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Vitamin D deficiency reduces the benefits of progesterone treatment after brain injury in aged rats

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Abstract

Administration of the neurosteroid progesterone (PROG) has been shown to be beneficial in a number of brain injury models and in two recent clinical trials. Given widespread vitamin D deficiency and increasing traumatic brain injuries (TBIs) in the elderly, we investigated the interaction of vitamin D deficiency and PROG with cortical contusion injury in aged rats. Vitamin D deficient (VitD-deficient) animals showed elevated inflammatory proteins (TNFα, IL-1β, IL-6, NFκB p65) in the brain even without injury. VitD-deficient rats with TBI, whether given PROG or vehicle, showed increased inflammation and greater open-field behavioral deficits compared to VitD-normal animals. Although PROG was beneficial in injured VitD-normal animals, in VitD-deficient subjects neurosteroid treatment conferred no improvement over vehicle. A supplemental dose of 1,25-dihydroxyvitamin D_3 (VDH) given with the first PROG treatment dramatically improved results in VitD-deficient rats, but treatment with VDH alone did not. Our results suggest that VitD-deficiency can increase baseline brain inflammation, exacerbate the effects of TBI, and attenuate the benefits of PROG treatment; these effects may be reversed if the deficiency is corrected.

Keywords: Aging; Inflammation; Progesterone; 1,25-Dihydroxyvitamin D_3; Traumatic brain injury; Vitamin D deficiency

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability among people of all ages in the United States. While the rate of death from TBI has declined for most age groups over the past ten years (due in large part to improved safety measures such as the use of safety belts), in the elderly it has risen by over 21% (CDC, 2004) and is currently more than twice that of the younger population (Mosenthal et al., 2002). The incidence of TBI is also increasing in older people as they live, drive, work, play, and continue to face the demands of a fast-paced and complex environment longer. Furthermore, the elderly are at higher risk of falls and accidents involving trauma and have more preexisting health problems that often contribute to this risk and complicate its effects. They are also often subject to alterations in systemic hormonal levels that may significantly affect their response to injury (Topinkova, 2008). Given that mortality and morbidity in many, if not most, patients with head trauma are not exclusively neurological in origin but rather a result of damage to multiple interacting organ systems (Zygun, 2005), any predisposing factor that contributes to systemic frailty (Lipsitz, 2004) could have a significant impact on the ability of aged patients to survive and recover from central nervous system (CNS) trauma.

Over the past decade, a number of studies have demonstrated that treatment with progesterone (PROG) and its metabolites significantly improves functional outcome after TBI in rats and humans (Gibson et al., 2008; Singh et al., 2008; Stein, 2008). A neuroactive steroid, PROG has been shown to improve behavioral and functional recovery and to reduce inflammation, oxidative damage, cerebral edema, and neuronal cell death (Djebaili et al., 2004; Grossman and Stein, 2000; He et al., 2004; Wright et al., 2001). Although specific modes of action have yet to be completely defined, PROG affects a variety of molecular mechanisms ranging from GABAergic and aquaporin modulation to complement C5a and iNOS inhibition (Pettus et al., 2005; Schumacher et al., 2007; VanLandingham et al., 2007), making it likely

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that interacting pleiotropic actions are responsible for its observed benefits. Despite the success of two recent clinical trials (Wright et al., 2007; Xiao et al., 2008) (100 and 159 patients, respectively) showing that administration of intravenous PROG within 8 h of injury can reduce mortality by 50% in severely injured patients and improve functional outcomes in moderately injured TBI patients at 1, 3, and 6 months post-injury, the effectiveness of such treatment in the elderly has not been specifically established. However, there is recent direct evidence that post-injury PROG treatment may be beneficial in aged rats (Cutler et al., 2007), especially in the acute phase after trauma.

Aside from advanced age, itself a major predictor of injury severity (Mosenthal et al., 2002), other potentially exacerbating factors in the aged include systemic issues such as kidney disease, hypertension, atherosclerosis and cardiovascular disease, diabetes, cancer, and hormonal imbalances such as hyperparathyroidism (Onyszchuk et al., 2008). While all these conditions can affect responses to injury, each has also been associated by a growing literature with insufficient serum levels of vitamin D as a key and often ignored underlying problem (Grant, 2006; Holick and Chen, 2008; Peterlik and Cross, 2005). According to the Third National Health and Nutrition Examination Survey, 61% of Caucasian- and 91% of African-Americans are vitamin D deficient (Khazai et al., 2008). Figures similar to these have been cited internationally for all segments of the population (Holick and Chen, 2008; MacFarlane et al., 2004), but they tend to be especially high in the old, the ill, and the institutionalized, with studies reporting prevalence statistics ranging from 65% to 74% in hospital inpatients (Chatfield et al., 2007; Corino et al., 2007; Thomas et al., 1998), to 87% in elderly institutionalized patients (Larrosa et al., 2001) and 86% in institutionalized postmenopausal women (Gaugris et al., 2005). Vitamin D deficiency (VitD-deficiency), defined by serum levels of 25-hydroxyvitamin D_3 ($25OHD_3$) below 50 nmol/L or 20 ng/mL (Grant and Holick, 2005), is associated with rickets in children and osteomalacia in adults, and has recently also been linked to a number of systemic conditions such as secondary hyperparathyroidism (Holick, 2005a; McCarty, 2005), metabolic syndrome (Peterlik and Cross, 2005), hypertension (Li et al., 2002; Wang et al., 2008), obesity (Rajakumar et al., 2008), and diabetes mellitus (Giulietti et al., 2004; Grant, 2006), as well as cardiovascular disease outcomes such as stroke and congestive heart failure (Michos and Melamed, 2008; Vieth and Kimball, 2006). Several recent studies also suggest that inadequate vitamin D may predispose towards Parkinson's and other neurodegenerative diseases, mood disorders (Garcion et al., 2002; Kalueff et al., 2004b), and even tuberculosis infection (Zasloff, 2006). A low level of vitamin D is one of the key markers of frailty, defined as a "global impairment of physiological reserves involving multiple organ systems" (Topinkova, 2008). Frailty often results in a reduced capacity to maintain physical and psychosocial homeostasis and greater vulnerability to internal and environmental stressors such as trauma (Markle-Reid and Browne, 2003; Topinkova, 2008).

