0.05$) and Sham ($p < 0.01$) groups with both diets. However, there was no significant difference between the Comb and PROG groups on this measure of long-term memory.

These findings can be interpreted to indicate that CCI significantly impaired spatial learning and memory function across all groups. Compared to Veh, PROG showed improvement in the probe test, and Comb therapy showed significant improvement in both training and the probe tests.

Necrotic cavity

At 22 days after injury, there were substantial necrotic cavities in the brains of all the CCI groups (Fig. 6A). While there was a trend of reduced cavity size with the combination therapy, there were no significant differences across the diet and treatment groups (Fig. 6B). The short course of hormone treatments did not significantly decrease the necrotic cavity caused by CCI.

F-Jc staining

TBI resulted in neurodegeneration as indicated by a significant increase in F-Jc positive cells. These cells were seen primarily on the edges of the necrotic cavity. There were no significant differences between the Veh, PROG, VDH, or combination-treated rats on this measure (Fig. 6C).

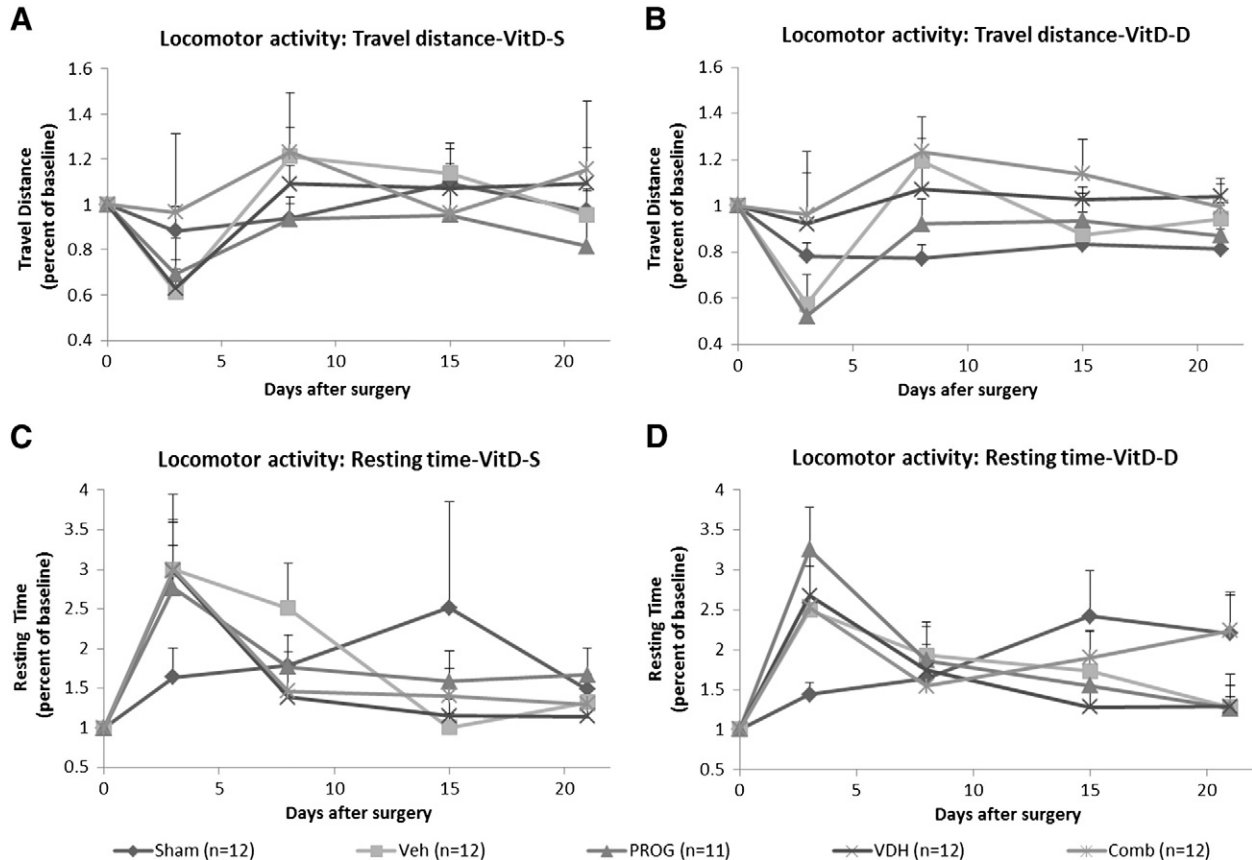


Fig. 3. Locomotor activity testing was done at 3 days (baseline) before surgery and at 3, 8, 15 and 21 days post-surgery or sham surgery. The average travel distance and resting time were presented as percentage of baseline. A. Travel distance of VitD-S cohort. B. Travel distance of VitD-D cohort. C. Resting time of VitD-S cohort. D. Resting time of VitD-D cohort.

Table 1
Mixed-model analysis of sensory neglect, grip strength and locomotor activity.

