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Progesterone, Vitamin D, and the Acute Inflammatory Response After Traumatic Brain Injury in the Aged Rat

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Progesterone, Vitamin D, and the Acute Inflammatory Response After Traumatic Brain Injury in the Aged Rat

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An abstract of
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Graduate Division of Biological and Biomedical Science
Neuroscience

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ABSTRACT

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By Milos Cekic

Traumatic brain injury (TBI) is the greatest single cause of death for people in the Western world younger than 45 and a significant cause of death and disability worldwide. The past decade has also seen a 21% increase in TBI events in people over the age of 65, with the mortality rate in this age group more than twice that of young adults. A large amount of recent evidence has shown that treatment with the neuroactive steroid progesterone (PROG) can attenuate many of the pathophysiological events following TBI in young adult animals as well as human patients, but this has not been specifically investigated in the aged. In this series of studies, we extend the potential application of progesterone (PROG) as a treatment for TBI to older subjects by demonstrating its effectiveness in reducing acute inflammation, cell death, and cerebral edema, and improving short-term behavioral outcome in aged rats after bilateral frontal cortical contusion injury. We also show that vitamin D deficiency, which is virtually endemic in the elderly population in industrialized countries and is associated with a number of systemic problems such as cardiovascular disease, atherosclerosis, and cancer, increases baseline inflammation prior to injury, exacerbates the acute inflammatory response to the injury itself, and attenuates the beneficial effects of PROG treatment after TBI in aged rats. These effects can be overcome by co-administration of PROG with 1,25-hydroxyvitamin D_3 (vitamin D hormone, VDH), the biologically active form of vitamin D and a neuroactive seco-steroid. Since TBI is a complex process affecting the entire organism and not just the nervous system, with the most common proximate causes of death after injury being edema, sepsis, or overwhelming systemic inflammation leading to multi-organ failure, these results have direct translational implications for treatment and early survival in the elderly human population with brain injury. We show that the endogenous systemic hormonal environment can affect brain injury and treatment outcome, and suggest that combination therapies, especially with pleiotropic agents that affect partially overlapping mechanisms, may be better suited than single targeted agents to the treatment of heterogeneous disease processes such as human TBI.

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O Muse, o alto ingegno, or m'aiutate;
o mente che scrivesti ciò ch'io vidi,
qui si parrà la tua nobilitate.
-Dante, *Inferno II*

"What was that? High C or vitamin D?"
-Groucho Marx, *A Night at the Opera*

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION 1

1.1. GENERAL INTRODUCTION 1

1.2. HYPOTHESES, EXPERIMENTAL DESIGN, AND ORGANIZATION 10

CHAPTER 2: TRAUMATIC BRAIN INJURY AND AGING 14

2.1. ABSTRACT 14

2.2. TRAUMATIC BRAIN INJURY: OVERVIEW 15

2.3. AGING: OVERVIEW 41

CHAPTER 3: PROGESTERONE AND TRAUMATIC BRAIN INJURY 51

3.1. ABSTRACT 51

3.2. PROGESTERONE AND TRAUMATIC BRAIN INJURY: OVERVIEW 52

CHAPTER 4: PROGESTERONE IMPROVES ACUTE RECOVERY AFTER TRAUMATIC BRAIN INJURY IN THE AGED RAT 66

4.1. ABSTRACT 66

4.2. INTRODUCTION 67

4.3. MATERIALS AND METHODS 71

4.4. RESULTS 76

4.5. DISCUSSION 90

CHAPTER 5: VITAMIN D DEFICIENCY REDUCES THE BENEFITS OF PROGESTERONE TREATMENT AFTER BRAIN INJURY IN AGED RATS 96

5.1. ABSTRACT 96

5.2. INTRODUCTION 97

5.3. MATERIALS AND METHODS 100

5.4. RESULTS 105

5.5. DISCUSSION 123

CHAPTER 6: COMBINATION TREATMENT WITH PROGESTERONE AND VITAMIN D HORMONE MAY BE MORE EFFECTIVE THAN MONOTHERAPY FOR NERVOUS SYSTEM INJURY AND DISEASE 126
6.1. ABSTRACT 126
6.2. INTRODUCTION 127
6.3. VITAMIN D AND NEUROPROTECTION 130
6.4. WHY COMBINE VITAMIN D AND PROGESTERONE? 146
6.5. CONCLUSION 152

CHAPTER 7: PROGESTERONE AND 1.25-DIHYDROXYVITAMIN D_3 IMPROVE OUTCOME IN YOUNG AND VITAMIN D DEFICIENT OLD RATS WITH BRAIN INJURY 154
7.1. ABSTRACT 154
7.2. INTRODUCTION 156
7.3. MATERIALS AND METHODS 159
7.4. RESULTS 164
7.5. DISCUSSION 184

CHAPTER 8: CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS 189
8.1. ABSTRACT 189
8.2. GENERAL SUMMARY AND CLINICAL IMPLICATIONS 190
8.3. POTENTIAL UNDERLYING MECHANISMS 203
8.4. CLOSING REMARKS AND FUTURE DIRECTIONS 215

REFERENCES 217

LIST OF TABLES

TABLE:

4.1. Summary of progesterone dosage outcomes compared to vehicle 95

LIST OF FIGURES

FIGURE:

4.1. IL-6 levels in aged animals 24 hours after injury 78

4.2. IκB levels in aged animals 24 hours after injury 78

4.3. COX-2 levels in aged animals 24 hours after injury..... 79

4.4. Activated caspase-3 levels in aged animals 24 hours after injury 79

4.5. NFκB p65 levels in aged animals 48 hours after injury 81

4.6. IκB levels in aged animals 48 hours after injury 81

4.7. COX-2 levels in aged animals 48 hours after injury..... 82

4.8. Activated caspase-3 levels in aged animals 48 hours after injury 82

4.9. Edema in aged animals 48 hours after injury..... 84

4.10. P-glycoprotein levels in aged animals 48 hours after injury..... 84

4.11. IL-6 levels in aged animals 72 hours after injury 86

4.12. TNFα levels in aged animals 72 hours after injury 86

4.13. COX-2 levels in aged animals 72 hours after injury..... 87

4.14. Activated caspase-3 levels in aged animals 72 hours after injury 87

4.15. Spontaneous locomotor behavior (total distance traveled) in aged animals 72 hours after injury..... 89

4.16. Spontaneous locomotor behavior (center time) in aged animals 72 hours after injury 89

5.1. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal sham uninjured animals 107

5.2. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal vehicle-treated injured aged animals 24 and 72 hours after injury..... 109

5.3. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal progesterone-treated injured aged animals 24 and 72 hours after injury 111

5.4. TNFα levels in vitamin D deficient compared to vitamin D normal aged animals 24 hours after injury 113

5.5. TNFα levels in vitamin D deficient compared to vitamin D normal aged animals 72 hours after injury 113

5.6. IL-1β levels in vitamin D deficient compared to vitamin D normal aged animals 24 hours after injury 114

5.7. IL-1β levels in vitamin D deficient compared to vitamin D normal aged animals 72 hours after injury 114

5.8. IL-6 levels in vitamin D deficient compared to vitamin D normal aged animals 24 hours after injury 115

5.9. IL-6 levels in vitamin D deficient compared to vitamin D normal aged animals 72 hours after injury 115

5.10. NFκB p65 levels in vitamin D deficient compared to vitamin D normal aged animals 24 hours after injury 116

5.11. NFκB p65 levels in vitamin D deficient compared to vitamin D normal aged animals 72 hours after injury 116

5.12. Activated caspase-3 levels in vitamin D deficient compared to vitamin D normal aged animals 24 hours after injury 117

5.13. Activated caspase-3 levels in vitamin D deficient compared to vitamin D normal aged animals 72 hours after injury 117

5.14. Spontaneous locomotor behavior (total distance traveled) 72 hours after injury expressed as the ratio of post-injury to pre-injury values 119

5.15. Spontaneous locomotor behavior (resting time) 72 hours after injury expressed as the ratio of post-injury to pre-injury values 119

5.16. Spontaneous locomotor behavior (stereotypy time) 72 hours after injury expressed as the ratio of post-injury to pre-injury values 120

5.17. Spontaneous locomotor behavior (movement time) 72 hours after injury expressed as the ratio of post-injury to pre-injury values 120

6.1. Vitamin D metabolism 134

7.1. TNFα levels in normal young animals 72 hours after injury 165

7.2. IL-1β levels in normal young animals 72 hours after injury 165

7.3. NFκB p65 levels in normal young animals 72 hours after injury..... 166

7.4. Activated caspase-3 levels in normal young animals 72 hours after injury 166

7.5. p53 levels in normal young animals 72 hours after injury..... 167

7.6. Serum progesterone levels in aged vitamin D deficient animals..... 169

7.7. Serum 25-hydroxyvitamin D_3 levels in aged vitamin D deficient animals 169

7.8. TNFα levels in vitamin D deficient aged animals 24 and 72 hours after injury 171

7.9. IL-1β levels in vitamin D deficient aged animals 24 and 72 hours after injury 172

7.10. IL-6 levels in vitamin D deficient aged animals 24 and 72 hours after injury 173

7.11. NFκB p65 levels in vitamin D deficient aged animals 24 and 72 hours after injury..... 174

7.12. Activated caspase-3 levels in vitamin D deficient aged animals 24 and 72 hours after injury..... 176

7.13. p53 levels in vitamin D deficient aged animals 24 and 72 hours after injury 177

7.14. Spontaneous locomotor behavior (total distance traveled) in vitamin D deficient animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values 179

7.15. Spontaneous locomotor behavior (resting time) in vitamin D deficient animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values 179

7.16. Spontaneous locomotor behavior (stereotypy time) in vitamin D deficient animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values 180

7.17. Spontaneous locomotor behavior (movement time) in vitamin D deficient animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values 180

8.1. A summary of VDH effects on T_H1/T_H2 cytokines and immune responses..... 210

CHAPTER 1

INTRODUCTION

1.1. GENERAL INTRODUCTION

1.1.1. Aging, Traumatic Brain Injury, and Neuroactive Steroids

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.

Increased mortality and morbidity after injury in the elderly are likely due to a variety of factors. First, endogenous levels of most circulating hormones are lower than

in younger groups, potentially decreasing the intrinsic ability of these subjects to respond to severe injury (Alkayed et al., 2000; Bounds et al., 2003; Gangula et al., 2002). Second, the aged are more likely to have complicating health factors such as altered metabolism or derangement of cardiovascular, hepatic, renal, and immune systems (Laumer et al., 1992; Lipsitz, 2004; Mosenthal et al., 2002). Specific to the nervous system and its ability to recover from injury are the documented age-related loss of blood brain barrier (BBB) function (Campbell et al., 2007), decrease in CYP enzyme activity (Meng et al., 2007), alteration in intracellular Ca^{2+} homeostasis (Mattson and Magnus, 2006; Raza et al., 2007), neuroinflammatory changes (Godbout and Johnson, 2004; Griffin et al., 2006; Kovacs, 2005; Maher et al., 2005), increased oxidative stress (Siqueira et al., 2005), altered neurotrophin metabolism and signaling (Williams et al., 2006), and alterations in basal forebrain cholinergic system function (Geula et al., 2003; Niewiadomska et al., 2002). All of these play key roles in TBI pathophysiology, and most have been implicated as mechanisms of potential neuroprotection in the development of treatments for TBI. This suggests a potential confound in the applicability of data obtained for normal adult subjects to the older population, since precisely the systems involved in the development and treatment of injury are those subject to age-related alteration.

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, 2008b). 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., 2004a; 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 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 hours 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.

1.1.2. Vitamin D Deficiency and Physiology in Aging and TBI

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 (D-deficiency), defined by serum levels of 25-hydroxyvitamin D_3 ($25OHD_3$) below 50nmol/L or 20ng/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), autoimmune conditions (Adorini and Penna, 2008; Baeke et al., 2008), neurodevelopmental disorders (Mackay-Sim et al., 2004; McGrath et al., 2004), diabetes mellitus (Giulietti et al., 2004; Grant, 2006), and 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). The widespread incidence of D-deficiency in the elderly could therefore be an exacerbating factor in TBI in this

population as well as an important confounding factor in data on PROG efficacy in aged subjects.