Calling vitamin D a "vitamin" is something of a misnomer. Although the name is still in use for historical reasons, vitamin D is more properly classed as a secosteroid because it consists of a cholesterol backbone and exerts steroid-like effects throughout the body, directly affecting the expression of over 1000 genes (Eelen et al., 2004) through the nuclear vitamin D receptor (VDR). Vitamin D is also a neurosteroid by definition, because it is both activated by and has direct effects in the CNS (Garcion et al., 2002). It has been shown to affect systems similar to those modulated by other neurosteroids such as PROG (Garcion et al., 2002), with which it may interact in a variety of physio-pathological contexts including TBI (Losem-Heinrichs et al., 2005). While vitamin D has classically been associated with systemic calcium homeostasis, there is now evidence that it is a potent modulator of the immune system that affects inflammation (Hayes et al., 2003), neuromuscular function (Pfeifer et al., 2002) and cell-cycle control (Banerjee and Chatterjee, 2003).

In this study we extend our investigation of the effects of PROG treatment on injury and recovery of function in the aged rat (Cutler et al., 2007) by evaluating the role that VitD-deficiency may play in short-term outcome in an attempt to develop a more realistic model of injury and illness for the elderly human population. Since vitamin D appears to be intimately related to a number of key processes that affect the extent of TBI, since it may interact with neurosteroid treatment, and since VitD-deficiency is virtually endemic in the elderly population, our approach investigated the efficacy of PROG treatment an aged model of TBI in the context of VitD-deficiency. We asked several questions: (1) Does VitD-deficiency cause increased baseline inflammation in the brain of uninjured aged rats, thereby establishing a potentially detrimental underlying condition? (2) Does VitD-deficiency exacerbate brain injury in animals treated with vehicle compared to vehicle-treated but nutritionally normal animals? (3) Does VitD-deficiency interact with PROG treatment, and does it affect the acute phase inflammatory response in treated VitD-deficient animals versus treated non-deficient animals? (4) Is it possible to improve functional outcome in VitD-deficient animals by correcting endogenous vitamin D status after TBI through administration of activated vitamin D hormone (1,25-dihydroxyvitamin D_3 [VDH])? The hypothesis was that VitD-deficiency will increase the inflammatory response even in sham but especially in injured animals, will attenuate the beneficial effects of PROG, and will be reversed by the co-administration with VDH.

2. Materials and methods

2.1. Subjects

Eighty-seven 22-month-old male Fischer 344 rats weighing 450–550 g at the time of injury were used in this

experiment. Animals were housed at Emory for 2 months prior to surgery and handled as previously described (Cutler et al., 2007). This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures we used were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), Protocol #146-2005.

2.2. Diet

The animals in this study were separated into two groups, vitamin D normal (VitD-normal) and vitamin D deficient (VitD-deficient). The VitD-normal group was given standard rat chow used in our animal care facility (Rodent Diet 5001, LabDiet®, St. Louis, MO). The VitD-deficiency group was fed a vitamin D-null version of the same diet (Diet 5A4Y, modified 5001 with no D_3, TestDiet®, Richmond, IN); all rats were weighed daily to ensure constant energy intake. Animals in the VitD-deficient group were maintained on the diet for at least 21 days prior to surgery. Eight days have been shown to be sufficient to induce a circulating $25OHD_3$ level consistent with deficiency (Narayanan et al., 2004), but we extended this interval to allow the sequelae of the VitD-deficiency to become apparent 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 rats assigned to the VitD-deficient group were maintained on it until they were killed for harvesting of brain tissue. Since vitamin D is activated by UVB light (280–315 nm wavelength), we ensured that the overhead lights in our animal colony did not produce radiation in this range.

2.3. Serum vitamin D and PROG levels

Blood (0.5–1.0 mL) was drawn directly from the right ventricle of the heart with a 21G needle after the rats were rendered unconscious from the Nembutal but before death or decapitation. The whole blood was allowed to coagulate for 30 min at RT, after which the clot was removed and the serum centrifuged for 5 min at $1000 \times g$ and stored at $-80\,^{\circ}C$. Vitamin D levels were determined with a $25OHD_3$ RIA double antibody method (DiaSorin Inc., Stillwater, MN). Serum $25OHD_3$ is a standard marker for determining vitamin D status (Heaney, 2004; Holick, 2005b; Holick et al., 2005; Tangpricha et al., 2004). PROG levels were measured with a solid-phase RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assays were performed independently by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center at Emory University in Atlanta, GA.

2.4. Surgery and contusion injury

Rats were anesthetized using isoflurane gas (5.0% induction, 1.0–1.5% maintenance, 700 mmHg N_2O, 500 mmHg O_2) and surgery was performed using aseptic techniques as previously described (Cutler et al., 2007). Briefly, 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 a pneumatic cortical contusion device (5 mm diameter) with impact velocity of 2.25 m/s, impact time of 500 ms, and depth of 3.5 mm ventral to bregma. The incision was sutured closed after all bleeding had stopped. In the sham group, the incisions were sutured closed after comparable time under anesthesia. Animals dehydrated due to blood loss were given 3 mL of lactated Ringer's solution subcutaneously within 6 h of injury.