A. Mixed model				
Effect	Locomotor distance	Locomotor rest time	Sticky task	Grip strength
Diet	0.14	0.57	0.78	0.52
Treatment	0.93	0.49	<0.01*	0.11
Time	<0.01*	<0.01*	<0.01*	<0.01*
B. Pairwise comparison of sticky task				
Pairwise groups	P-value			
Veh vs. PROG	0.18			
Veh vs. Comb	0.13			
Veh vs. Sham	<0.01*			
Veh vs. VDH	0.36			
Sham vs. PROG	0.04*			
Sham vs. Comb	0.04*			
Sham vs. VDH	<0.01*			
PROG vs. Comb	0.90			
PROG vs. VDH	0.66			
Comb vs. VDH	0.57			

Neuron loss and astrocyte activity

In brain tissue from the Sham groups, the individual astrocytes occupied contiguous non-overlapping domains (Fig. 7B). In all TBI groups, there were extensive overlaps and interdigitations of processes of highly reactive astrocytes on the border of the injury site (Fig. 7C). There were other individual non-overlapping domains of reactive astrocytes with hypertrophy of the cell body and the processes at the sites near the injury, especially in subcortical regions. Quantitative evaluation at 22 days post-surgery showed that the expression of GFAP increased

significantly compared to Shams both in the cortex close to the injury site and in the sub-cortex ventral to the injury site in both diet cohorts (Figs. 8, 9). There was no significant difference in GFAP expression in the cortex across diet cohorts (Fig. 8) or among the Veh, PROG, VDH, and Comb treatment groups. Comb treatment significantly decreased GFAP levels compared to the Veh group in the sub-cortex of both diet cohorts ($p < 0.01$, Fig. 9).

At 22 days post-surgery, there was no significant difference across diet cohorts or among the Veh, PROG, VDH, and Comb treatment groups in MAP-2 expression in the cortex. MAP-2 in all CCI groups decreased significantly compared to Sham in the cortex of both diet cohorts (Fig. 8). There was a significant difference in MAP-2 in the sub-cortex among treatment groups in both diet cohorts but no difference across diet cohorts (Fig. 9). Thus, all of the PROG, VDH, and Comb-treated rats had higher MAP-2 expression than the Veh group. The expression of MAP-2 in the PROG and Comb treatment groups was higher than that of the Veh and VDH treatment groups ($p < 0.05$). MAP-2 expression in the Comb treatment groups was greater than that of the PROG treatment group ($p < 0.05$).

Discussion

Because our previous work has shown the efficacy of combination treatment in long-term behavioral outcomes in 3-month-old (Hua et al., 2012) and aged rats in the acute phase of TBI (Cekic et al., 2011), here we expanded our research, first to determine whether 21 days of VDH deficiency can impair functional recovery in middle-aged, 13–14-month-old subjects with TBI, and then to determine whether VDH deficiency and supplementation affects the outcome of TBI in older animals. The issue of applying the same combination treatment regimen to TBI subjects across different age categories is of

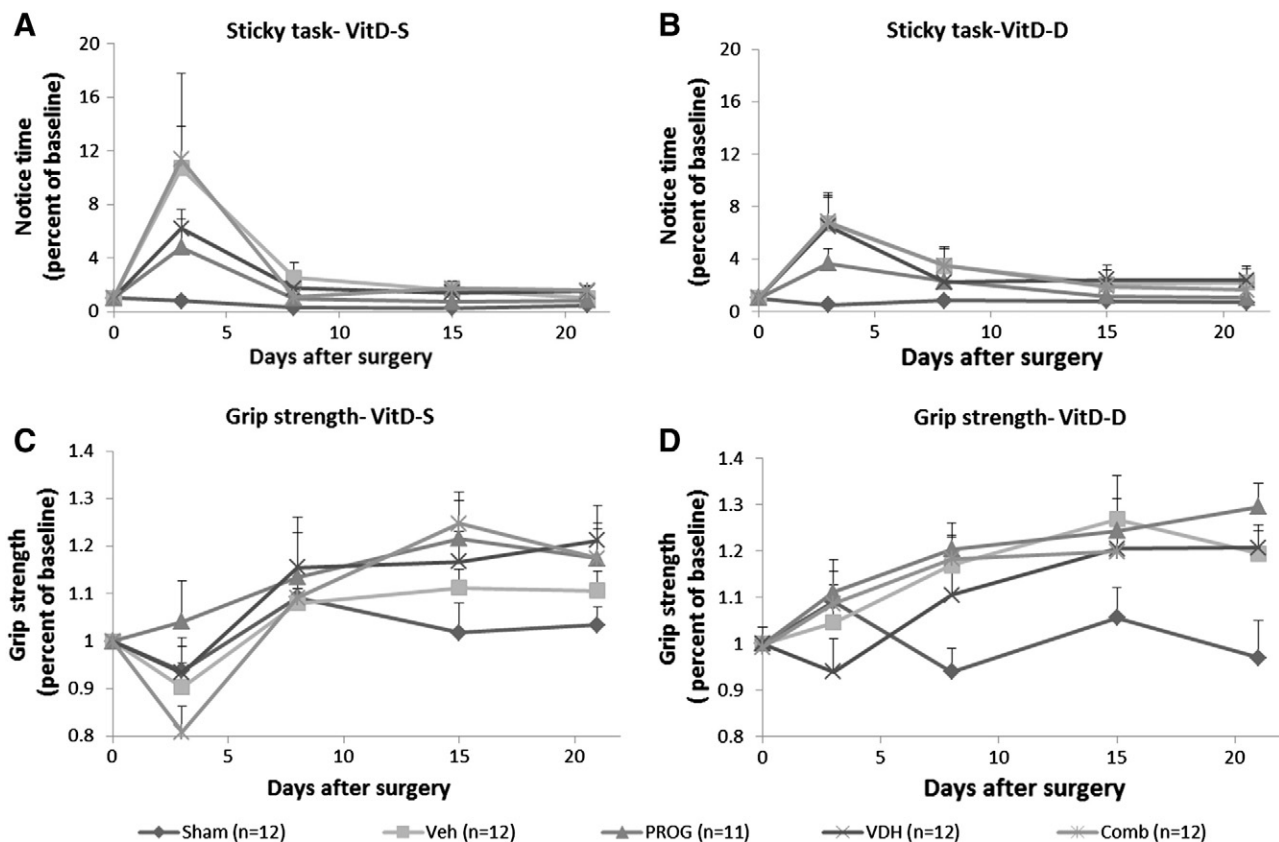


Fig. 4. Sticky task and grip strength tests were conducted at 3 days (baseline) before surgery and at 3, 8, 15 and 21 days post-surgery or sham surgery. The average latency to notice the sticker and grip strength were presented as percentage of baseline response. A. Latency to notice the sticker of VitD-S cohort. B. Latency to notice the sticker of VitD-D cohort. C. Grip strength of VitD-S cohort. D. Grip strength of VitD-D cohort.