This prevalence of D-deficiency is especially notable in the context of neurosteroid treatment for TBI since vitamin D in its biologically active form, 1,25-dihydroxyvitamin D_3 (VDH), is not really a vitamin, but rather a steroid in its own right. The term "Vitamin D" is something of a misnomer. Although the name is still in use for popular and historical reasons, VDH 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 1,000 genes (Eelen et al., 2004b) through the nuclear steroid vitamin D receptor (VDR). It has been shown to affect systems similar to those modulated by other hormones and steroids (Garcion et al., 2002), with which it may interact in a variety of physio-pathological contexts (Losem-Heinrichs et al., 2005; Magrassi et al., 1993; Somjen, 2007; Somjen et al., 2007; Weigel, 2007). VDH is also a neuroactive steroid, because both the final activating enzyme and its nuclear receptor are known to be widely distributed throughout the CNS (Garcion et al., 2002).

The physiological role of VDH was long believed to be limited to Ca^{2+} and phosphate homeostasis and the formation and maintenance of bone (DeLuca, 2004; DeLuca and Zierold, 1998; Garcion et al., 2002; Holick, 2003a, b; Ylikomi et al., 2002). Recent evidence, however, suggests a much wider role for this compound, including modulation of the immune system (Cantorna et al., 2004; DeLuca and Zierold, 1998; Griffin et al., 2004; Hayes et al., 2003; Holick, 2003a, b; Mahon et al., 2003), the renin-angiotensin system (Rammos et al., 2008), cardiovascular function (Martins et al., 2007; Melamed et al., 2008), neuromuscular function (Pfeifer et al., 2002), cell cycle control

(Banerjee and Chatterjee, 2003; Zhu et al., 1999), and cancer (Peterlik and Cross, 2005). Since it affects many of the inflammatory and cell death mechanisms involved in TBI and appears to overlap to some extent with the anti-inflammatory and neuroprotective actions of PROG, it may be a potential treatment in its own right or in combination with PROG; it might also be useful in counteracting D-deficiency acutely, potentially making it an appropriate adjuvant treatment to PROG in the context of D-deficiency.

In this series of projects, we explore the effectiveness of PROG as a treatment for TBI in aged rats in the acute phase after injury. This is appropriate since there is evidence that short-term responses can have significant predictive value on long-term survival and recovery of function (Kovacs, 2005; Pape et al., 2007; Stamatovic et al., 2006). While longer-term studies are clearly necessary to confirm short-term results, this series of experiments attempts to provide the groundwork for such studies. We also examine the interactions between TBI, D-deficiency, and PROG treatment in aged subjects. Finally, we explore the question of whether VDH treatment, alone or supplemental to PROG administration, might improve outcome in D-deficient aged rats. These are very important questions in the development of a treatment for TBI that is effective in the aged human population, which may be more vulnerable than younger individuals due to age-related physiological alterations and widespread prevalence of D-deficiency. The results could have significant impact on the clinical management of TBI in the elderly.

1.1.3. Scientific Context and Rationale

No pharmacologic treatment currently exists for TBI. Although a number of agents have shown promise in animal models, every clinical trial to date has failed to show benefits in human patients (Margulies et al., 2008; Narayan et al., 2002) with the exception of two

small clinical trials demonstrating the effectiveness of PROG (Wright et al., 2007; Xiao et al., 2008). Despite the lack of translational success, the general methodology for TBI research is well established, with several standard models of injury in use (Narayan et al., 2002). There are several conspicuously missing questions at the frontiers of experimental TBI treatment, however, and we attempt to begin addressing some of these in this series of studies:

1. *The very young and the very old.* While it may seem commonsense that the very young and very old are not identical with young adults, this fact is largely ignored in TBI research. Given the vastly different physiological milieux these groups at divergent ends of the life-cycle present, any treatment that shows promise in normal adults should be tested specifically in these populations, as the effects may not be the same.
2. *Endogenous physiologic context.* Most experimental TBI research is concerned with the effect of a treatment on the outcome after injury without regard for the endogenous context and the way it may be affected through nutrition, hormonal balance, environmental modification, etc. Given the heterogeneity of the human population in this regard even within a single age group, it is important to consider these variables and control for them or risk significant confounds that might obscure the benefits of potential treatments. This is even more important when considering age groups at either end of the life cycle.
3. *Combination therapy.* Most TBI drug development focuses on monotherapies using a single compound, usually to target a specific mechanism or closely related cluster of mechanisms. While this approach appears reasonable, its lack of

translational success suggests that other alternatives should be explored. These potentially include the use of pleiotropic agents singly targeting multiple divergent mechanisms (such as PROG), or the use of several agents that affect different mechanisms in combination. The heterogeneous and highly complex nature of TBI suggests that successful treatments may be more forthcoming if multiple targets in the injury cascade are targeted, possibly even non-synchronously.

4. *Gender*. Most TBI research is performed on young adult males, but this is hardly an appropriate model of the heterogeneous clinical population. The physiology of females is very different from that of males, and is likely to interact with treatment modalities in a significantly different way. Especially in the case of female sex hormones, the importance of hormonal physiology cannot be overstated. In relation to the age issue as well, females have a very different aging phenotype than males do, with much more significant and abrupt changes such as menopause taking place during the aging process.

In this series of studies, we address the first three of these concerns. We attempt to extend the results demonstrating the effectiveness of PROG treatment for TBI to senescent subjects with TBI in order to determine whether PROG is beneficial in this population and, if so, whether it works according to the same dosing parameters as in young adult animals. We also examine the endogenous context of D-deficiency and its potential interactions with the injury as well as PROG treatment in aged rats. Finally, we investigate the interactions of combined PROG and VDH in the context of D-deficiency

and TBI in aged animals. In light of human studies showing systemic levels of pro-inflammatory cytokines to be the most reliable parameters for monitoring and prognostic purposes after severe trauma (Pape et al., 2007), we focus on the development of inflammation in the acute phase as an indicator of survival and potentially long-term outcome. We do not specifically explore mechanisms of interaction, but rather focus on the proteomic and behavioral effects as an attempt to create a more viable translational model for the human population. The development of a safe and effective clinical treatment for TBI for the human population ultimately motivates this research project, and to that end we primarily focus on a broader systemic understanding of the interactions of TBI, the aging process, and neuroactive steroids.

1.2. HYPOTHESES, EXPERIMENTAL DESIGN, AND ORGANIZATION

These experiments test the overarching hypothesis that PROG will be an effective treatment in aged animals, and that endogenous factors such as D-deficiency will affect this outcome and require supplemental agents to maximize the effect. The specific hypotheses tested were:

1) PROG will exert beneficial effects in the attenuation of acute phase inflammation in aged subjects, although these effects may require a different dosing regimen than that used in younger conspecifics. The benefits observed in terms of cytokine expression will also be evident in more global measures such as edema and open-field behavioral parameters.
2) D-deficiency, as a confounding endogenous factor, will increase baseline inflammation in uninjured aged animals, creating a potentially exacerbating underlying condition.
3) D-deficiency will result in an exaggerated inflammatory response in vehicle-treated injured aged animals compared to vitamin D normal subjects, and this will also be evident in open-field behavior.
4) D-deficiency will attenuate the benefits of PROG treatment after TBI in aged animals.
5) Co-administration of VDH with PROG will counteract the effects D-deficiency and will restore the effectiveness of PROG treatment, but VDH or PROG alone will not have a beneficial effect.

To answer these questions we used a controlled cortical impact (CCI) injury model in senescent rats, some of which were made vitamin D deficient through the administration

of a special vitamin D-null diet. Deficiency was confirmed through serum analysis. Treatments were given in accordance with previously established protocols. All animals were tested on open-field behavioral parameters, which were later correlated to the expression levels of inflammatory cytokine proteins (TNFα, IL-1β, IL-6), transcription factors (NFκB p65), and markers of cell death (cleaved caspase-3) and DNA damage (p53) in each animal as determined by gel electrophoresis and quantified using densitometry. Protein data were obtained at 24 h and 72 h after injury, timepoints chosen as representative of peak early injury and the short-term beginning of recovery. Although our approach to protein analysis is semi-quantitative, all our comparisons were relative and therefore absolute measures were not necessary. We felt that this was a more accurate global approach than specific immunoassays, as the latter, although fully quantitative, are incapable of distinguishing between different isoforms of individual proteins.

We do not attempt to elucidate specific mechanisms for several reasons: 1) we were more concerned with the global impact of our experimental interventions since we were attempting to create a "more" realistic and potentially translational model of the human population; 2) since both PROG and VDH have multiple pleiotropic effects, we reasoned that more than one mechanism is responsible for the observed responses; 3) similarly, since both D-deficiency and TBI are extremely complex processes, we believe that more than one mechanism is involved in the development of injury, and it would be entirely too complicated to attempt to elucidate all such possible mechanisms in a study of this length; and finally, 4) we believe that the effects of treatment are best understood and described at the systemic level where very notions such as "injury" and "behavior"

apply as emergent properties that would be difficult if not impossible to understand through a more reductionist approach. Since the ultimate test of a treatment is the effect it has on the organism as a whole, we limited our observations to that level.

This thesis is organized according to the logical progression of experiments. Chapter 2 provides a somewhat detailed overview of the pathways involved in the evolution of a traumatic CNS injury, with special emphasis on the individual processes suggested as possible points of therapeutic intervention. This chapter includes an overview of the aging process and the associated neuro-immuno-endocrine alterations that may affect the injury process.

Chapter 3 contains a review of PROG physiology, its benefits in the treatment of both experimental and clinical TBI, and its known mechanisms of action. There is also a brief discussion relating PROG to aging and TBI.

Chapter 4 describes the first experiment, which examines several doses of PROG and their effects on the expression of inflammatory cytokines and cell death at 24 h, 48 h, and 72 h after TBI in aged animals. We also look at edema, p-glycoprotein (P-gp) expression as a marker of BBB integrity, and open-field behavioral measures.

Chapter 5 builds on the results of the previous chapter to examine acute inflammation after TBI under conditions of D-deficiency in aged animals. Here we examine the effects of D-deficiency on inflammatory cytokine levels in uninjured animals, vehicle-treated injured animals, and PROG-treated injured animals and compare the results to animals that were vitamin D normal. We also examine cell death, DNA damage, open-field behavioral parameters, and construct several statistical models that demonstrate interaction effects between deficiency, injury, and treatment.

Chapter 6 discusses the rationale for using VDH as a combination treatment with PROG, both as a supplement under vitamin D deficient conditions and on its own in nutritionally normal subjects. We explore the pathways affected by VDH and discuss the benefits of using several pleiotropic agents in combination.

Chapter 7 explores the use of PROG, VDH, and combined PROG and VDH treatments after TBI. First we present proof-of-concept data combining VDH and PROG in young, nutritionally normal animals with TBI. We then investigate this treatment combination in vitamin D deficient aged subjects with TBI using the same experimental parameters as in Chapter 5. Chapter 7 is an elaboration of the experiments in Chapter 5, but with an extended treatment arsenal including VDH and PROG-VDH combination. Here we also provide statistical models that incorporate all the data across nutritional conditions and different treatments.

Finally, Chapter 8 discusses the general conclusions and future directions for this series of experiments, provides some suggestions for the potential mechanisms that may be involved, and outlines a general systems theoretical framework for conceptualizing steroid effectiveness, vitamin D effects, and the injury process. We sincerely hope that some of this research will lead to the development of improved treatments for people with TBI, and to that end have structured the studies to be as translational as possible.