2.5. Treatment

Animals were assigned to VitD-normal or VitD-deficient groups. VitD-normal animals were assigned to one of three groups ($n = 5$/group): sham (SHAM), vehicle (VH), and progesterone (PROG). Deficient animals were assigned to one of five groups ($n = 5$/group): sham (SHAM), vehicle (VH), progesterone (PROG), progesterone with VDH (D + PROG), and VDH alone (D). The same assignment was followed for both 24 and 72 h survival groups. The treatments were VH: 22.5% 2-hydroxypropyl-β-cyclodextrin; PROG: 16 mg/kg PROG (P0130, Sigma–Aldrich, St. Louis, MO); D + PROG: 16 mg/kg PROG combined with 5 μg/kg VDH (D1530, Sigma–Aldrich) for the first injection and 16 mg/kg PROG with equivalent volume VH for the rest; D: 5 mg/kg VDH for the first injection and vehicle for the rest. We used our previously published treatment protocol (Cutler et al., 2007) consisting of an intraperitoneal injection 1 h post-injury followed by subcutaneous injections at 6 h, 24 h, and every 24 h thereafter until the animals were killed. All drug treatments were dissolved in vehicle, and injection volume was proportional to each animal's weight across all groups. The intact sham (SHAM) groups served to provide baseline data and therefore received no injury or injections. We used 16 mg/kg PROG because previous research demonstrated it to be the most effective dosage in young (Goss et al., 2003) and aged (Cutler et al., 2007) rats. Animals receiving VDH treatment were given only a single 5 mg/kg VDH injection 1 h post-injury based on the evidence that a single megadose of VDH can reverse deficiency (Diamond et al., 2005); since the focus of this study was correction of VitD-deficiency rather than vitamin D supplementation, animals receiving a normal diet were not given any VDH.

2.6. Activity testing

Spontaneous locomotor activity was performed as previously described (Cutler et al., 2007). This task has previously been shown to be sensitive to our model of TBI and to the effects of PROG treatment (Cutler et al., 2007), as well as to potential behavioral and motor derangements due to VitD-deficiency in open-field testing (Kalueff et al., 2004a).

2.7. Tissue preparation and Western blot analysis

Animals were killed 24 or 72 h after surgery with a lethal dose of Nembutal (1 mL) and decapitated. Their brains were prepared for protein analysis and Western blots were performed as previously described (Cutler et al., 2007). Briefly, the brains were homogenized in ice cold Tper (Pierce, Rockford, IL) and protease inhibitor cocktail and a bicinchoninic acid protein assay was performed to determine the concentration of total protein. Protein concentration curves were calculated to confirm that the analysis would be performed within the linear range of detection. Fifteen microliters of each sample (at a concentration of 2 μg/μL, for 30 μg total protein per well) were run in each well of an 18-well 4–20% Tris–HCL acrylamide Criterion Gel (BioRad, Hercules, CA), blotted, and blocked in milk solution before being incubated with primary antibodies. The primary antibodies used in this experiment were TNFα (AB1837P, Millipore/Chemicon, Temecula, CA), IL-1β (ab9787, Abcam Inc., Cambridge, MA), IL-6 (Abcam, ab6672), NFκB p65 (#3034, Cell Signaling Inc., Danvers, MA), COX-2 (Abcam, ab6665), p53 (Cell Signaling, #9282), cleaved caspase-3 (Asp175; Cell Signaling, #9661S), and β-actin (Abcam, ab37063). Bands were detected with enhanced chemiluminescence on a Kodak (Rochester, NY) Image station 440CF scanner and analyzed with the accompanying Kodak1D densitometry image analysis software. Band intensity was compared only between treatment groups run on the same blots and when more than one blot was used due to the large number of samples, all data were normalized to the average of a reference group run on all blots in question. β-Actin was used as a loading control.

2.8. Statistical analysis

All results were expressed as the mean ± the standard error of the mean (SEM). Statistical significance was set *a priori* at $p < 0.05$ and data were analyzed using *t*-tests, Pearson correlations, one-way analysis of variance (ANOVA) with Tukey-HSD *post hoc* tests, and general linear models (GLMs). All analyses were calculated using SPSS™ 17.0 statistical analysis software.

3. Results

3.1. General observations of frailty in vitamin D deficient aged rats

The deficient animals were observed to be more "frail" compared to rats fed a normal diet. Although these observations were not always blinded, deficient animals generally bled longer (indicating a possible coagulation problem), displayed softer bones (i.e., the skull was easier to drill through), showed less stable vital signs during surgery, and required a lower concentration of isoflurane to become unconscious. They also took longer to recover after surgery and were observed to be less active when handled for treatment, injections and weighing.

3.2. Serum 25-hydroxyvitamin D_3 levels confirmed vitamin D deficiency

Serum level assay data for $25OHD_3$ showed that rats fed a normal diet averaged 27.45 ± 0.81 ng/mL ($n = 16$), while rats fed a vitamin D-null diet averaged 10.50 ± 0.80 ng/mL ($n = 38$). This is a significant difference ($p < 0.0001$) and confirms that our protocol resulted in a dramatic decrease in serum $25OHD_3$. These levels are generally consistent with VitD-deficient status in rats (Rojanasathit and Haddad, 1977).