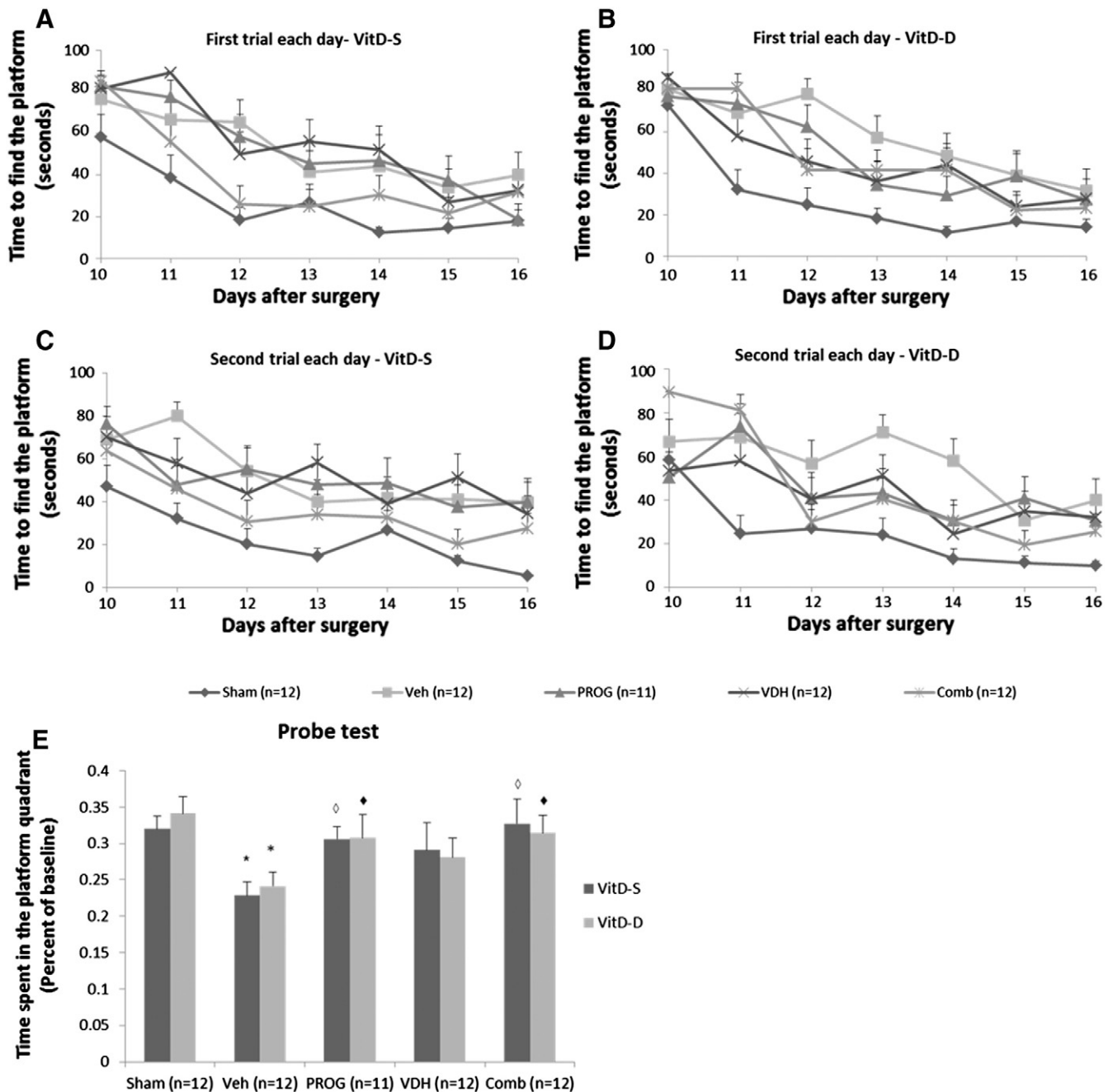


Fig. 5. Morris water maze testing began 10 days after TBI or sham surgery. Rats received 2 trials per day over 7 consecutive days of testing with different starting locations. A–B. Time to find the platform in MWM training trial 1 each day with both diets. The latency to reach the platform in the Sham group was significantly better than that of the other treatment groups over time in trial 1 with both diets. The overall MWM latency of the Comb group was significantly better than that of the Veh group. C–D. Time to find the platform in MWM training trial 2. The results of trial 2 were consistent with trial 1 and showed that combination therapy improved acquisition of the task compared to Veh with both diets. E. A probe test without a platform was conducted on the 18th day after surgery or sham surgery to evaluate time spent in the platform quadrant. With both diets, Veh rats spent significantly less time in the platform quadrant than Sham rats. PROG and Comb rats spent more time in the platform quadrant compared to Veh rats. There was no significant difference between PROG and Comb groups. There was no significant difference in probe test results across diet cohorts. *: compared to Sham with VitD-S, $p < 0.05$; †: compared to Sham with VitD-D, $p < 0.05$; ‡: compared to Veh with VitD-S, $p < 0.05$; ♦: compared to Veh with VitD-D, $p < 0.05$.