CHAPTER 2

TRAUMATIC BRAIN INJURY AND AGING

2.1. ABSTRACT

Traumatic brain injury (TBI) is the greatest single cause of death for people in the Western world younger than 45 and a significant cause of death and disability worldwide. It is also on the rise in the aged population. TBI is a complex process that develops from a primary injury and evolves via a secondary cascade involving multiple mechanisms such as neurovascular damage and ischemia, ionic imbalance and metabolic failure, glutamate excitotoxicity, oxidative stress, and an exaggerated inflammatory response. TBI is not limited to the central nervous system (CNS), but is rather a problem affecting the entire organism, with the proximate cause of death after brain injury most often being edema, sepsis, or an overwhelming systemic inflammatory response that leads to multi-organ failure. The elderly are further subject to a variety of physiological and metabolic alterations that can affect recovery after major trauma. These specifically include changes in the organization of the CNS, immunosenescence or "inflammaging," and reduced physiological reserve, or frailty. The evolution of and recovery from TBI in senescent subjects should be considered specifically with reference to characteristic and age-related physiology.

2.2. TRAUMATIC BRAIN INJURY: OVERVIEW

2.2.1. Traumatic Brain Injury: Prevalence and Demographics

TBI is a significant cause of death and disability worldwide. Its sequelae comprise a wide range of functional and behavioral changes that may affect sensation, language, thinking, emotional expression or a combination of these. TBI is further associated with the development of epilepsy and increased risk for degenerative conditions such as Alzheimer's and Parkinson's diseases, as well as other brain disorders that become more prevalent as people age (NINDS, 2002). TBI is the most significant single cause of death for individuals in the Western world younger than 45 (Okonkwo and Stone, 2003), and as many as half of all trauma deaths in the United States involve significant injury to the brain (Sosin et al., 1995).

According to recent data, at least 1.4 million Americans sustain a TBI annually; of these, 50,000 die, 235,000 are hospitalized (Adekoya et al., 2002), and 1.1 million are treated and released from an emergency department (Langlois et al., 2004). The Centers for Disease Control (CDC) further estimates that at least 5.3 million Americans (about 2% of the population) currently have a lifelong or long-term need for assistance with daily activities due to TBI (Thurman et al., 1999). Data collected in various areas around the United States suggests that the incidence of hospitalizations due to any kind of brain injury regardless of severity is 175 to 200 per 100,000 individuals (McArthur et al., 2004), and the average brain injury death rate is about 22 per 100,000 people per year (Kraus and McArthur, 2003). In 2000, the direct and indirect costs associated with TBI exceeded $60 billion in the United States (Finkelstein et al., 2006).

The age distribution of hospitalizations due to TBI shows several discrete peaks: the first is in the late teenage years, a second smaller one occurs in the late 30s, and the most pronounced peak occurs in the late 70s (McArthur et al., 2004). The mortality data for moderate or severe brain injury follow a different curve, and studies show a decrease from the very youngest (40.6%) to those aged 5-10 (26.6%), further decreasing in people aged 25-30 (21.8%) (McArthur et al., 2004). This is followed by a monotonic increase culminating with 75-80 year-olds, who demonstrate the highest mortality rates. These rates vary by study, but range from 60.9% (McArthur et al., 2004) to 86.8% (Gomez et al., 2000).

2.2.2. Traumatic Brain Injury: Causes

The causes of TBI also vary across different age groups and studies. According to the CDC, the most frequent causes of TBI are falls (28%), motor vehicle accidents (MVAs) (20%), struck by/against events including colliding with a moving or stationary object (19%), and assaults (11%) (Langlois et al., 2004). An examination of the Healthcare Cost and Utilization Project (HCUP) Nationwide Inpatient Sample datasets for 1998 to 2000 placed MVAs as the most common cause of TBI, accounting for 36.2% of the sample. In this study, falls were second with 20.1%, suicides and self-inflicted injuries third with 9.4%, and assaults were fourth with 7.4% (McArthur et al., 2004). Of note in the HCUP study is the fact that MVAs were four times more frequent than falls as a cause of TBI in people below 50; in those older than 50, however, falls were 3 times more frequent as causes of injury. This age stratification is consistent with more recent data from the CDC showing that death and hospitalizations from fall-related TBI increase with age: in 2005, TBIs accounted for one half of fall-related deaths and 8% of non-fatal hospitalizations

due to falls among older adults (Thomas et al., 2008). These statistics demonstrate the extent of the problem in the older population. While deaths due to TBI have generally decreased in most populations due to improved safety measures, they have increased in the elderly, making it necessary to consider this population specifically in the development of pharmacological interventions for TBI.

2.2.3. The Complex Injury Process

TBI is a complex phenomenon that involves a number of cell types, structures, and processes including brain parenchymal (neurons, astrocytes, oligodendrocytes, microglia), cerebrovascular (endothelial cells, vascular smooth muscle cells), and blood components (platelets, leukocytes, complement, coagulation), as well as more general vascular, endocrine, and neurotransmitter function. A fundamental and emerging concept is the fact that TBI is not a static event but rather a set of processes occurring simultaneously but not always on the same time scale. These processes interact extensively, creating multiple layers of complexity for both the description of the mechanisms of injury and the development of treatment. “Thus, experiments and arguments that attempt to single out one process as important for cell survival … require convincing proof and thus should be viewed with cautious consideration” (Gingrich and Traynelis, 2000).

An effective clinical treatment has been slow in coming despite dozens of phase II and phase III trials. One reason for this clinical failure lies in the complexity of the injury itself, as well as in the fact that in human TBI patients multiple co-morbidities may be present. Another reason lies in the extreme heterogeneity of human brain injuries, which are often multi-systemic. As noted by Margulies et al. (Margulies and Hicks, 2009), most

of the failed clinical drug trials have focused on agents typically designed to target a specific mechanistic pathway in the injury process; the patients, however, were subject to a multitude of toxic events requiring a pleiotropic agent that could act simultaneously or even sequentially on the injury cascade without producing serious side-effects and additional complications (Margulies and Hicks, 2009). Although an understanding of the individual mechanisms involved in TBI is important, the authors conclude that "the heterogeneity of TBI provides a strong rationale for the hypothesis that combination therapies will improve clinical outcomes compared to current single agent interventions" (Margulies and Hicks, 2009).

2.2.4. Approaches to Treating TBI

Approaches to treating TBI can be classed as primary, secondary, and tertiary. The primary intervention program is focused on preventing the cause of the trauma and includes public policy adjustments such as requiring seat belt and helmet use and establishing speed limits. Secondary intervention attempts to minimize the consequences of the trauma once it is experienced. Since much of the damage sustained due to a TBI occurs in the hours and days following the initial trauma, this approach attempts to provide neuroprotection in the short term. Finally, tertiary intervention refers to maximizing functioning and optimizing daily life activities through symptom management and rehabilitation in the long term following an injury. (Park et al., 2008).

In this study we are mostly concerned with the second, neuroprotective, approach, with two fundamental assumptions: 1) that the cascade of secondary damage causes significant and delayed exacerbation of the injury, and 2) that attenuation of this

secondary injury through intervention will result in improved function and recovery (Park et al., 2008).

2.2.5. The Primary Injury

2.2.5.1 Diffuse Axonal Injury (DAI)

The primary insult in closed head TBI has traditionally been categorized into two basic types, focal and diffuse injury, differentiated by different mechanisms of induction and distinct clinical characteristics (Okonkwo and Stone, 2003). Traumatic axonal injury (TAI), or diffuse axonal injury (DAI) as it is more commonly termed in humans (Maxwell et al., 1997), is known to be associated with TBI and is responsible for at least 35% of the morbidity and mortality of patients without mass lesions (Gennarelli et al., 1994; Gennarelli et al., 1982). DAI consists of widespread white matter injury defined as "the scattered destruction of axons throughout the brain of animals and humans that have sustained traumatic brain injury typically involving acceleration/deceleration of the head" (Sahuquillo et al., 2001). Already in the late 1960s and early 1970s, Ommaya and co-workers showed that acceleration alone is sufficient for injury to occur, and that a brain experiencing both translational and rotational acceleration is likely to generate focal damage, but that DAI was induced primarily by rotational and not translational acceleration (Ommaya, 1995; Ommaya et al., 1968; Yarnell and Ommaya, 1969), making DAI a "shear" rather than an impact injury (Wang and Ma, 2010). At its most severe, DAI is commonly associated with severe disability, coma, and death (Cordobes et al., 1986).

DAI was classically thought to occur due to traumatic tearing of axons at the moment of impact, retracting to expel a portion of axoplasm and forming an "axonal

retraction ball" (Gennarelli et al., 1982; Stich, 1961). More recently, however, a far more complex process has been described, with only a subpopulation of axons sustaining the most severe injury rupture on impact and conform to classical descriptions (Maxwell et al., 1993). DAI is rather currently considered to be a process evolving from focal axonal alteration to eventual disconnection (Povlishock et al., 1983). This progressive damage was first described by Pettus et al. (Pettus et al., 1994; Pettus and Povlishock, 1996), who demonstrated microscopic mechanoperforation of the axolemma by observing influx of normaly excluded extracellular tracers such as horseradish peroxidase. An increase in axonal permeability is thought to allow for an influx of calcium that triggers the activation of a variety of cysteine proteases such as calpain and caspase that locally degrade the axonal cytoskeleton and are considered the key players in subsequent axonal pathology (Bartus et al., 1999; Buki et al., 1999). Degradation of the neurofilament and microtubular support of local intraaxonal transport further leads to vesicular and organelle accumulation that results in axonal detachment (Buki and Povlishock, 2006). Local cytoskeletal collapse does not always lead to impaired axonal transport nor to the classical findings of axonal swelling, however, in part because the massive influx of Ca^{2+} in more severe axonal injury induces a converson of anterograde to retrograde transport (Marmarou et al., 2005). The intracellular Ca^{2+} overload also induces mitochondrial injury and the activation of cytochrome-c (Cai et al., 1998), with futher activation of caspase and eventual axonal failure and disconnection (Stone et al., 2001).

DAI is common in both diffuse and focal trauma at all severity levels (Adams et al., 1989; Cordobes et al., 1986), and has been shown to reliably predict poor survival and long-term outcome (Fork et al., 2005). It is often underdiagnosed, especially in cases of

mild TBI, but nonetheless leads to significant long-term behavioral deficits (Li et al.). Since DAI is an evolving progressive process, it is potentially amenable to therapeutic intervention through a number of mechanisms also involved in the secondary injury cascade such as Ca^{2+}-induced mitochondrial damage and caspase activation (Buki and Povlishock, 2006). These mechanisms are further discussed below.

2.2.5.2. Focal Injury

In contrast to the "shearing" forces of DAI, focal injury is generally more apparent and is caused by contact or inertial forces resulting from an impact to the skull. Whereas the primary injury in DAI is itself a process, the primary lesion in focal injury is essentially "dead on impact," in that mechanical forces can cause immediate destruction of neurons and their processes, glia, and vascular tissue. Here treatment must be relegated to the cascade of secondary injury initiated by the primary insult (Sahuquillo et al., 2001). The effect of focal lesions on mortality and morbidity is primarily dependent on their extent, location, and progression in the acute post-TBI period (Okonkwo and Stone, 2003). They are generally characterized by cortical contusions and/or hemorrhage inside and outside the brain parenchyma, where they usually lead to local necrosis due to ischemia. Contusions are typically found at the apices of gyri, and most frequently occur at the "frontal poles, orbital frontal lobes, the lateral and inferior surfaces of temporal lobes, and cortex above the Sylvian fissure" (Gaetz, 2004). They can be classified as contusions that occur immediately below skull fractures, coup contusions at the impact site, contrecoup contusions in regions distant to and sometimes opposite the coup, herniations, and gliding contusions frequently associated with DAI.