3.3. Vitamin D deficiency elevates baseline levels of inflammatory cytokines in uninjured brain

We first asked whether VitD-deficiency would increase baseline inflammation in intact animals, as this would suggest a general systemic inflammatory state even *before* injury. Fig. 1 shows the relative levels of inflammatory proteins (TNFα, IL-1β, IL-6, NFκB p65, COX-2) in the MFC of SHAM animals maintained on a VitD-deficient diet compared to animals fed a normal diet. All cytokines were normalized respectively to those found in normal shams (vertical axis value = 1) and are shown as the ratio of deficient:normal ± SEM. *t*-Test *p*-values comparing deficient versus normal animals were TNFα ($p = 0.026$), IL-1β ($p = 0.002$), IL-6 ($p = 0.047$), NFκB p65 ($p = 0.036$), COX-2 ($p = 0.26$). With the exception of COX-2, all inflammatory cytokines measured were significantly elevated in the intact VitD-deficient rats compared to intact VitD-normal animals. While the data shown were from brains extracted 72 h after

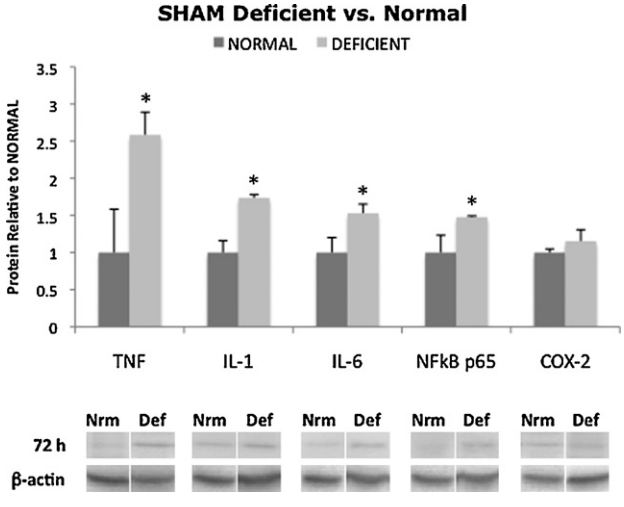

Fig. 1. Uninjured (SHAM) deficient animals show elevated levels of inflammatory cytokine proteins (light) compared to nutritionally normal animals (dark). Values are normalized to normal uninjured animals (vertical axis = 1). Asterisks denote a significant *t*-test with $p < 0.05$. Western blot images refer to the cytokine located in the graph above, and β-actin images indicate loading controls. Nrm: normal; Def: vitamin D deficient.

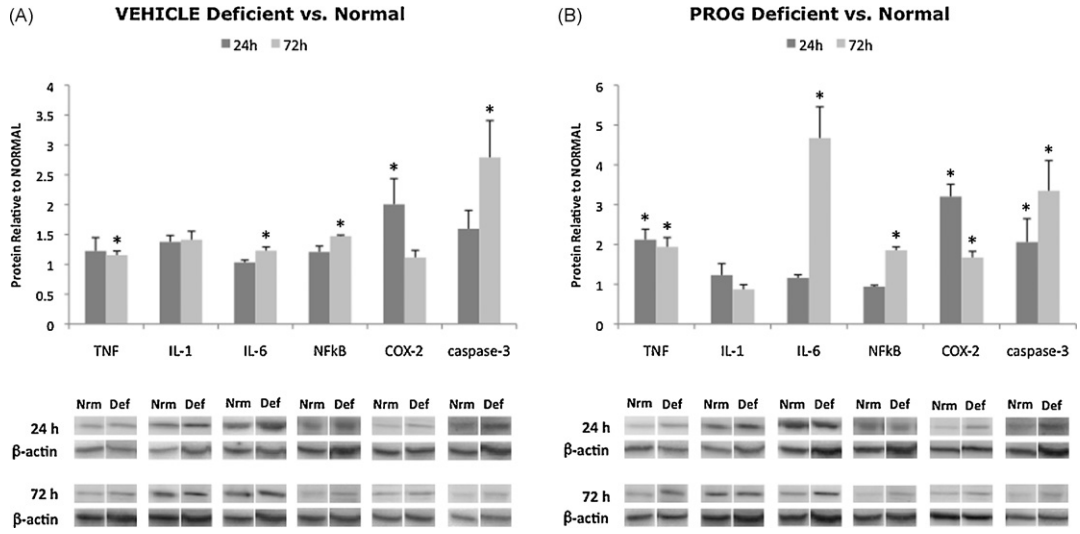

Fig. 2. (A) Injured deficient animals treated with vehicle show elevated levels of inflammatory proteins at 24 h (dark grey) and 72 h (light grey) after injury compared to nutritionally normal animals. (B) Injured deficient animals treated with PROG also show increased inflammation at 24 and 72 h compared to normal animals. All protein results are normalized to the values for nutritionally normal animals at the same time-point, i.e., all normal values (not shown) are at value = 1 on the vertical axis. Asterisks denote a significant *t*-test with $p < 0.05$. Western blot images refer to the cytokine located in the graph above, and β-actin images indicate loading controls. Nrm: normal; Def: vitamin D deficient.

the sham surgery, similar results were obtained at 24 h as well (not shown).

3.4. Vitamin D deficiency exacerbates injury in vehicle-treated animals with TBI

Our second question was whether VitD-deficiency exacerbates inflammation in injured, vehicle-treated animals. Fig. 2A shows the results for the same proteins as in Fig. 1 as well as activated caspase-3 at 24 and 72 h after injury. The data were normalized to the respective cytokine at the same time-point in VitD-normal animals (vertical axis value = 1) and are shown as the ratio deficient:normal ± SEM. At 24 and 72 h, respectively, the *t*-test *p*-values comparing normal and deficient animals were TNFα ($p = 0.029$; $p = 0.039$), IL-1β ($p = 0.023$; $p = 0.078$), IL-6 ($p = 0.35$; $p = 0.013$), NFκB p65 ($p = 0.22$; $p < 0.001$), COX-2 ($p = 0.035$; $p = 0.20$), cleaved caspase-3 ($p = 0.11$; $p = 0.009$). At 24 h after injury, only COX-2 was significantly elevated in VitD-deficient animals treated with vehicle compared to their VitD-normal counterparts. By 72 h, however, all inflammatory markers with the exception of IL-1β and COX-2 were significantly higher in vehicle-treated VitD-deficient versus VitD-normal animals.