considerable clinical relevance, especially if it is determined that such treatments might be beneficial in some age groups, detrimental in others and superfluous in yet others. In part, this is what we sought to determine here: to compare effects we observed in young adult and aged subjects to effects in mature conspecifics that could be considered “middle-aged.” However, in hindsight, one serious limitation of the current study is that we found that dosing parameters may vary as a result of age and/or environmental conditions. Having learned this, it would have been useful to have done a dose–duration–response study with the VDH to determine whether higher or repeated doses would have

led to better results on functional outcomes. Another issue we noted in hindsight was that for older animals with bilateral brain injury, a much longer period of VDH deficiency might be needed to produce significant impairments in the recovery process. These weaknesses should be addressed in future research to address this clinically relevant question. It will also be worthwhile to determine whether sex differences in outcome are important in older female animals that are still cycling normally.

Should we also have tested intact animals with PROG and/or VDH? Both hormones have been used in clinics to treat a variety of illnesses

Table 2
Mixed-model analysis of MWM.

A. Mixed-model analysis of MWM training period		
Effect	P-value	
	Trial 1	Trial 2
Diet	0.88	0.71
Treatment	<0.01*	<0.01*
Time	<0.01	<0.01*
B. Pairwise analysis of MWM training period		
Veh vs. PROG	0.37	0.16
Veh vs. Comb	0.02*	0.01*
Veh vs. Sham	<0.01*	<0.01*
Veh vs. VDH	0.36	0.14
Sham vs. PROG	<0.01*	<0.01*
Sham vs. Comb	<0.01*	<0.01*
Sham vs. VDH	<0.01*	<0.01*
PROG vs. Comb	0.18	0.28
PROG vs. VDH	0.99	0.95
Comb vs. VDH	0.17	0.30

for several decades, and both have well-documented safety profiles. None of the clinical trials (including those sponsored and reviewed by the NIH) administered PROG to intact patient controls because safety levels were not at issue. Although it could be interesting and important to study long-term effects of VDH deficiency and treatment (or combined treatment with PROG) in intact subjects, it was not the purpose

of this project. This could be considered a study limitation, but the issue of primary concern in a translational study like this one is to determine whether the treatments produce any benefits compared to controls with the same extent of injury but no treatment.

We do believe it could be important to investigate, at some point, whether PROG affects the cognition of intact older animals. There have been papers investigating hormone treatment with PROG or estrogen in an aging model to see if they could be cognitive enhancers to intact animals (Chisholm and Juraska, 2013; Chisholm et al., 2012; Vallée et al., 2001). Galea et al. (2000) reported that naturally increasing levels of PROG during pregnancy in rats improved their performance on a spatial learning task. One recent study in humans found that when women were in the luteal phase of their menstrual cycle, when PROG was high, consolidation of memory to a threatening, emotionally laden stimulus was better and their memories (measured one week after exposure to the task) were enhanced compared to women in non-luteal phases of their cycle (Felmingham et al., 2012). Apparently, PROG levels in healthy women can be used to predict memory recall for emotional stimuli, and the hormone may help to mediate cortisol response to stress. The study can be taken to indicate that PROG has a role in normal learning and memory in the absence of any known brain pathology. It is also interesting to find that young adult female rats with ovariectomy and combined treatment with estrogen and PROG were impaired in the acquisition of the MWM (Chesler and Juraska, 2000), while their middle-aged counterparts with the same treatment had facilitated performance of the same task (Markham and Juraska, 2002). This suggests that the effects of hormone treatment in aging female animals are often not the same as in the young. However, the duration of treatment needed for the hormone to induce long-term cognitive changes can be very long, ranging from months to more than a year. In all our studies, we have administered PROG only for one week—a very different model seeking very different outcomes from studies looking at long-term hormonal effects on learning and memory. The objective of the present paper was to determine the effect of combination therapy with PROG and VDH on acute TBI models. We suggest that the effect of these hormones alone or in combination in intact models is a separate topic requiring a much larger study than could be done in the context of our project.

The effect of VDH deficiency on long-term behavior and morphological outcome of TBI

What we learned in this experiment is that a 21-day VDH-deficient diet can produce low serum VDH levels in 13–15-month-old rats, but this may not be sufficient to induce long-term morphological changes and cognitive decline in these younger, as compared to senescent, rats. Either a more severe deficiency or a longer course of treatment appears to be needed. This has important clinical relevance for treatment paradigms, although the findings do not address the molecular mechanisms that would explain why younger subjects are better able to deal with a deficiency that more dramatically affects brain injury outcome in older animals. It is very likely that their immune-inflammatory responses are better adapted and more resilient than those of very young or very old animals. The present study shows that, for our 14-month-old rats, at least 21 days on a VDH-deficient diet does not change the behavioral baseline of the animals compared to conspecifics on a VDH-normal diet. In these older rats, a 21-day, short-course VDH-deficient diet did not exacerbate behavioral outcomes or contribute to the extent of necrotic cavity 22 days post-TBI compared to VDH-sufficient animals. We also observed that 3 weeks of chronic VDH dietary deficiency did not affect MAP-2 expression compared to the VDH-normal diet cohort. Whether middle-aged human patients with TBI and a more acute VDH deficiency would have worsened outcomes than if they were VDH sufficient, remains to be determined in future clinical examinations. The consequences of the deficiency may just not manifest itself until much later in life when the consequences of a stroke or TBI would also be worse.