2.2.5.3. Hemorrhaging

Several types of hemorrhage are associated with TBI, including "intracranial haematoma associated with a direct rupture of a blood vessel, extradural haematoma associated with skull fracture, and acute subdural haematoma caused by a rupture of the bridging veins of the dura or possibly cortical arteries" (Gaetz, 2004). In addition to direct damage, the disruption of neuronal and vascular tissue causes a local ischemic area that can be a significant factor in the induction of concentric zones of progressive secondary injury (Gennarelli, 1993). These zones constitute the perilesional area or traumatic penumbra and extend from the primary lesion to include regions with traumatic damage without destruction of neural tissue, structural disruption, and areas potentially affected by edema and ischemia caused by the primary lesion that may be further vulnerable to inflammatory and cytotoxic phenomena (Gennarelli, 1993).

2.2.6. The Secondary Injury Cascade

"Secondary injuries are multiple, parallel, interacting and interdependent cascades of biological reactions caused by primary injury" (Park et al., 2008). The secondary pathophysiological events that occur in the short-term after TBI can be divided into three categories: those that occur immediately, those that develop over the first 24 hours, and those that continue to evolve in the period between 24 and 72 hours after injury. These events are mediated by several basic mechanisms at the cellular level, including metabolic derangement, calcium- and free radical-induced damage, receptor and channel dysfunction, and inflammation.

It is interesting to note that secondary injury mechanisms are almost identical in both TBI and stroke-related focal injuries, suggesting that a pharmacological agent that interferes with these destructive processes may be beneficial in both types of insult. In the

case of TBI, these phenomena lead to pathological events on a more global scale such as altered cerebral blood flow, brain swelling and edema, elevated intracranial pressure, and multi-organ system dysfunction, all of which ultimately determine patient outcome (Sahuquillo et al., 2001; Zygun, 2005). Long-term consequences of secondary damage include deafferentation, demyelination, glial scarring, cell death, altered neurotransmission, neuroplasticity that may be either adaptive or pathological, and, on a more global scale, seizures as well as cognitive and sensorimotor behavioral impairment (Povlishock and Katz, 2005; Raghupathi, 2004). Gene expression changes related to glucose metabolism, oxygen utilization, ionic channels, membrane transporters, receptors, cytoskeletal integrity, and inflammation tend to mirror the pathophysiological process and the subsequent molecular and biochemical pathways of secondary damage (Marklund et al., 2006).

2.2.6.1. Neurovascular Damage and Ischemia

The initial result of a primary traumatic event is an impairment of regional cerebral blood flow (rCBF) that severely limits the availability of metabolic substrates such as glucose and oxygen to the brain. A reduction in rCBF has been associated with unfavorable outcomes after TBI (Golding, 2002). Although rCBF and brain metabolism are tightly coupled under normal conditions, injury results in neurovascular uncoupling, with increased glucose demand being accompanied by decreased local rCBF (Ginsberg et al., 1997; Richards et al., 2001). The interruption of blood flow after TBI results in an ischemic state as the availability of ATP, used for maintenance of ionic gradients across cell membranes, especially in neurons and astrocytes, is exhausted. An early post-TBI increase in glycolysis is the result of cellular efforts to maintain membrane gradients,

which, along with the decreased rCBF due to mechanical damage, leads to energy failure, alterations in intracellular pH, failure of the energy-dependent Na^+/K^+-ATPase pump and other ion channels, membrane depolarization and an influx of Na^+, and increased intracellular Ca^{2+}, which seems to be the "final common pathway" (Dumont et al., 2001) for development of irreversible secondary damage. This makes control of cerebral perfusion pressure (CPP) of key importance in the clinical management of TBI patients, although optimal CPP is still a matter of debate (Golding, 2002).

Mechanical injury also causes disruption of the blood-brain barrier (BBB), which further contributes to ischemia. In fact, the "immediate and prolonged opening of the BBB is a hallmark of TBI pathophysiology and results in extravasation of blood components, including RBCs, plasma proteins and water (vasogenic edema). BBB opening be brought about by transient elevation of arterial pressure (which occurs at the moment of injury) which forces apart tight junctions in the most vulnerable part of the circulation (the large arteries and arterioles), shear stresses and also by the release of substances from damaged or activated cells" (Golding, 2002). The key consequences of BBB breakdown in the short term are further reduction in blood flow (through swelling of astrocyte endfeet, a primary component of the BBB, and endothelial vasoconstriction), the opening of the BBB to immune infiltration and inflammation (through chemoattraction of neutrophils and monocytes to the damaged area and subsequent diapedesis into the brain parenchyma), and initiation of the coagulation cascade through the exposure of contact proteins such as tissue factor on damaged endothelial cells and activated platelets (Varga-Szabo et al., 2008). Initial cerebrovascular damage therefore

results in the key processes that mediate further local and systemic damage: edema, ischemia, inflammation, and coagulopathy.

2.2.6.2. Edema

A characteristic presentation in TBI patients is "brain edema," defined as "an increase in net brain water content which leads to an increase in tissue volume" and further described as "a concert of complex molecular and cellular, structural and functional changes in blood-brain barrier (BBB) function, microcirculation, cell volume regulation and autodestructive mediators" (Pappius, 1974; Unterberg et al., 2004). Since the volume of the intracranial space is fixed, any increase in brain volume leads to an increase in intracranial pressure (ICP), which can further reduce CPP and rCBF, and, more importantly, lead to herniation of parts of the brain that can be fatal. In addition to maintenance of adequate CPP, therefore, ICP is another major issue in the emergency management of TBI patients. The paradox here is that an attempt to increase CPP by increasing blood pressure (to cope with reduced compliance and increased vascular resistance) can lead to edema formation and increased ICP, further exacerbating the situation (Unterberg et al., 2004).

There are several types of edema of significance in the secondary injury after TBI. One type, vasogenic edema, occurs due to breakdown in the integrity of the BBB, specifically the increased permeability of capillary endothelial cells due to tissue necrosis, which allow an increase of fluid in the interstitial space. The second and more significant type of edema in TBI, cytotoxic edema, occurs without BBB damage and results in the swelling of individual neuronal or glial cells (Marmarou et al., 2000), potentially reducing the extracellular space from 20% to 4% in experimental models of

ischemia (Klatzo, 1967). Since astrocytes can swell five-fold and outnumber neurons by 20 to 1 in humans (10 to 1 in rats), most brain swelling appears to be due to glial pathology (Unterberg et al., 2004). Cytotoxic edema is caused by ion-pump failure due to energy depletion after ischemia, increased membrane permeability to Na^+ and K^+, and cellular uptake of osmotic solutes. Water follows these increased concentrations of ions and solutes inside the cell, causing swelling (Unterberg et al., 2004). Additional factors contributing to edema after injury are various inflammatory cytokines that can cause both cell death and increased vascular permeability, further amplifying the problem.

2.2.6.3. Protein Channels and Edema

An important and emerging concept in edema management is control of expression levels of the Aquaporin4 (AQP4) bi-directional water channel. AQP4 is highly expressed in astrocytic endfeet (Vajda et al., 2002), the part of the cells involved in contacting capillaries and maintaining the BBB, and also the part with the majority of observed swelling after injury (Bullock et al., 1991). AQP4 knockout mice in fact show reduced mortality and brain edema after middle cerebral artery (MCA) ligation (Manley et al., 2000). Another channel that may be involved is P-glycoprotein (P-gp), which is an integral part of a healthy BBB and serves to extrude toxic substances from cells (Miller et al., 2008). Modulating the expression of these channels may provide a mechanism by which to control the development of edema post-TBI

2.2.6.4. Glutamate Excitotoxicity

First described by Olney (Dumont et al., 2001; Olney, 1978), excitotoxicity is a major contributor to secondary damage after brain trauma. Neuronal depolarization due to hypoxia causes a dramatic increase in extracellular glutamate, the main excitatory

neurotransmitter in the CNS (Gaetz, 2004). This happens in two ways: 1) release of glutamate from neurons injured by the primary insult and those whose ionic homeostasis has been disrupted by energy failure, and 2) disruption of glutamate reuptake and recycling by astrocytes, one of their major functions, due to damage and edema (Dumont et al., 2001). The presence of abnormally high extracellular glutamate levels activates various neuronal receptors (such as the N-methyl-D-aspartate, or NMDA, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or AMPA, glutamate receptors) that cause depolarization and activation of voltage-sensitive Ca^{2+} channels (VSCCs), especially L- and N-type VSCCs (Park et al., 2004). Influx of Ca^{2+} can further amplify the glutamate cascade by stimulating more glutamate release via a positive feedback loop (Choi, 1988). Increases of glutamate up to 10-15-fold have been reported in focally adjacent extracellular fluid for up to 4 days after injury in humans (Schroder et al., 1995). The consequence is, once again, massively increased intracellular calcium. Since oligodendrocytes and astrocytes also express AMPA and kainate glutamate receptors, they are affected by the extracellular increases of this neurotransmitter as well (Sahuquillo et al., 2001). Oligodendrocytes appear to be the cell type most vulnerable to excitotoxic mechanisms, partly because their AMPA receptors appear to be more permeable to Ca^{2+} and partly because they have a significantly poorer ability to buffer calcium (Profyris et al., 2004).

2.2.6.5. Calcium-Mediated Secondary Injury Cascade

Increased intracellular calcium is critical in the progression of secondary damage after injury. It is elevated not only by membrane depolarization (due to metabolic failure and glutamate signaling) and influx, but also by massive release from the mitochondrial

matrix into the cytoplasm and by the inability of cells to extrude excess Ca^{2+} due to metabolic failure (Nicholls and Ward, 2000). Regardless of the mechanism, derangement of intracellular calcium homeostasis leads to "cell death via secondary cascades including activation of protein kinases, phospholipases, proteases, ROS [reactive oxygen species] and mitochondrial dysfunction" (Park et al., 2004).

High intracellular calcium interferes with the normal functioning of mitochondria, leading to both morphological and physiological impairments that eventually lead to a breakdown in cellular respiration (Okonkwo and Stone, 2003). Excessive Ca^{2+} leads to mitochondrial swelling (due to mitochondrial sequestration of intracellular calcium), opening of the permeability transition pore, and the release of cytochrome c, a key initiator of the apoptotic signaling cascade (Park et al., 2008). In addition, increased intracellular calcium leads to activation of calcium-dependent proteases such as calpains, caspases, phospholipase A2, lipoxygenase, and cyclooxygenase. Calpain activation leads to degradation of the cytoskeleton, while activation of caspases initiates apoptotic cell death, and other calcium-dependent proteases destroy cell membranes and dissolve ultrastructural cellular components such as neurofilamanets (Dumont et al., 2001). The activation of lipase, lipoxygenase, and cyclooxygenase further leads to the presence of arachidonic acid metabolites such as thromboxanes, prostaglandins, and leukotrienes, which in turn lead to reduced blood flow due to vasoconstriction and platelet aggregation, increased inflammatory response, and the development of reactive oxygen species (ROS). All of these processes further amplify the cascade of secondary damage and exacerbate injury.

2.2.6.6. Oxidative Stress

Oxidative stress occurs when the antioxidant ability of cells to cope with ROS, normally natural byproducts of cellular metabolism, is overwhelmed. Important sources of ROS are arachidonic acid metabolites upregulated by injury and immune processes. The reperfusion phase of ischemia/reperfusion injury is also key in the development of oxidative stress. During the hypoxemia caused by ischemia, ATP is degraded to ADP and AMP, leading to ion pump failure and disturbances of membrane permeability. Glycolysis is increased during this time, leading to partial proteolysis of xanthine dehydrogenase, an enzyme involved in glycolysis, to xanthine oxidase (Profyris et al., 2004). The presence of AMP leads to the production of hypoxanthine, which is degraded by xanthine oxidase to xanthine and finally to uric acid during the reperfusion phase, generating superoxide anions (O_2^-) from the newly available oxygen. Superoxide can be further reduced to hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^-). These ROS further amplify the Ca^{2+} disturbance and can be extremely destructive to cell components such as lipids, proteins, and nucleic acids (Keel and Trentz, 2005).