3.5. Vitamin D deficiency exacerbates damage with PROG treatment after TBI

The third question was whether VitD-deficiency exacerbated injury-related inflammation in animals treated with PROG. Fig. 2B shows the results for the same proteins in deficient versus normal PROG-treated animals 24 and 72 h after TBI. The data are normalized to the respective cytokine at the same time-point in normal animals (vertical axis value = 1) and are shown as the ratio deficient:normal ± SEM. At 24 and 72 h, respectively, the *t*-test *p*-values were TNFα ($p = 0.015$; $p = 0.006$), IL-1β ($p = 0.22$; $p = 0.30$), IL-6 ($p = 0.15$; $p < 0.001$), NFκB p65 ($p = 0.21$; $p = 0.003$), COX-2 ($p = 0.001$; $p = 0.017$), cleaved caspase-3 ($p = 0.012$; $p = 0.019$). TNFα, COX-2, and caspase-3 are elevated 24 h after injury in VitD-deficient versus VitD-normal animals treated with PROG, but by 72 h all except IL-1β are higher in the deficient group. This may suggest that effects of VitD-deficiency become more pronounced as the injury evolves over time.

3.6. Vitamin D deficiency attenuates the beneficial effects of PROG after TBI, but co-treatment with VDH improves outcome in deficient animals

Since cytokine profiles were in general significantly worse in deficient than in normal animals by 72 h after injury, the next question we asked was twofold: (1) Does PROG lose its therapeutic effectiveness under conditions of VitD-deficiency? (2) If so, would PROG regain its efficacy if it were given with VDH? Our results show that PROG treatment in VitD-deficient animals results in mild improvement compared to vehicle-treated VitD-deficient animals, but these effects are minimal compared to the significant improvements seen when it is given with VDH. Panels A–D in Fig. 3 show the relative levels for several cytokines and proteins 24 and 72 h after TBI in VitD-deficient animals. All values are normalized to the vehicle-treated group average for each time-point: TNFα (Fig. 3A, 24 h: $F_{4,20} = 8.530$, $p = 0.001$; 72 h: $F_{4,20} = 26.931$, $p < 0.001$), IL-1β (Fig. 3B, 24 h: $F_{4,20} = 5.911$, $p = 0.010$; 72 h: $F_{4,20} = 15.393$, $p < 0.001$), IL-6 (Fig. 3C, 24 h: $F_{4,20} = 16.481$, $p < 0.001$; 72 h: $F_{4,20} = 23.538$,

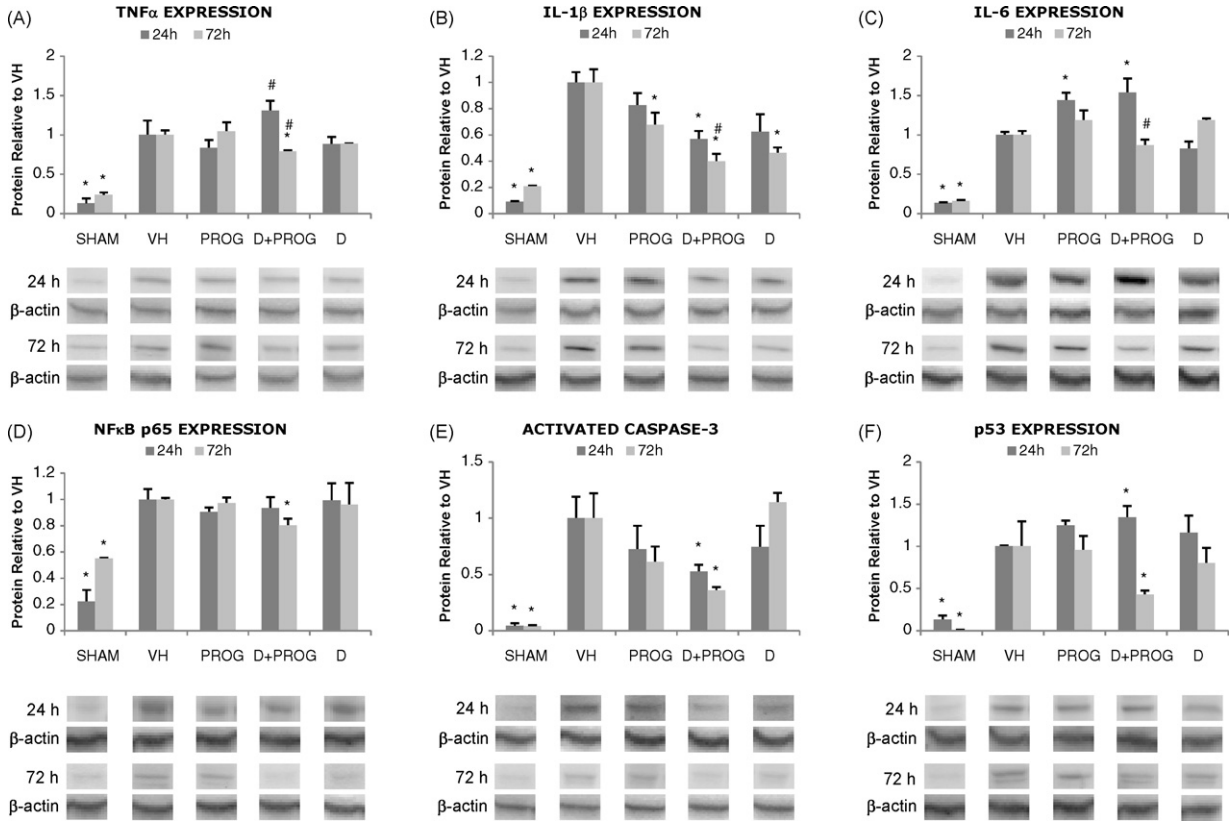

Fig. 3. Levels of individual inflammatory cytokine proteins, cleaved caspase-3, and p53 in deficient injured animals under different treatment conditions at 24 h (dark grey) and 72 h (light grey) after injury. Results are normalized to the vehicle (VH) group within each time-point (vertical axis value = 1). Asterisks (*) denote *post hoc* $p < 0.05$ significance relative to VH, and (#) denotes $p < 0.05$ relative to PROG. The major treatment effect significantly different from vehicle in most cases is only D + PROG, suggesting a reversal of the injurious effect of deficiency. Western blot images refer to the treatment group in the graph above, and β-actin images indicate loading controls.