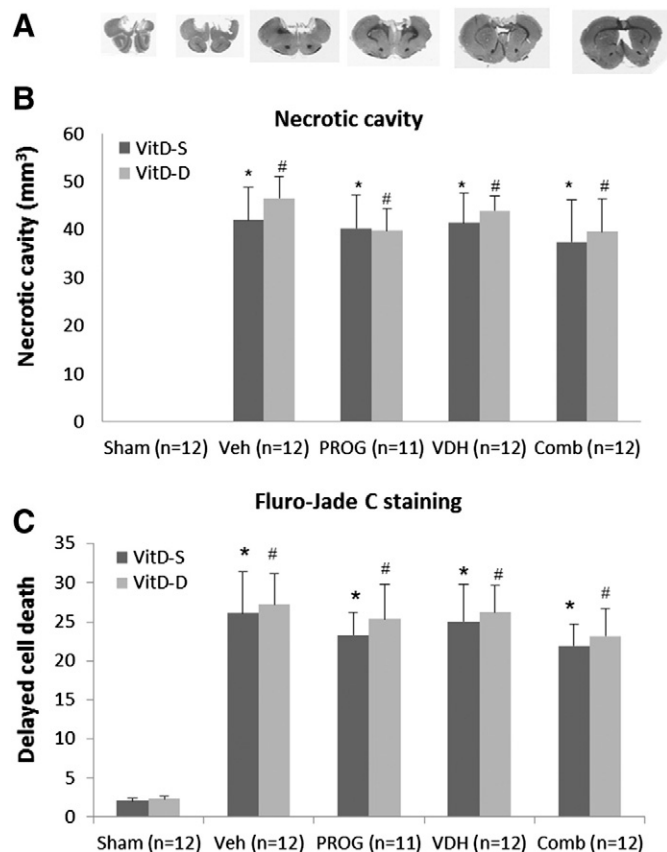


Fig. 6. A. Infarct site. Brain sections from 6 coronal levels from bregma to 5 mm anterior to bregma were selected for Nissl staining 22 days after injury. Nissl staining showed that CCI resulted in substantial brain damage at the site of pre-frontal cortex. B. Necrotic cavity. All the CCI groups had more than 40 mm³ necrotic cavity which was significantly different from Sham groups with both diets ($p < 0.01$). There were no significant differences in necrotic cavity among the CCI groups. C. Number of F-Jc positive cells. All the CCI groups with both diets had more delayed neuron death than the Sham groups. However, there were no significant differences among all CCI groups in delayed cell death. *, $p < 0.01$, compared to Sham with VitD-S. #, $p < 0.01$, compared to Sham with VitD-D.

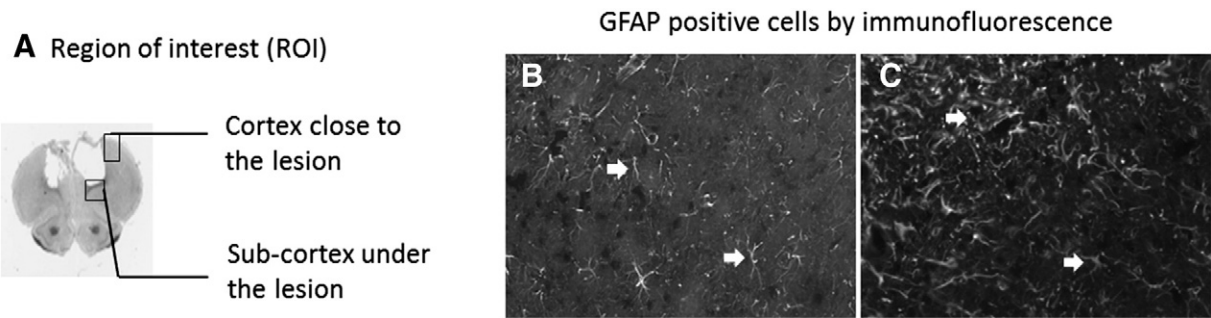


Fig. 7. A. Region of interest: Pictures were taken at the cortex close to the lesion and at the sub-cortex under the lesion to analyze the expression of MAP-2 and GFAP. B. GFAP expression in Sham. Immunofluorescence staining showed that at 22 days post-injury, the appearance of normal astrocytes in healthy cerebral cortex of Sham rats was evenly dispersed without overlapping (indicated by white arrows). C. Reactive astrocytosis in response to CCI. In the cortex of Veh animals at 22 days post-injury, immunofluorescence staining showed extensive overlaps and interdigitations of processes of severely reactive astrocytes on the border of the injury site (indicated by the arrow at upper left). There are other individual non-overlapping domains of reactive astrocytes with hypertrophy of the cell body and the processes near the injury site (indicated by the arrow at lower right).

A growing body of evidence suggests that low VDH levels may increase the risk of cognitive decline or dementia in elderly people (Annweiler et al., 2010; Evatt et al., 2008; Newmark and Newmark, 2007). Unfortunately, many of the human studies are limited by the fact that one cannot determine whether the patients have been deficient for very long times or whether the progression of the disease is caused by the VDH deficiency or is the result of the complexity of the patient's diseases that unfold over time. Rodent studies relevant to a possible causal relationship between VDH deficiency and adverse cognitive or behavioral effects have used one of two model systems: VDH receptor knockout animals, or restriction of UV light and dietary VDH (McCann and Ames, 2008). Both systems were designed to impair the ability to utilize VDH during brain development, and only a few of these studies specifically

examined learning and memory. The results in several spatial learning tests were negative (Becker et al., 2005; Minasyan et al., 2007), and only one recent study on developmental VDH deficiency in C57Bl/6J mice reported a positive finding (Fernandes de Abreu et al., 2010). A stroke study in rats also found that 8 weeks on a VDH-deficient diet did not change the baseline (pre-stroke) measure of forelimb sensorimotor response (Balden et al., 2012).