When ROS interact with lipids, peroxides are produced that can further exacerbate membrane damage. Interaction of ROS with proteins extends the damage by causing protein fragmentation, oxidation of amino acid side chains, and forming protein cross-linkages. Finally, by interacting with thymine, ROS can produce single strand breaks in DNA, causing irreversible damage and leading to necrotic and apoptotic cell death (Profyris et al., 2004). Further sources of ROS are necrotic cells (which upon disintegration release ROS into the interstitial space) as well as neutrophils and macrophages, which produce these species during their characteristic “oxidative burst” in the early phase of the inflammatory response.

In addition to ROS, reactive nitrogen species (RNS) further contribute to oxidative damage. These species are created by the actions of inducible nitric-oxide synthase (iNOS) in the cerebrovascular endothelium, which enhances the production of nitric oxide (NO), a potent vasodilator, to counter the vasoconstriction due to edema, inflammation, and the presence of arachidonic acid metabolites. While NO production is of benefit in this case, it also results in the presence of highly reactive moieties that can still further exacerbate the secondary injury cascade by oxidation of cellular components (Rees et al., 2008; Valko et al., 2007).

2.2.6.7. Apoptosis and Necrosis

The two main ways a cell can die are by necrosis and apoptosis. Necrosis is a chaotic and disorganized path to cellular death, caused by massive failure, either through direct (mechanical) damage or the overwhelming action of ROS. This type of death also leaves many fragments and adds to local damage by creating conditions for local inflammation. Necrosis is the type of cell death usually created by the primary injury, and there is currently very little that can be done clinically to affect it.

The other pathway is apoptosis, or programmed cell death, which proceeds through a highly ordered sequence of cell dissolution and can be activated by a variety of mechanisms, culminating in the activation of caspase-3, the final effector in the cascade. "The modes of cell death are distinct but combine to create a picture of cytological chaos; this is an inevitable outcome of a cell's attempt to superimpose a highly structured process (apoptosis) onto a more chaotic, non-linear process" (Roy and Sapolsky, 1999). Since it requires energy, apoptotic cell death is most frequently seen in the ischemic penumbral regions and rarely at the core of an injury (Sahuquillo et al., 2001). Intrinsic

initiators such as mitochondrial damage and the release of cytochrome c (and related mitochondrion-associated proteins like Bax and Bad) and DNA damage can cause cells to react to the extracellular presence of a death signal (e.g., tumor necrosis factor-α—TNFα—or iNOS) or withdrawal of a survival signal (e.g., nerve growth factor, NGF) (Dumont et al., 2001; Okonkwo and Stone, 2003). In addition to activating the caspase cascade, DNA damage can initiate apoptosis by activating cellular repair/reproductive machinery and proteins such as cyclin and cyclin-dependent kinases (CDKs), which lead to cell death in terminally differentiated neurons due to the inability of these cells to enter mitosis (Zhu et al., 1999). Since apoptosis is an important part of the development of secondary injury, it does afford a potential target for treatment.

Apoptosis occurs biphasically after TBI: the first phase occurs in the early period after injury alongside necrosis and affects multiple cell types, while the second is predominantly confined to white matter and involves oligodendrocytes, which are more susceptible to the loss of trophic support (Beattie et al., 2000), and leads to demyelination. The first wave of apoptosis occurs around 6h after injury near the central region of the lesion and continues for several days during the evolution of the acute phase (Profyris et al., 2004). After this, the presence of apoptotic cells at the center decreases, with a concomitant apoptotic increase in more distant regions, potentially due to loss of trophic support or death signaling from the region of primary injury (Crowe et al., 1997). An example of this is the clinically significant degeneration of cells in the nucleus basalis magnocellularis (NBM), the main source of acetylcholine (ACh) to the cortex, after frontal cortical injury. The loss of NBM cells occurs as part of the secondary injury and is

a significant event in post-injury memory impairment (Connor et al., 1992; He et al., 2004b; Murdoch et al., 2002).

2.2.6.8. Inflammation

Inflammation is one of the key contributors to secondary injury after TBI. While a number of studies have demonstrated that initial immune system response is essential to recovery (Chan, 2008; Morganti-Kossmann et al., 2001; Shohami et al., 1999b; Stahel et al., 2000), prolonged or overactive inflammation is a known mechanism of significant damage. Although the CNS has traditionally been considered "immune privileged," recent data show that this is not the case (Skaper, 2007), especially after trauma-induced BBB dysfunction. Immune system response to injury is biphasic: it consists of an initial activation of microglia and infiltration by polymorphonucleocytes, mostly neutrophils, through the damaged BBB (Holmin et al., 1998), followed by monocyte and macrophage infiltration and activation of astrocytes and various other immune components. Initial neurovascular damage exposes tissue factors and initiates the inflammatory/coagulation cascade. It also upregulates endothelial cell adhesion molecules (CAMs) such as intercellular adhesion molecule 1 (ICAM-1), vascular (V)-CAM, platelet endothelial (PE)-CAM, P-selectin and E-selectin, which attract passing neutrophils, assist in their binding to endothelial cells, and initiate diapedesis into brain parenchyma. Coagulation proteins also activate complement, which can cause membrane damage and apoptosis. Complement C5a further interacts with actin, a cytoskeletal protein released into the bloodstream in response to massive injury (Gomme and Bertolini, 2004), and becomes a potent chemotactic signal for further inflammation. Various proteins released by necrotic or damaged cells further amplify this response. By 24h after injury, the neutrophils enter

the lesion and phagocytose debris, further generating pro-inflammatory cytokines and proteases that enhance leukocyte infiltration and chemotaxis, activate glia, and cause greater neuronal damage (Profyris et al., 2004). As mentioned before, they also generate a respiratory burst that produces ROS.

By 48 hours after injury, however, the infiltrate shifts from neutrophils to monocytes, macrophages, reactive microglia, natural-killer cells, T-helper cells, and T-cytotoxic suppressor cells (Sahuquillo et al., 2001). As monocytes and microglia differentiate into macrophages by 72h after injury, they further phagocytose debris and release key pro-inflammatory cytokines such as TNFα, Interleukin-1 (IL-1), and Interleukin-6 (IL-6) (Leskovar et al., 2000). Binding of both IL-1 and TNFα to receptors initiates nuclear factor κB (NFκB) signaling: "[T]his is crucial as active NFκB stimulates the production of inflammatory mediators such as ROS, cytokines, inducible nitric oxide synthase (iNOS), prostaglandin synthase-2, arachidonic acid, proteases and endothelial cell adhesion molecules (CAMs)" (Profyris et al., 2004).

All these cytokines and metabolites serve to increase further recruitment of leukocytes and thrombocytes, increasing BBB permeability and amplifying the entire cascade. The release of inflammatory mediators causes astrocytes to release neurotrophic factors such as NGF (Heese et al., 1998), potentially also serving to control the initial damage. Delayed induction of anti-inflammatory TGFβ, which begins more slowly than the pro-inflammatory TNFα and IL-1 and peaks at 7 days after injury (Semple-Rowland et al., 1995), also tends to promote survival. These data are consistent with observations that inflammation seems to be required for recovery, but that it can be extremely

destructive when it is overactive and prolonged (Chan, 2008; Morganti-Kossmann et al., 2001).

This pro-inflammatory versus anti-inflammatory dynamic seems to be an important factor in the development of extended and damaging inflammation and in the genesis of the most likely cause of death for TBI victims, multi-organ system (MOS) dysfunction and failure (Lee et al., 2001). Induced by high levels of IL-1, macrophages function as antigen presenting cells (Cassatella, 1995), which then cause undifferentiated $CD4^+$-expressing T cells to mature and differentiate. These cells are critical in the mounting of an adequate response to damage and infection. Naïve, or T_H0, $CD4^+$ cells can differentiate into one of two phenotypes: in the presence of Interleukin-12 (IL-12) they develop pro-inflammatory T_H1 characteristics, which consists of production and release of TNF and Interferon-γ (IFN-γ), further attraction of macrophages and monocytes, and activation of cell-mediated immunity and inflammation. In the presence of Interleukin-4 (IL-4), T_H0 cells develop an anti-inflammatory T_H2 phenotype, characterized by further production of IL-4 as well as Interleukin-5 (IL-5) and Interleukin-13 (IL-13), binding to B cells, and activation of antibody-mediated immunity (Desmedt et al., 1998; Kidd, 2003; Onoe et al., 2007; Tausk et al., 2008). These two general phenotypes mutually inhibit each other.

A T_H1 response activates cell-mediated immunity against intracellular pathogens such as viruses and certain bacteria. An overactive T_H1 response, however, can lead to excessive inflammation and autoimmune conditions such as multiple sclerosis (MS) and Type 1 diabetes mellitus. A T_H2 response, on the other hand, is optimal for mounting an antibody-mediated defense against extracellular pathogens, but this can manifest

pathologically in the development of conditions such as asthma and other IgE-mediated allergies. A recent addition to this bilateral scheme consists of a Treg phenotype, stimulated by combinations of TGFβ, IL-6, and Interleukin-10 (IL-10) (Costantino et al., 2008), which by itself inhibits the activity of NFκB. Treg cells are also anti-inflammatory, and the divergence of action dependent on the chemical milieu and timing of cytokine expression may account for the fact that IL-6 has shown both inflammatory and anti-inflammatory activity in different studies (Keel and Trentz, 2005). The key point here is that T_H differentiation is of fundamental importance in the development of pathological inflammation in the hours and days after injury as the damaged system attempts to establish a dynamic equilibrium between the T_H1 and T_H2 populations and pro- and anti-inflammatory activity.

This balancing act has consequences not only for the local injury environment, but for the organism as a whole. "It seems that the host defense response tries to strike a fine balance between SIRS [systemic inflammatory response syndrome] and CARS [compensatory anti-inflammatory response syndrome], to induce reparative mechanisms and limit entry or overload of microorganisms, on the one hand, and to avoid autoaggressive inflammation, with secondary tissue damage and susceptibility to infections, on the other hand" (Keel and Trentz, 2005). In fact, "dysregulated inflammatory mechanisms are thought to play a crucial role in the development of multiple organ dysfunction syndrome" (Zygun, 2005).

2.2.7. Systemic Dysfunction and Traumatic Brain Injury

Severe trauma is the most frequent cause of death in people below age 40 (Demetriades et al., 2004). Deaths occurring in the immediate and very early aftermath of the injury are

generally caused by severe primary injuries to the brain or by hemorrhagic shock caused by severe blood loss (Gennarelli et al., 1994). Later mortality is caused by secondary CNS injuries and failure of host defense that occurs as a result of "imbalance between these dual immune responses, with an overwhelming release of pro- or anti-inflammatory mediators...responsible for organ dysfunction…Endothelial cell damage, accumulation of leukocytes, disseminated intravascular coagulation (DIC) and microcirculatory dysfunction finally lead to programmed cell death (apoptosis) and necrosis of parenchymal cells (microenvironment theory) with the development of multiple organ dysfunction syndrome (MODS) or multiple organ failure (MOF)" (Keel and Trentz, 2005).

In many cases this is what seems to be the final cause of death. For example, a study of TBI patients by Zygun and colleagues found that 89% developed dysfunction and 35% failure of at least one non-neurological organ (Zygun et al., 2005). In this study, failure of one non-neurological organ system was associated with a 40% death rate, and this increased to 47% with the failure of two systems. In another study, 80% of TBI patients developed respiratory and 82% developed cardiovascular failure (Zygun et al., 2003). Multi-organ damage as a consequence of neurologic injury is not limited to TBI patients: in another study of patients with aneurismal subarachnoid hemorrhages, 81% developed at least one extra-neurologic organ system dysfunction, and 26% developed failure; mortality was 31% for one organ failure, 91% for two, and 100% for patients who developed three or more (Gruber et al., 1999). The important point here is that TBI does not occur in isolation and that the systemic effects of brain injury may be important modulators of a patient's ability to survive severe trauma.