$p < 0.001$), NFκB p65 (Fig. 3D, 24 h: $F_{4,20} = 9.960$, $p = 0.001$; 72 h: $F_{4,20} = 6.847$, $p = 0.003$). In most cases, only D + PROG treatment resulted in significant reduction of inflammation by 72 h after injury, suggesting vitamin D may interact with both the injury process and PROG treatment.

3.7. Administration of VDH with PROG in vitamin D deficient animals reduces cell death and DNA damage compared to vehicle, PROG, or VDH alone

The two molecular endpoints examined in this study were levels of activated caspase-3, the final effector in the apoptotic pathway, and p53, a cell-cycle control protein elevated by DNA damage and involved in the cellular choice between apoptotic cell death and DNA repair processes (Offer et al., 2002). Since vitamin D is known to increase p53 expression (Gupta et al., 2007), we measured the ratio of altered to normal p53 as an indicator of DNA damage (Offer et al., 2002). Our results (Fig. 3, panels E and F) show a significant decrease in activated caspase-3 (Fig. 3E, 24 h, $F_{4,20} = 6.332$, $p = 0.008$; 72 h, $F_{4,20} = 11.634$, $p < 0.001$) and a bidirectional effect on p53-DNA interaction (Fig. 3F, 24 h, $F_{4,20} = 6.563$, $p = 0.003$; 72 h, $F_{4,20} = 6.181$, $p < 0.001$) only in animals treated with D + PROG. This effect seems to be dependent on the time after injury. These results suggest that the combined D + PROG treatment is most effective in reducing cell death and DNA damage after TBI in VitD-deficient animals.

3.8. Combined treatment with PROG and VDH improves behavioral function compared to treatment with vehicle, PROG, or VDH alone

In addition to molecular measures of inflammatory cytokines, we examined the behavioral effects of the various treatments. Since this study was limited to the short-term effects on inflammation, only short-term spontaneous locomotor activity was used. The results are shown by the panels in Fig. 4 as the ratios of post-injury:pre-injury measurements and are normalized to sham animals to control for the variability in different animal squads. The basic parameters examined were total distance (Fig. 4A, $F_{7,32} = 7.709$, $p < 0.001$), resting time (Fig. 4B, $F_{7,32} = 26.340$, $p < 0.001$), stereotypy time (Fig. 4C, $F_{7,32} = 3.749$, $p = 0.007$), and movement time (Fig. 4D, $F_{7,32} = 5.464$, $p = 0.001$) 72 h after injury. For all parameters except movement time, PROG showed a significant improvement in VitD-normal animals ($p < 0.05$). In deficient animals, however, PROG treatment failed to show

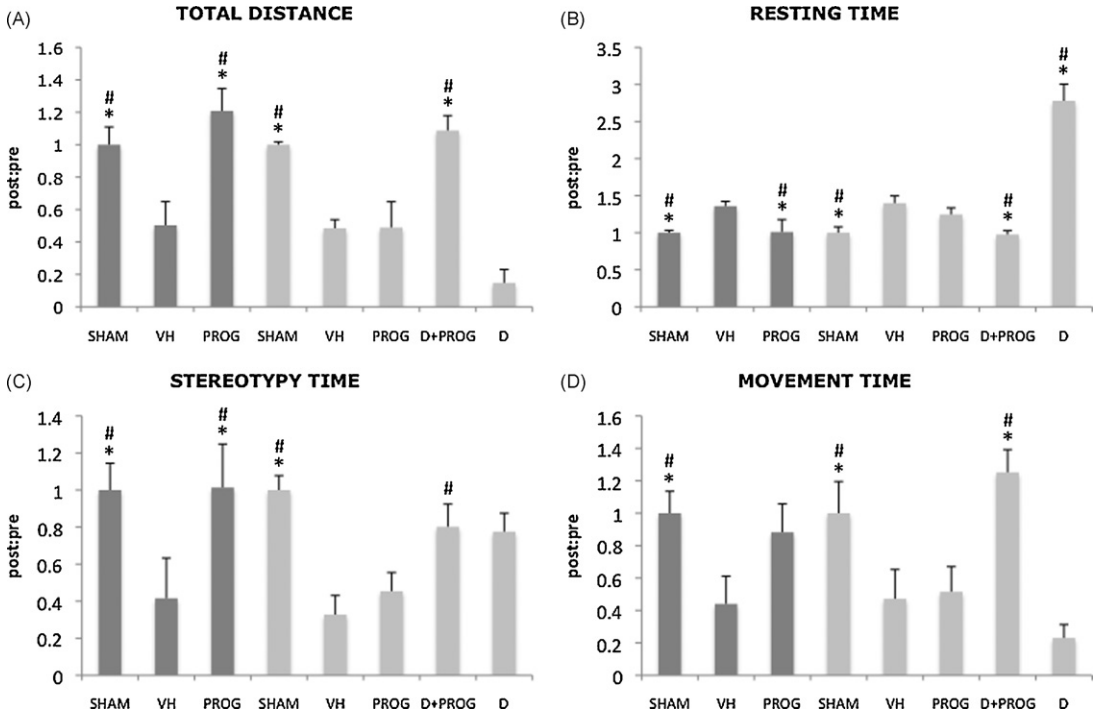

Fig. 4. Open-field activity results for normal (dark grey) and deficient (light grey) animals showing a beneficial effect with combined D + PROG treatment in all cases. All results are normalized to the SHAM group for each nutritional condition. Asterisks (*) denote *post hoc* $p < 0.05$ vs. VH (normal) and hash marks (#) denote $p < 0.05$ vs. VH (deficient).

improvement and only the animals treated with the D + PROG combination showed a significant difference ($p < 0.05$) from animals treated with vehicle. VDH alone was also not beneficial, so the improvement is not a behavioral response to VDH administration.