Effects of combination therapy vs. PROG alone on behavioral outcome after TBI

We expected that PROG combined with VDH would improve behavioral outcomes more effectively than PROG alone in VDH-sufficient and

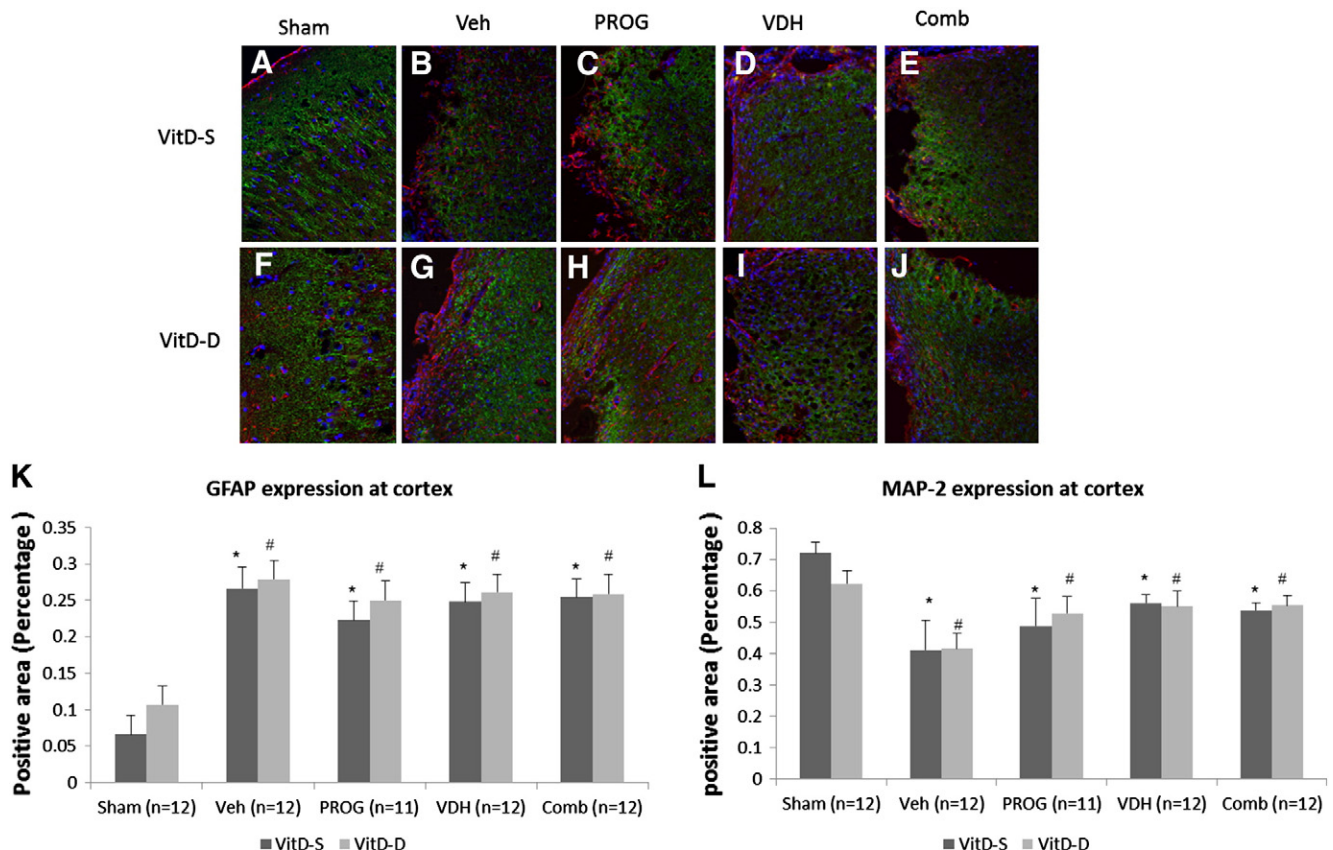


Fig. 8. GFAP/MAP-2 co-label staining in cortex close to the injury. A–J. GFAP-positive cells were labeled in red (Alexa Fluor 594 (ab')) and MAP-2 in green (Alexa Fluor 488 (ab')). The cell nuclei were labeled in blue by DAPI. K. The expression of GFAP in cortex close to the injury. *, $p < 0.01$, compared to Sham with VitD-S. #, $p < 0.01$, compared to Sham with VitD-D. L. MAP-2 expression in cortex of both diet cohorts. *, $p < 0.01$, compared to Sham with VitD-S. #, $p < 0.01$, compared to Sham with VitD-D.