The non-neurologic systems most frequently affected by CNS injury appear to be the cardiovascular and pulmonary. The key etiology in the development of dysfunction in these two systems appears to be the massive release of catecholamines that occurs after neurologic injury and can lead directly to myocardial dysfunction (Dujardin et al., 2001) and the development of severe pulmonary edema and respiratory failure (Macmillan et al., 2002). Other mechanisms of fundamental importance are inflammation, coagulation, and infection (Zygun, 2005).

Significant elevations of inflammatory cytokines have been observed in cerebrospinal fluid (CSF), the systemic circulation (Hirashima et al., 1997; McKeating et al., 1997), and even in the gut (Chen et al., 2007) after TBI. Given the significant blood flow to the brain under normal conditions, and a damaged BBB after injury, the brain essentially acts as a filter for the blood, allowing the systemic dissemination of inflammatory mediators away from the area of local injury. This eventually leads to systemic inflammatory response syndrome (SIRS), "characterized by the local and systemic production and release of different mediators, such as pro-inflammatory cytokines, complement factors, proteins of the contact phase and coagulation systems, acute phase proteins, neuroendocrine mediators and an accumulation of immunocompetent cells at the local site of tissue damage" (Keel and Trentz, 2005). Concurrent with this response is a systemic anti-inflammatory response, and, as mentioned above, it appears that it is the ability to strike a balance between these two opposing directions that determines eventual survival. The presence of inflammation at and near the site of injury therefore has much more significant effects than just the

contribution to local secondary damage; it appears to be a key mechanism in TBI-associated mortality (Lim and Smith, 2007).

2.2.7.1. Activation of the Plasmatic Cascade

The systemic presence of inflammatory factors further leads to activation of the plasmatic cascade and the acute phase reaction. The plasmatic cascade consists of complement, coagulation, and kinin-kallikrein systems (Keel and Trentz, 2005). Complement factors are innate immune system components in the blood that serve to destroy invading bacteria by forming opsonins, anaphylatoxins, and the membrane attack complex (MAC) (Stahel et al., 1998). The opsonins C3b and C4b are involved in phagocytosis of cellular debris, while C3a and C5a are inflammatory mediators, supporting phagocytic cell recruitment and chemotaxis, adhesion of leukocytes to endothelium, and release of vasoactive substances, ultimately leading to an increase in vascular permeability and the development of edema (Mollnes and Fosse, 1994). C5a can also induce apoptosis and necrosis of parenchymal cells via MAC activation (Stahel et al., 1998).

Massive injury and inflammation also induce the coagulation cascade, which results in both intravascular fibrin clots (DIC) and concomitant anti-coagulation activity that leads to wasting of clotting factors and coagulopathy in the first 24 hours after injury (Harhangi et al., 2008; Stein and Smith, 2004). DIC can lead to ischemic damage and further disturbances of the microcirculation, while consumptive coagulopathy can lead to significant rebleeding (Stein and Smith, 2004). Additionally, the kinin-kallikrein system, which further activates both complement and coagulation, leads to the release of systemic bradykinin, a potent vasodilator, which increases vascular permeability and contributes to the genesis of edema (Sugimoto et al., 1998).

2.2.7.2. Systemic Release of Inflammatory Cytokines

Systemic release of inflammatory cytokines such as TNFα, IL-1β, and especially IL-6 induces the acute phase reaction, a systemic response to inflammation, mainly executed by the liver, that resets a number of homeostatic set points in order to improve defense and adaptation (Gabay and Kushner, 1999). These changes exhibit considerable variability, from maintaining or modulating inflammation to playing adaptive roles. During the acute phase, a number of acute phase proteins (APPs) are upregulated (positive APPs such as C-reactive protein, fibrinogen, prothrombin) while others are downregulated (negative APPs such as albumin, high-density lipoproteins (HDL), antithrombin III (ATIII), and protein C). Although these changes are presumed to improve survival, in cases of severe injury such as TBI an increased proportion of positive to negative APPs has been shown to accelerate the development of DIC and to have generally pathophysiologic effects (Keel and Trentz, 2005). Acute inflammation has also been observed to increase levels of whole-body oxidative stress, another important mediator of systemic injury (Shohami et al., 1999a).

2.2.8. Potential Pathways of Intervention

All the pathways involved in secondary and systemic injury discussed above are potential points of clinical intervention. Although a number of phase II and III clinical trials of potential treatments for moderate and severe TBI based on these specific mechanisms have been attempted, however, most of them have failed (Narayan et al., 2002). Major reasons for these failures lie in an incomplete understanding and insufficient appreciation of the complexity of the secondary injury process and consequent attempts to develop a "magic bullet" to interfere with isolated mechanisms rather than multiple interacting

processes (Margulies et al., 2008; Narayan et al., 2002). The most promising therapies are therefore turning out to be pleiotropic agents that can either act across multiple mechanisms simultaneously, or affect one or more mechanisms of injury at different time points, or both (Vink and Van Den Heuvel, 2004).

A potentially even more powerful alternative is to use multiple pleiotropic agents together, in that they may affect some of the same mechanisms but with potentially different kinetics in addition to expanding the number of mechanisms affected. If one or both of these compounds is further a common hormone, it would be important to establish how exogenous administration of one may interact with the physiological context provided by the presence or absence of another. In this research project we consider the effects of two common hormones, progesterone and 1,25-dihydroxyvitamin D_3, when they are administered exogenously after TBI in old rats both individually and in combination. We also examine their effectiveness in the acute reduction of neuroinflammation after TBI in the context of vitamin D deficiency, also in old rats.

2.3. AGING: OVERVIEW

Defining aging explicitly has proven difficult, but at least three definitions of the concept are currently in use (Mangel, 2001):

1. "*Physiological definition*: Aging is a decline of state or repair with increasing age.
2. *Actuarial definition*: Aging is an increase of mortality rate with increasing age.
3. *Evolutionary definition*: Aging is the persistent decline in components of fitness (rates of survival and reproduction) with increasing age."

These definitions are based on the specific characteristics of an aged organism: "a decline in physiological repair, an increase in probability of death and a decline in fertility with advancing adult age" (Mangel, 2001). It has already been noted (section 2.2.1) that TBI in the aged is a significant problem with public health implications in that both the incidence of hospitalizations and mortality rates due to TBI are highest in individuals over 70 (McArthur et al., 2004), and while deaths due to TBI have in general been reduced in most demographics with improvements in safety, they have increased significantly in the older population.

Aside from demographic considerations the elderly are subject to physiological and metabolic alterations that can affect recovery after major trauma and therefore need to be considered specifically with respect to both treatment modalities and characteristic underlying physiology. In the context of TBI, these alterations due to aging include changes in the organization of the CNS (Mattson and Magnus, 2006), immunosenescence or "inflammaging" (Franceschi et al., 2007), and the concept of reduced physiological reserve, or "frailty" (Walston et al., 2006).

2.3.1. Effects of Aging on the CNS

Although it is established that there is no overt loss of neurons in the brain with normal aging, a number of more subtle structural, chemical, and metabolic changes occur (Dickstein et al., 2007; Mattson and Magnus, 2006), both at the level of individual neurons and in medium-scale neuronal networks, that can significantly affect the ability of the CNS to adapt to internal and environmental changes. This alteration in the "plasticity" (Stein and Hoffman, 2003a) of the aging brain affects not only the maintenance of normal cognitive and psychosocial function but also the ability to recover from injury.

The alterations in neuronal cells due to aging include a reduction in the size of the neuronal soma, a reduced number of dendrites and dendritic spines, alterations in the levels or neurotransmitters and their receptors, and changes in the electrophysiological properties of neurons (Chang et al., 2005; Hof et al., 2002; Jacobs et al., 1997; Jacobs et al., 2001; Nakamura et al., 1985). A variety of studies have demonstrated that age induces a regression of dendritic arbors as well as a reduction in the complexity of dendritic branching (Dickstein et al., 2007), and this loss has been observed specifically in the pyramidal neurons of the prefrontal, superior temporal, and precentral cortices in human (Nakamura et al., 1985; Scheibel et al., 1975) and animal (Cupp and Uemura, 1980; Mervis, 1978) subjects. The specific figures suggest a 25% loss of dendritic spines (Cupp and Uemura, 1980) and up to a 50% decrease in spine density, accompanied by a 10% decrease in total dendritic length (Jacobs et al., 1997; Jacobs et al., 2001), and a 40-55% reduction in the number of synapses (Peters et al., 1998). This loss is especially important as the synapses are considered to be the most vulnerable parts of neurons (Mattson and Magnus, 2006). Decline in dendritic detail is accompanied at a larger scale

with changes in dendritic diameters and lengths that can significantly alter the passive electrical structure and therefore the excitability and signaling properties of aged neurons (Chang et al., 2005; Mattson and Magnus, 2006). Since the architecture of dendritic arborizations appears to be the key structural element in neuronal network plasticity (Kolb et al., 1998), this disruption in cortical connectivity and normal signaling mechanisms may provide a substrate not only for the development of age-related dementia, but also for the reduced capacity with age to adapt to injury by either invoking new learning or uncovering connections that could compensate for the loss (Stein and Hoffman, 2003a).

A number of neurochemical changes also occur during normal aging, including a significant reduction in the number of neurons expressing ionotropic and NMDA glutamate receptors (Gazzaley et al., 1996; Hof et al., 2002) and reduced excitatory neurotransmission associated with an increase in inhibitory GABA release from pre-synaptic interneurons (Dickstein et al., 2007). In addition to these specific changes, it also appears that all the neuromodulatory systems are also significantly affected by aging (Mattson and Magnus, 2006), including those implicated in TBI-induced dysfunction such as the cholinergic system of the NBM (Dickstein et al., 2007).

Other changes n the CNS in response to aging are similar to the changes that occur in other cells: “increased amounts of oxidative stress, perturbed energy homeostasis, accumulation of damaged proteins, and lesions in their nucleic acids” (Mattson and Magnus, 2006). The cellular events that occur during normal aging also make neurons increasingly susceptible to excitotoxic damage (Mattson and Magnus, 2006), specifically through the impairment of ion pumps (Mattson, 2003), dysregulation

of Ca^{2+} homeostasis (Mattson and Magnus, 2006), and decreased mitochondrial function (Melov, 2004). Since these are all processes that are involved in the evolution of the injury after traumatic insult, it seems reasonable that the very process of aging would significantly increase vulnerability and impair the potential for recovery in aged individuals.

2.3.2. Immunological Effects of Aging/Immunosenescence

In addition to specific alterations in the CNS, age-related changes in the function of the immune system are also common and have been implicated in virtually all age-associated disease processes (Bulati et al., 2008). Aging is associated with a general activation of the inflammatory response, which, due to the chronic antigenic stress on innate immunity experienced over a lifetime, becomes the basis for the onset of inflammatory diseases (Vasto et al., 2007). This effect is not just systemic, but also has specific effects within the CNS (Godbout and Johnson, 2006). Given the importance of inflammatory cytokines such as TNFα, IL-1β, and IL-6 in both behavioral modulation and the evolution of traumatic injury, it is likely that "an exacerbated neuroinflammatory cytokine response in the aged disrupts neuronal synaptic plasticity, creating a brain environment that is permissive to severe long-lasting mental health complications" (Godbout and Johnson, 2006) as well as an inability to recover from CNS trauma. In fact, the suggestion is that "the aged brain resides in a chronic state of neuroinflammation, characterized by increased reactivity upon immune stimulation and low-level production of central cytokines" (Dilger and Johnson, 2008).