A number of significant correlations were also observed between our behavioral and molecular data. Total distance was negatively correlated with expression of TNFα ($r = -0.640$, $p < 0.001$), IL-6 ($r = -0.619$, $p = 0.001$), NFκB p65 ($r = -0.551$, $p = 0.004$), and caspase-3 ($r = -0.639$, $p < 0.001$), but not IL-1β ($r = -0.269$, $p = 0.174$). Resting time correlated positively with the expression of TNFα ($r = 0.457$, $p = 0.019$), IL-1β ($r = 0.169$, $p = 0.382$), IL-6 ($r = 0.494$, $p = 0.010$), NFκB p65 ($r = 0.616$, $p = 0.001$), and caspase-3 ($r = 0.630$, $p < 0.001$). Stereotypy time was negatively correlated with TNFα ($r = -0.558$, $p = 0.003$), IL-1β ($r = -0.251$, $p = 0.174$), IL-6 ($r = -0.585$, $p = 0.001$), NFκB p65 ($r = 0.390$, $p = 0.040$), and caspase-3 ($r = -0.429$, $p = 0.018$). Movement time was also negatively correlated with TNFα ($r = -0.544$, $p = 0.003$), IL-1β ($r = -0.382$, $p = 0.034$), IL-6 ($r = -0.506$, $p = 0.007$), NFκB p65 ($r = -0.487$, $p = 0.009$), and caspase-3 ($r = -0.559$, $p = 0.001$).

To explore the interaction between inflammatory molecular events and behavior in more detail, we constructed a GLM with deficiency/injury/treatment as fixed factors (Type I SS due to the logical priority within the factors) and normalized molecular measures as covariates using total distance traveled and resting time as outcomes. Most of the variability in total distance outcome (corrected model: $F = 5.069$, $p = 0.043$, $R^2 = 0.918$) was accounted for significantly ($p < 0.05$) by TNFα ($\eta_p^2 = 0.860$; η_p^2 denotes partial eta squared, an estimate of effect size). Resting time (corrected model: $F = 16.930$, $p = 0.001$, $R^2 = 0.971$) showed significant effects of TNFα ($\eta_p^2 = 0.881$), NFκB p65 ($\eta_p^2 = 0.842$), caspase-3 ($\eta_p^2 = 0.757$), deficiency ($\eta_p^2 = 0.903$), and treatment ($\eta_p^2 = 0.883$). Since much of the variability in behavioral outcome can be accounted for by inflammatory molecular measures, we suggest that this cluster of cytokines is a major component of the behavioral alterations that often occur in the short term after brain injury.

We also looked at GLMs with each molecular measure (at 72 h) as outcome using only the fixed factors (deficiency/injury/treatment). TNFα (corrected model: $F = 32.401$, $p < 0.001$, $R^2 = 0.919$) showed significant main effects for deficiency ($\eta_p^2 = 0.554$), injury ($\eta_p^2 = 0.893$), and treatment ($\eta_p^2 = 0.477$), and a deficiency–treatment interaction ($\eta_p^2 = 0.410$). Also significant here was the model for IL-6 (corrected model: $F = 37.690$, $p < 0.001$, $R^2 = 0.930$), which showed main effects for deficiency ($\eta_p^2 = 0.771$), injury ($\eta_p^2 = 0.865$), and treatment ($\eta_p^2 = 0.492$) as well as interactions effects for deficiency–treatment ($\eta_p^2 = 0.642$) and deficiency–injury ($\eta_p^2 = 0.401$). We interpret these results to indicate a complex relationship of interactions between VitD-deficiency, injury, treatment, and outcome, and one that varies significantly with regard to the different cytokines in the acute phase response. It appears that TNFα and IL-6 play the most prominent roles.

4. Discussion

In this study we examined the interaction of VitD-deficiency with TBI and PROG treatment in aged rats. We know from the literature that levels of vitamin D have systemic effects that may affect recovery; we also know that vitamin D can be neuroprotective and that it interacts with other neurosteroids (Garcion et al., 2002; Losem-Heinrichs et al., 2005). Our results show that: (1) VitD-deficiency increases baseline inflammation in the brains of uninjured aged rats, potentially establishing a detrimental underlying condition; (2) VitD-deficiency increases a number of inflammatory markers after injury in aged rats treated with vehicle at both 24 and 72 h; (3) VitD-deficient animals treated with PROG also have an elevated acute phase inflammatory response compared to rats with normal vitamin D status at 24 and 72 h after TBI; and (4) brain-injured, VitD-deficient animals do not show significant improvement over vehicle when treated with PROG or VDH alone, as measured by levels of inflammatory proteins (TNFα, IL-1β, IL-6, NFκB p65), cell death (cleaved caspase-3), DNA damage (p53), and open-field activity. When PROG treatment is combined with a single 5 μg/kg dose of VDH, however, VitD-deficient animals with TBI show benefits similar to those seen in non-deficient, brain-injured rats treated with PROG.