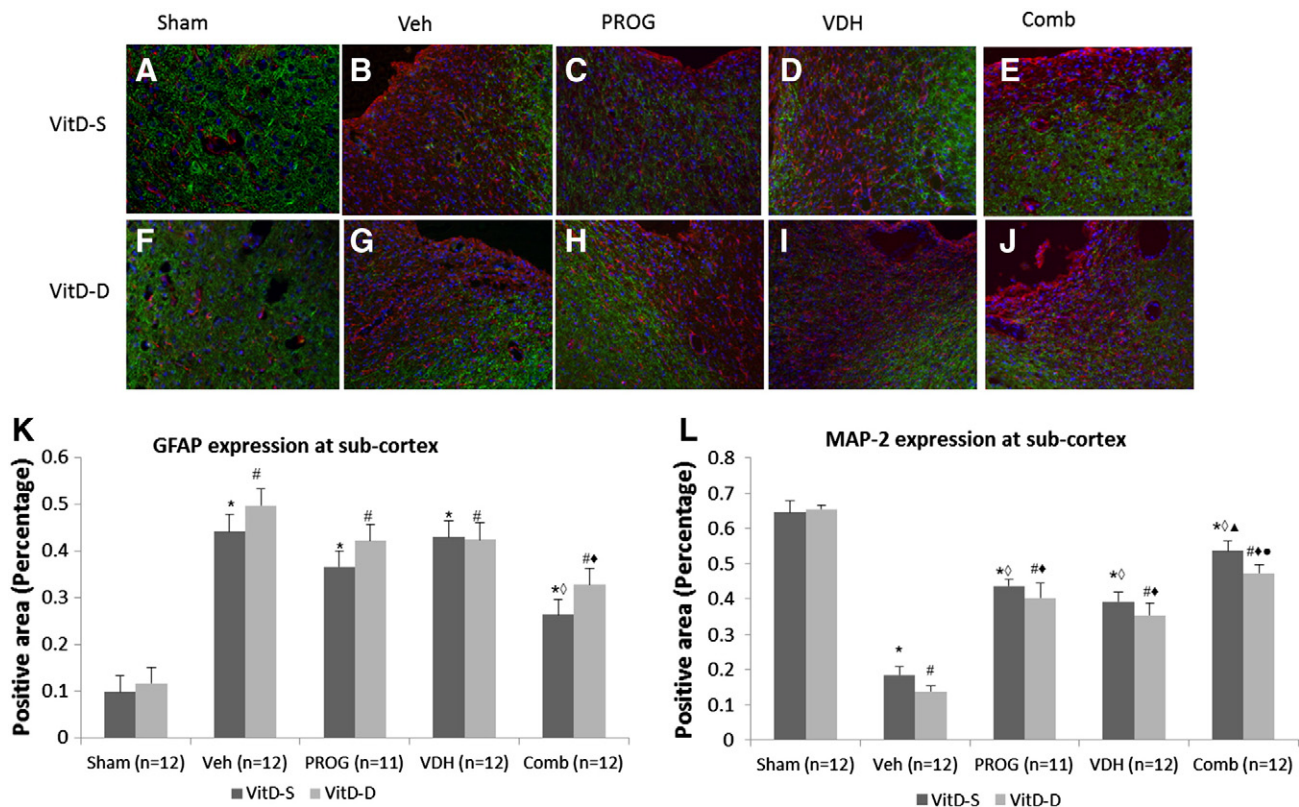


Fig. 9. GFAP/MAP-2 double staining in sub-cortex under the injury. A–J. GFAP-positive cells were labeled in red (Alexa Fluor 594 (ab')) and MAP-2 in green (Alexa Fluor 488 (ab')). The cell nuclei were labeled in blue by DAPI. K. The expression of GFAP in sub-cortex vertical to the injury of both diets. *, $p < 0.05$, compared to Sham with VitD-S. #, $p < 0.05$, compared to Sham with VitD-D. ♦, compared to Veh with VitD-S, $p < 0.05$. ♦, compared to Veh with VitD-D, $p < 0.05$. L. MAP-2 expression in sub-cortex of both diet cohorts. *, $p < 0.05$, compared to Sham with VitD-S. #, $p < 0.05$, compared to Sham with VitD-D. ♦, compared to Veh with VitD-S, $p < 0.05$. ♦, compared to Veh with VitD-D, $p < 0.05$; ▲: compared to PROG and VDH with VitD-S, $p < 0.05$; ●: compared to PROG and VDH with VitD-D, $p < 0.05$.

VDH-deficient older rats with bilateral injury of the MFC. However, we were surprised to find that the rats given combination therapy did not differ on sensorimotor and locomotor activity tests from rats given PROG or VDH alone. During the training period on the MWM, the rats with combination treatment did learn to remember the position of the hidden platform faster than the rats in the CCI group with either diet. In the MWM probe test for longer-term memory, our results also showed that PROG can significantly improve cognitive function, but combination therapy with VDH did not lead to better efficacy than PROG alone (Fig. 5E). In our hands, a single large dose of VDH was not enough to elevate serum VDH levels in either VDH-S or VDH-D rats and did not produce significant effects on cognitive deficits post-brain injury. In hindsight and because the work in senescent rats did not, as expected, extrapolate to younger but fully mature rats (14 months of age), it would have been appropriate to test both higher doses of VDH and a longer duration of treatment to determine if a slightly different combination therapy could have produced a more salubrious effect on functional and morphological outcomes.

As noted, our results here are in contrast with our previous findings in young adult (2–3 months old) VDH-sufficient rats that combination therapy can improve cognitive function during acquisition training as well as in memory probe testing in the MWM (Hua et al., 2012). Age may be only one reason we found no significant difference between PROG alone and combination therapy on the behavioral tests. Cognitive decline in memory constructs can occur during aging (Gage et al., 1989; Markowska et al., 1989), particularly on frontal cortex-dependent tasks (Frick et al., 1995; Gallagher and Rapp, 1997). An earlier study found that 25-month-old mice perform worse on a spatial water maze than do 5-month-old mice, but significant differences were not observed on a non-spatial water maze test (Frick et al., 2000). These findings can be interpreted to suggest that the baseline performance of older

animals can be worse than that of their younger counterparts, but middle-aged rats may still be too “robust” to show the latent deficits.