The specific changes in immune function affect most of the cellular components of immunity, including T cells, B cells, natural killer (NK) cells, antigen-presenting cells

such as dendritic cells (Gruver et al., 2007), as well as microglia, the CNS-specific immune population (Dilger and Johnson, 2008; Griffin et al., 2006). The most important factor in the dysregulation of systemic cytokine and hormone networks appears to be the decline in T cell function associated with thymic atrophy and the consequent reduced output of naïve T cells (Gruver et al., 2007). This leads to an immune repertoire skewed towards previously encountered antigens where an increased proportion of the niches in peripheral tissues become occupied by terminally differentiated cells (Nikolich-Zugich, 2008). Additional studies indicate that B cell production in the bone marrow also decreases significantly with age (Dorshkind et al., 2009), and this is associated with the production of antibodies with a decreased affinity for antigen and an impaired ability for class-switching recombination (Frasca et al., 2008). Innate immunity is also affected by age, and a number of studies have indicated declines in monocyte and macrophage function in old animals, including reduced expression of Toll-like receptors, impaired production of cytokines, impaired respiratory burst and RNS production, and reduced ability to activate T and B cell populations (Candore et al., 2008). Along with this inability to mount an appropriate immune response to antigenic stimulation, with aging there is also a decrease in the production of anti-inflammatory hormones (Straub et al., 2000) as well as a tendency towards production of elevated amounts of pro-inflammatory cytokines by peripheral blood mononuclear cells (Franceschi et al., 2007). This general tendency towards a fulminating inflammatory state with increasing age has been dubbed "inflammaging" (Franceschi et al., 2007).

The immune system communicates with the CNS through several mechanisms: 1) passive diffusion of cytokines from the blood to the brain through the circumventricular

organs, bypassing the BBB; 2) energy-dependent transport across the BBB; 3) the secretion of immune-related molecules by endothelial cells comprising the BBB on peripheral immune stimulation; and 4) direct transmission of immune signals via the vagus nerve (Dilger and Johnson, 2008). These signals converge in the brain and result in the production of inflammatory cytokines by microglial cells (Griffin et al., 2006), leading to sickness behavior, which under normal conditions is an adaptive response to peripheral immune activation, and consists of fever, lethargy, reduced social interaction, increased sleep, and reduced appetite (Godbout and Johnson, 2006).

2.3.2.1. Microglial Activation and Neuroinflammation

There is now evidence that the brains of aged animals exhibit gene expression profiles characteristic of microglial activation and neuroinflammation (Frank et al., 2006; Godbout et al., 2005; Perry et al., 1993; Sheffield and Berman, 1998). Microglia themselves also appear to respond to stimulation with an amplified inflammatory response in aged animals (Godbout et al., 2005). This neuroinflammatory "priming" can significantly affect the development of neurological dysfunction: "Normally these neuroinflammatory changes are transient, with microglia returning to a quiescent state after the resolution of the immune challenge. Aging, however, may provide a brain environment in which microglia activation is not resolved, leading to a heightened sensitivity to immune activation; this lack of resolution may contribute to the pathogenesis of neurologic disease" (Godbout and Johnson, 2006). This situation is likely to exacerbate secondary injury in older subjects after TBI, as trauma can lead not only to a severe systemic immune response (Lenz et al., 2007), but also to immune factor release from the damaged cells within the brain itself (Dilger and Johnson, 2008).

Exaggerated neuroinflammation can also interfere with neuroplasticity during the recovery phase, and inflammatory cytokines such as TNFα, IL-1β, and IL-6 appear to directly interfere with long-term potentiation (Griffin et al., 2006; Li et al., 1997; Vereker et al., 2000), memory consolidation (Barrientos et al., 2006), neurite outgrowth (Neumann et al., 2002), and hippocampal neurogenesis (Vallieres et al., 2002). "Taken together these results suggest that the presence of reactive glia in the aged or diseased brain is permissive to an amplified and prolonged neuroinflammatory response, which may lead to subsequent behavioral and cognitive complications" (Godbout and Johnson, 2006), especially after TBI.

2.3.3. Frailty

Disruptions of the function of the nervous, immune, and endocrine systems and their interactions can lead to a loss of physiological reserve, conceptualized under the notion of "frailty" (Fried et al., 2001; Topinkova, 2008). Frailty has been variously defined as "1) a wasting syndrome; 2) a physiological state of vulnerability to increased morbidity and mortality; 3) a constellation of signs and symptoms that include weight loss, decreased activity, muscle weakness, fatigue, and slow gait; 4) a loss of physiological reserve; and 5) altered homeostatic capacity" (Lipsitz, 2004). Although the specific definition of the concept is still under discussion, it nonetheless has heuristic value for understanding the loss of resilience to external and internal perturbations, which is frequently associated with the aging process and can increase vulnerability to injury.

"The concept that frailty is a biological syndrome with a decreased functional response reserve to events, resulting from cumulative declines of multiple subsystems and vulnerability to adverse outcomes also emerged from the analysis of conceptual

models developed over the last decade. A biological model, the "cycle of frailty," that includes sarcopenia (the loss of muscle mass and strength), neuroendocrine decline, and immune dysfunction as potential causes, was proposed. The cycle or downward spiral can be precipitated by "trigger events"" (Paganelli et al., 2006). In other words, the condition is a multi-systemic dynamic maintenance of homeostasis that is more easily disrupted, leading to irreversible decline, in frail versus non-frail individuals. The prevalence of clinical frailty rises from about 7% in those aged 65 - 74 to 34% in those over 85 years old (Rockwood et al., 2004a; Rockwood et al., 2004b), but the concept, even sub-clinically, provides a unifying view of the effect of various age-related dysfunctions on the ability to recover from major trauma.

2.3.3.1. Aging and Decreased Activity of Systemic Hormones

In addition to structural and immune decline, human aging is associated with a decreased activity in a number of systemic hormones, including thyroid hormone (TH) (Vermeulen and Kaufman, 1995), sex steroids (Araujo et al., 2004; Jiang and Huhtaniemi, 2004; Vermeulen et al., 2002), growth hormone (GH) (Lanfranco et al., 2003), insulin-like growth factor-I (IGF-I) (Lanfranco et al., 2003), and 25-hydroxyvitamin D_3 (Elmadfa and Meyer, 2008; Holick, 2004; Rammos et al., 2008). Especially intriguing is the relationship of endocrine decline with the change in immunological function described above. Some research suggests that proinflammatory cytokines may downregulate the physiological responses to a variety of hormones including insulin, GH, IGF-I, TH, and estrogens (Ferrucci et al., 2004). More specifically, TNFα, IL-1, and IL-6 can specifically modulate the release of GH, growth hormone releasing hormone (GHRH), and somatostatin (Talhouk et al., 2004), and are known to reduce concentrations of IGF-I

(Priego et al., 2003) and increase the concentrations of glucocorticoids (Beishuizen and Thijs, 2003) in the serum of human patients. Lower serum IGF-I and DHEA-S levels were found in frail compared to non-frail elderly individuals (Fried et al., 2001), and an inverse correlation between serum IL-6 and IGF-I was noted only in frail individuals (Fried et al., 2001). Vitamin D deficiency has also been associated with both higher levels of inflammatory cytokines (Moore et al., 2005; Nagpal et al., 2005; Richards et al., 2007; Topinkova, 2008) and the development of frailty in older adults (Boxer et al., 2008; Morley et al., 2006). These data can be taken to suggest that the immune system and its age-associated dysfunction may be intimately related to the functioning of the neuroendocrine system, and that this relationship may further contribute to the development of frailty or at least systemic instability that makes it more difficult to recover from serious injury.

2.3.4 Conclusion

At a more conceptual level, frailty has been associated with the loss of physiological complexity and complex system dynamics of the organism as a whole. In essence, frailty leads to a weakening of the adaptability and resilience of a complex interacting system (such as a human being) makes it more vulnerable to disruption (Lipsitz, 2004). “In vital and resilient organisms, complex physiological pathways allow a wide variety of adaptive responses that are qualitatively and quantitatively modified to specific events. This complexity helps keep multiple systems in balance with minimal fluctuations in the homeostatic equilibrium. Aging results in the decline of normal interactions and the redundancy of communication between these physiological systems. It was hypothesized that frailty, both the phenotype and its latent vulnerability, results from reaching a

threshold of decline in one or more systems that triggers a cascade of dysregulation in multiple systems and that this dysregulation may influence many clinical domains, as well as comorbid conditions and disability" (Walston et al., 2006). This is especially important for our context of developing a treatment for TBI in the elderly: given the multi-faceted nature of injury and the complex aging process and loss of resilience, it would be unreasonable to attempt to elucidate a single or even a cluster of mechanisms that account for system-level effects. Rather, any observed effect of either aging or injury at the organismic level is likely to be a product of multiple interacting and intersecting systems, and a purely reductionistic explanation would be incomplete due to the complex nature of the processes in question and their emergent properties.

CHAPTER 3

PROGESTERONE AND TRAUMATIC BRAIN INJURY

3.1. ABSTRACT

Traumatic brain injury (TBI) is a significant clinical problem for which no effective treatment currently exists. Although dozens of pharmacological agents, many designed to attack specific molecular targets such as a particular neurotransmitter or receptor mechanism, have shown promise in animal models of TBI, all have failed to perform better than placebo when administered to human patients. Recent laboratory and clinical data have demonstrated a potentially beneficial role for progesterone (PROG) in the treatment of TBI, ischemic stroke, and certain neurodegenerative disorders such as multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS). Unlike single-target agents, PROG exerts pleiotropic effects on a number of the molecular and physiological processes in the cascade of secondary damage that follows a TBI.

NOTE: Sections of this chapter were published previously in Cekic M, Sayeed I, and Stein DG (2009) "Combination treatment with progesterone and vitamin D hormone may be more effective than monotherapy for nervous system injury and disease." *Front Neuroendocrinol* 30(2):158-72.

3.2. PROGESTERONE AND TRAUMATIC BRAIN INJURY: OVERVIEW

3.2.1. Progesterone and Traumatic Brain Injury in Human Patients

A number of recent publications have demonstrated effectiveness of PROG treatment in experimental models of TBI and stroke (Sayeed and Stein, 2009; Schumacher et al., 2007; Stein, 2008a; Stein, 2008b; Stein and Hurn, in press). Based on positive pre-clinical data, two single-center Phase II clinical trials using PROG to treat TBI were recently completed, with promising results. The ProTECT ("Progesterone for Traumatic Brain Injury, Experimental Clinical Treatment") trial was a randomized, double-blind, placebo-controlled trial of 100 patients with moderate to severe brain injury (Glasgow Coma Scale (GSC) scores of 4-12) performed at Grady Hospital in Atlanta, Georgia, a Level I trauma center (Wright et al., 2007). No serious adverse effects were observed, and the severely injured patients receiving three days of intravenous PROG beginning 6-8 hours after injury showed a greater than 50% reduction in mortality at 30 days over those receiving placebo. The moderately injured group also showed statistically significant "encouraging signs of improvement" on Disability Rating Scale outcome compared to controls at 30 days. The conclusion was that PROG helped patients with both severe and moderate injuries, but that the effect was confounded in the severely injured by the fact that many in the group given PROG survived who otherwise would not have, so the overall recovery process took longer than for the moderately injured group.

These results were supported by another single-center trial of 159 severely brain-injured subjects (GCS ≤ 8) (Xiao et al., 2008) in which patient outcomes were tracked for a longer time. The patients in this study received a five-day treatment with intramuscular injections of PROG within 8 hours of injury and showed substantially better survival and

functional outcomes at both 3 and 6 months than controls. It is important to note that in both studies, PROG not only decreased mortality, but also significantly enhanced functional outcome measures. The patients did not just survive to be consigned to a vegetative existence. Although these two reports need to be confirmed in larger multi-center studies, taken together they are the first to show a substantial benefit for TBI in human patients (Doppenberg et al., 2004), making PROG among the most promising of the candidates that have been proposed (Vandromme et al., 2008).

3.2.2. Progesterone Signaling Mechanisms

PROG is produced by the ovaries and the corpus luteum in females during normal reproductive cycling (Schumacher et al., 2007) and by the adrenal glands, which are its main source in men (Puder et al., 2000). PROG is also locally synthesized in both the peripheral and central nervous systems by neurons and glia. Its synthetic enzymes, cytochrome cholesterol side-chain cleavage enzyme (P450scc), which generates pregnenolone from cholesterol, and 3β-hydroxysteroid dehydrogenase (3β-HSD), which synthesizes PROG from pregnenolone, are both present throughout the brains of animals as diverse as fish and humans (Guennoun et al., 1995; Mellon et al., 2001; Mensah-Nyagan et al., 2001). This makes it a neuroactive steroid, or "neurosteroid," defined as a steroid hormone that is synthesized in and has effects on the nervous system (Baulieu et al., 2001).