The first general result is consistent with previous data showing that VitD-deficiency increases systemic inflammation and thereby predisposes to the development of associated conditions such as cardiovascular disease (Martins et al., 2007; Melamed et al., 2008), atherosclerosis (Rammos et al., 2008), multiple sclerosis (Munger et al., 2004; Spach and Hayes, 2005), diabetes mellitus (Giulietti et al., 2004; Grant, 2006), and inflammatory bowel disease (Peterlik and Cross, 2005; Zhu et al., 2005). This elevated inflammation may potentially worsen outcome in deficient animals by causing systemic damage and reducing the victim's ability to cope with the injury; it may also lead to an elevated inflammatory response to injury and more significant secondary damage, as our second general result in vehicle-treated VitD-deficient rats indicates. While only COX-2 is elevated in these animals at 24 h, the acute response at 72 h after injury is increased for most of the markers examined, suggesting an amplified extended inflammatory response. This same effect is seen in PROG-treated VitD-deficient animals, which show significantly increased acute response at 72 h post-TBI. Of note especially is the result for IL-6, as this cytokine is known to interact with vitamin D deficiency (Gurlek et al., 2002; Thien et al., 2005) and hyperparathyroidism (McCarty, 2005), and is a key protein in the acute phase response to injury (Keel and Trentz, 2005; McCarty, 2005). IL-6 and TNFα also figured prominently in regression models connecting short-term behavioral outcomes with the molecular data and deficiency/injury/treatment interactions. This analysis can be interpreted to suggest that the expression cluster of acute inflammatory factors, and especially TNFα and IL-6, can account for the short-term functional impairments observed after TBI and thus they are likely to be major components of the alterations in sickness behavior that often occur after brain injury. Since multiple factors contribute to the injury response, we suggest that the end result of VitD-deficiency is the sum effect of these derangements in individual physiological functions and that extended VitD-deficiency exacerbates the underlying state of vulnerability and frailty already induced by the aging process, making it more likely that the injury cascade will overwhelm endogenous defenses even with PROG treatment. This issue is further complicated by the well-established alteration in steroid and VDH signaling and metabolism with age (Charalampopoulos et al., 2006; Elmadfa and Meyer, 2008), which is consistent with previous findings (Cutler et al., 2007) that a higher dosage of PROG (16 mg/kg vs. 8 mg/kg) is necessary for maximum benefit in older animals.

VDH itself has been reported to be neuroprotective in a variety of *in vitro* and *in vivo* models including cortical infarction (Wang et al., 2000), zinc-induced neurotoxicity (Chen et al., 2003; Lin et al., 2003), experimental autoimmune encephalomyelitis (Garcion et al., 2003), LPS-induced oxidative stress (Asakura et al., 2001), animal models of Parkinson's disease (Shinpo et al., 2000; Wang et al., 2001), and multiple sclerosis in human patients (Hayes et al., 1997; Munger et al., 2004). VDH also affects a number of molecular pathways that, singly or in synergy, may help explain our results. For example, VDH is known to powerfully modulate the innate immune system (Cantorna and Mahon, 2005) and to induce a skew towards anti-inflammatory T_H2 responses (Hayes et al., 2003). It is also known to influence neuronal survival through cell-cycle control and inhibition of neuronal apoptosis (Eelen et al., 2004), MAP kinase activity modulation (Moore et al., 2007), improved DNA stability and repair (Polek et al., 2003), and maintenance of normal intracellular Ca^{2+} levels through downregulation of L-type voltage-sensitive Ca^{2+} channels (Brewer et al., 2001) and upregulation of intracellular Ca^{2+} buffering by calbindin-D28k, parvalbumin, and calretinin (Kutuzova and Deluca, 2004). VDH has further been shown to induce a number of proteins involved in growth and regeneration such as nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) (Samina Riaz and Tomlinson, 2000; Sanchez et al., 2002).

There is also evidence that vitamin D may interact specifically with other neurosteroids such as PROG and estradiol in various tissues. VDH has been found to stimulate estradiol and PROG secretion in human placenta (Barrera et al., 2007), and it is known to interact with PROG and estrogen in maintaining bone health, especially in post-menopausal women (Gaugris et al., 2005; Holick, 2004). VDR gene polymorphisms have also been associated with breast and prostate cancer risk (Lowe et al., 2005; Robsahm et al., 2004), suggesting not only that there may be crosstalk among the different steroid signaling pathways, but also that the hormonal context within which a compound operates may modulate the end effect. Especially intriguing in this respect is the finding that xenobiotic activation of the pregnane X receptor (PXR),

for which PROG is a ligand and by way of which it may exert some of its neuroprotective effects (Bauer et al., 2004; Langmade et al., 2006), can lead to drug-induced osteomalacia by upregulating the expression of CYP24 (Pascussi et al., 2005; Xu et al., 2006), the chief metabolizing enzyme of VDH. Given that PROG is a promising treatment for TBI (Stein et al., 2008), the possibility that it may in some way interact with vitamin D and especially VitD-deficiency could have serious consequences for patients with TBI. It is also possible that, since PROG and VDH activate different neuroprotective mechanisms, a combination of these compounds might improve outcome not only in VitD-deficient subjects but also in those that are nutritionally normal. This is consistent with our results showing a nonlinear synergistic effect of PROG and VDH combination treatment on acute inflammation after experimental TBI.

Our results suggest that in aged rats, chronic VitD-deficiency can significantly exacerbate acute CNS inflammation and attenuate the benefits of PROG treatment after TBI. PROG regains its efficacy, however, when the deficiency is corrected by co-treatment with VDH. Increased inflammation is also observed in the brains of deficient uninjured animals, demonstrating that the previously reported effects of VitD-deficiency on systemic inflammation extend to the CNS and may provide a confounding and potentially detrimental context for both injury and putative treatment. These results may have important implications for the clinical management of TBI in the aged human population.

Disclosure statement

Dr. Donald Stein is entitled to royalty derived from BHR Pharmaceuticals's sale of products related to the research described in this paper and may receive research funding from BHR, which is developing products related to this research. In addition, the author serves as consultant to BHR and receives compensation for these services. The terms of this agreement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

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