The difference in sensitivity to hormone treatment between young and older animals might be another reason for our inconsistent findings. A recent study showed that higher levels of PROG in the prefrontal cortex and hippocampus were associated with better Y-maze performance in middle-aged female rats, and suggested that “the capacity for cortico-limbic PROG utilization may influence cognitive performance over the aging process” (Paris et al., 2011). The genetic variance in the VDH receptor gene is associated with cognitive function in old age (Kuningas et al., 2009). One report suggests that “two cognitive domains, *Bsm1* and *Taq1*, are vulnerable and tend to decline constantly across the lifespan” (Hedden and Gabrieli, 2004). Furthermore, the carriers of the *Bsm1* and *Taq1* polymorphisms are susceptible to age-related decline of cognitive functioning (Kuningas et al., 2009). Although we did not compare the behavioral performance of our middle-aged rats to that of their younger counterparts, our data indicate that the middle-aged animals may be less sensitive to hormone treatment than the younger ones (~2.5–3.0 months old), even with a higher dose.

Combination therapy and reactive astrogliosis

It has been shown that regardless of diet, brain injury stimulates the expression of GFAP compared to shams (Staffa et al., 2012). Our results show that regardless of diet, brain injury significantly stimulated the expression of GFAP compared to Shams, and the combined therapy decreased GFAP expression at subcortical structures. Many researchers have found that following brain injury, inflammatory cytokines such as TNF- α , IL-6, Toll-like receptor ligands, reactive oxygen species, and glutamate are molecular triggers and modulators of high levels of reactive astrocyte expression and proliferation (Di Giorgio et al., 2007;

Farina et al., 2007; John et al., 2003). We know from our previous research that PROG can modulate inflammatory cytokines, Toll-like receptors, and oxidative stress (Hua et al., 2011; Pettus et al., 2005). In addition, VDH itself is able to regulate excitotoxicity by modulating L-type voltage-sensitive Ca^{2+} channels (L-VSCCs) (Brewer et al., 2001). We think that the combined hormone treatments could attenuate some of the chronic (or sub-chronic) inflammatory mechanisms induced by cortical contusion injury (Hua et al., 2012). We also found in this experiment that combination therapy does better in reducing astrogliosis by modulating astrocyte over-proliferation, which in turn could minimize the potential side effects of scar formation.

Combination therapy and neuron loss

We used multiple methods to evaluate neuronal loss caused by cortical contusion and the resulting secondary neurodegeneration. As shown in Fig. 6C, F-Jc positive cells increased significantly compared to intact brain tissue in the Sham group, but the differences among the groups were not significant.

F-Jc staining was used to label degenerating but not healthy neurons (Ehara and Ueda, 2009). We therefore conducted MAP-2 staining in the cortex close to injury to estimate the number of surviving neurons after injury. MAP-2 is one of many important neuronal cytoskeletal proteins that are expressed in axons and dendrites (Drewes et al., 1998). MAP-2 is required for microtubule stability and dendritic elongation as well as for the interaction of microtubules with other organelles because it facilitates the polymerization of pure tubulin into microtubules (Sharma et al., 1994). Therefore, MAP-2 immunostaining has been widely applied to label the dendritic meshwork and to estimate the dynamic changes in dendritic architecture. Previous studies have shown that MAP-2 degeneration is related to lipid peroxidation and calcium-related calpain activation (Atalay et al., 2007; Ercan et al., 2001). PROG is protective against excitotoxicity by reducing N-methyl-D-aspartate (NMDA), glutamate and excitatory cholinergic signaling, and by balancing Ca^{2+} mobilization (Cai et al., 2008; Hu et al., 2009). It is worth noting that by itself, VDH can down-regulate L-type calcium channel expression (Brewer et al., 2001) and maintain calcium levels (Chatterjee, 2001). We found no differences between groups in MAP-2 expression in the cortex close to the lesion. However, in the sub-cortex ventral to the lesion, MAP-2 expression after PROG, VDH and combination treatment was higher than that seen in the Veh-alone group. The combination treatment produced more neuronal sparing compared to either VDH or PROG treatment alone in preventing MAP-2 degradation subcortically. Our current data show that combination therapy has the potential to contribute to restoration of brain function in long-term disorders of consciousness and cognitive function by increasing expression of MAP-2 in the chronic phase after TBI. The benefits of such therapy, especially after brain injury, might not be seen until much later in life. This speculation was not tested in the current experiments but could be an important factor in learning how combination therapies work to protect the brain from subtle damage caused initially by a past TBI.

Conclusion

Here we report that relatively acute VDH deficiency in middle-aged (13-month-old) rats does not significantly impair pre-TBI baseline behavioral functions, and at the levels we obtained, does not worsen impaired cognitive outcome seen after TBI in this age cohort. Our animals were apparently more resilient to dietary modifications than we expected. However, combination therapy with PROG and VDH did show moderate improvement in preserving spatial and reference memory, but it was not significantly better than the results obtained with PROG alone. In hindsight, a longer period of VDH deficiency and/or a higher or more repetitive dose of VDH could have been tested and should be examined for clinical relevance in a future experiment. It is

worth noting that our combination therapy regimen did reduce reactive astrocyte proliferation, which in the very long term might prevent the appearance of functional deterioration later in life. Importantly, the combination treatment did show better efficacy compared to VDH and PROG monotherapies in preventing MAP-2 degradation, an important marker of tissue loss.

Disclosure

DG Stein is party to a licensing agreement with BHR Pharmaceuticals, Ltd. related to PROG usage in TBI. This agreement has been approved by Emory University, who receives the largest share of fees in accordance with its conflict of interest policies. DGS also serves as consultant to BHR and may receive royalties and research funding from BHR occasionally.

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