Like all steroids, PROG exerts its cellular effects by regulating gene transcription in the nucleus. These "classical" actions are mediated by the cytoplasmic progesterone receptor (PR), which occurs in two main splice isoforms, PR-A and PR-B. Ligand binding to these receptors recruits nuclear receptor coregulators such as members of the

steroid receptor coactivator (SRC) family, which have been found to be limiting factors in steroid-induced responses in the brain (Charlier et al., 2005). The entire complex then migrates to the nucleus, where it binds to progesterone response elements (PREs) in the promoters of genes and initiates or inhibits gene expression. The PR is also capable of interacting with the Src tyrosine kinase family in the cell membrane (Edwards, 2005).

In addition to the classical cognate PR, PROG also interacts with other signal transduction mechanisms such as the σ1 receptor, for which it is a competitive inhibitor and through which it may reduce N-methyl-D-aspartate (NMDA) glutamate signaling (Bergeron et al., 1999; Hanner et al., 1996). PROG also signals at the nicotinic acetylcholine receptor (nAChR) (Valera et al., 1992) and affects gamma-aminobutyric acid (GABA), the main inhibitory transmitter in the brain, through its 5α-reduced metabolite allopregnanolone (or 3α,5α-tertrahydroprogesterone; ALLO) and positive modulation of the $GABA_A$ receptor (Belelli et al., 2002). Both these mechanisms may contribute to the neuroprotective effects of PROG, as they inhibit the excitotoxic response to injury. PROG metabolites have indeed been shown to be neuroprotective in their own right in models of kainic acid-induced hippocampal injury (Ciriza et al., 2004; Ciriza et al., 2006) and after experimental TBI (Djebaili et al., 2005; Djebaili et al., 2004; VanLandingham et al., 2007). PROG is also known to activate the pregnane X receptor (PXR), which may also be responsible for some of its protective effects in addition to those achieved through the PR. Finally, recent evidence suggests that PROG may exert direct signaling effects through activation of a membrane surface receptor, the 25-Dx (Guennoun et al., 2007; Meffre et al., 2005).

All these modes of action—gene transcription, neurotransmission, and signal transduction—are affected by PROG, and are likely to be responsible for its effects in the nervous system. Further complexity is added by the fact that both the synthetic enzymes and receptor/signaling systems are modulated by physiological context such as injury and, potentially, aging (Schumacher et al., 2007). For example, not only may receptors be upregulated (25-Dx) or downregulated (PR) in response to TBI, but certain genes affecting neuronal functioning may develop responsiveness to PROG only after injury (De Nicola et al., 2003; Schumacher et al., 2004; Schumacher et al., 2007).

3.2.3. Progesterone as a Neuroprotective Agent

One reason PROG shows benefits where other drugs have failed is that it is a pleiotropic drug with complex effects on multiple systems. Since the concept of rational drug design was presented, most attempts at developing treatments have been focused on dissecting the most obviously relevant mechanism and then pharmacologically targeting it. The less-than-spectacular results of this research program have stimulated a rethinking of the approach itself, and this is nowhere more obvious than in the case of brain injury.

Attention was first drawn to PROG as a treatment for TBI when it was observed that females exhibited less edema after injury than males, with pseudopregnant females (high in PROG) exhibiting virtually none (Attella et al., 1987; Roof et al., 1993). A number of subsequent studies have shown that PROG can reduce edema and excitotoxic cell death in the perilesional area of secondary injury (He et al., 2004a), and protect against ischemia (Coughlan et al., 2005; Sayeed et al., 2007). One major problem with central nervous system (CNS) damage (in both TBI and stroke) is disruption of blood flow to the local area of injury, leading to loss of oxygen and glucose, energy failure, and

eventual cell death. PROG has been shown specifically to protect neurons against cerebral ischemia (Cervantes et al., 2002; Gonzalez-Vidal et al., 1998) and to decrease infarct size (Jiang et al., 1996; Kumon et al., 2000). There are several observed effects in this resistance to ischemia: 1) maintenance of mitochondrial function, 2) increased pro-survival signaling, and 3) reduced internal and exogenous pro-apoptotic signaling (Alkayed et al., 2001; Cervantes et al., 2002; Gonzalez-Vidal et al., 1998; Sayeed et al., 2007; Schumacher et al., 2007). PROG appears to affect mitochondria in multiple ways. It restores them to normal morphology even after severe vacuolation (De Nicola et al., 2003), it inhibits pro-apoptotic cytochrome c release (Sayeed et al., 2009) and it upregulates the expression of anti-apoptotic mitochondrial proteins such as B-cell lymphoma 2 (Bcl-2) while decreasing the levels of pro-apoptotic signals such as Bcl-2-associated X protein (Bax), Bcl-2-associated death-promoter (BAD), and caspase-3 activation (Alkayed et al., 2001; Djebaili et al., 2005; Garcia-Segura et al., 1998; Nilsen and Brinton, 2002; Sayeed et al., 2009; Wise, 2006; Yao et al., 2005). PROG may affect the expression of these proteins through activation of the extracellular signal regulated kinase (ERK) signaling pathway, which phosphorylates the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), upregulates bcl-2, and confers improved resistance to ischemia (Finkbeiner, 2000; Freeland et al., 2001).

PROG and its metabolites have also recently been shown to modulate mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) signaling in the hypothalamus, hippocampus, and cerebellum of ovariectomized rats *in vivo* (Guerra-Araiza et al., 2009). Finally, PROG has also been shown to reverse the alterations in mitochondrial respiration (Robertson et al., 2006) and normalize the expression of the

Na^+,K^+-ATPase in experimental autoimmune encephalomyelitis (EAE) and models of spinal cord and nerve crush injury (Garay et al., 2008; Labombarda et al., 2002; Roglio et al., 2008). Since both of these are important issues in the energy failure and loss of ionic gradients that lead to cell death, this normalization of cellular metabolism is a key step in the attenuation of secondary injury.

Increased survival of glial and neuronal cells is associated with elevated levels of trophic factors. PROG has been shown to increase levels of both nerve growth factor (NGF) (Oyesiku et al., 1999; Tometten et al., 2005) and brain-derived neurotrophic factor (BDNF) (Gonzalez et al., 2005; Gonzalez et al., 2004; Scharfman and Maclusky, 2005) after injury. These proteins are especially necessary for glial survival and remyelination (Labombarda et al., 2008; Schumacher et al., 2007). Most importantly, however, PROG is known to reduce microglial activation and the production of pro-inflammatory and pro-apoptotic cytokines such as tumore necrosis factor a (TNFα) and Interleukin-1 (IL-1) (Drew and Chavis, 2000; He et al., 2004a; Miller and Hunt, 1998; Pettus et al., 2005). This is very significant, since prolonged inflammation is the main cause of extended secondary injury (Bramlett and Dietrich, 2004; Morganti-Kossmann et al., 2002; Skaper, 2007). PROG also inhibits activation of complement factors (Pettus et al., 2005; VanLandingham et al., 2007), and modulates the coagulation cascade (VanLandingham et al., 2008), both of which are important mechanisms of inflammatory amplification. PROG has also been shown to push helper T cell (T_H) cell differentiation towards the T_H2 phenotype, which may also play a role in its anti-inflammatory activity (Matsuzaki et al., 2005).

Improvement of mitochondrial function, increased pro-survival factors, and reduced inflammation are not only beneficial in the injury penumbra but have important systemic effects. TBI-associated systemic inflammation is a key mechanism in mortality, and can lead to multi-organ failure and infection (Lee et al., 2001; Zygun et al., 2005). After TBI, catecholamine-induced necrosis of cardiomyocytes leads to cardiopulmonary dysfunction and is also a significant cause of mortality (Zygun et al., 2005). A compound like PROG that increases survival signaling and attenuates inflammation has a role in recovery that extends well beyond the brain and even as far as the gut (Chen et al., 2007; Peltier et al., 2008).

There is also evidence that PROG treatment after TBI reduces lipid peroxidation (Roof et al., 1997), perhaps through upregulation of antioxidant enzymes such as superoxide dismutase (SOD) (Moorthy et al., 2005), although the mechanisms of action are not completely understood (Schumacher et al., 2007). A reduction of the damage caused by reactive oxygen and nitrogen species (ROS/RNS) can improve cell survival by maintaining membrane integrity, and helps to maintain the blood-brain barrier (BBB) by limiting oxidative damage to the endothelium. PROG has also been shown to help maintain BBB function by upregulating P-glycoprotein (P-gp), an efflux pump transporter and marker of BBB health that serves to eliminate xenobiotic and toxic substances; in the case of traumatic injury, these consist of inflammatory cytokines and ROS-producing compounds (Cutler et al., 2007). PROG can also protect neurons from direct toxicity of glutamate, $FeSO_4$, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and β-amyloid (Callier et al., 2001; Goodman et al., 1996; Nilsen and Brinton, 2002; Ogata et al., 1993), the last two of which may have implications for the

development and prevention of Parkinson's and Alzheimer's diseases, respectively. As mentioned previously, PROG also attenuates glutamate excitotoxicity through conversion to ALLO and subsequent activation of $GABA_A$ and σ1 receptors (Belelli et al., 2002; Bergeron et al., 1999).

These mechanisms—reduced inflammation and lipid peroxidation, maintenance of BBB integrity, and ionic stability–all serve to reduce edema after TBI (Roof et al., 1996; Roof, 1992) and stroke (Bach-y-Rita, 2001; Coughlan et al., 2005; Jiang et al., 1996). Recent findings also indicate that PROG can regulate expression of aquaporin-4 (AQP4), the water channel present in astrocyte endfeet and thought to be important in edema formation (Guo et al., 2006). Since brain swelling is one of the main neurological causes of mortality after TBI, this is an important issue in the clinical management of brain-injured patients.

Both the ProTECT trial (Wright et al., 2007) and that reported by Xiao et al. (Xiao et al., 2008) demonstrated improved functional recovery for patients receiving PROG after TBI. This is an important issue because ultimately the test of a pharmacological intervention is its effect on functional outcome and quality of life. In this context, PROG has shown improved long-term recovery in a number of behavioral paradigms including cognitive, sensory, and spatial learning and memory (Roof et al., 1994). These effects have been seen in different models of mild, moderate, and severe experimental injury (Brinton et al., 2008; Schumacher et al., 2007). The ability to sustain the neuronal circuitry implicated in complex behaviors is an important component of recovery. As expected, PROG has also been shown to attenuate retrograde neuronal degeneration in the nucleus basalis of Meynert (NBM) (He et al., 2004a) and to maintain

ACh homeostasis by regulating choline acetyltransferase (ChAT) levels in both TBI (Djebaili et al., 2005) and spinal cord injury (Labombarda et al., 2002). In addition to affecting connectivity, PROG also helps in signal transmission by promoting myelination and remyelination of injured neurons, and maintaining myelin basic protein (MBP) at control levels (Labombarda et al., 2006). MBP levels are associated with the establishment of a glial scar. These facts suggest that rather than just stopping further damage, PROG in fact initiates repair mechanisms (Labombarda et al., 2006; Labombarda et al., 2008).

3.2.4. Other Steroids and Interactions

PROG is not the only neurosteroid nor is it the only one with neuroprotective effects. While some of the mechanisms affected by PROG are similar for most steroid hormones, others vary widely, as do the effects when the steroids are used in combination (Schumacher et al., 2007).

3.2.4.1. Estrogen

Estradiol (EST) has shown significant promise in the treatment of brain injury and has been extensively examined in a variety of *in vitro* and *in vivo* models of neuronal injury (Schumacher et al., 2007). Like PROG, EST is synthesized both systemically and locally, and thus also qualifies as a neurosteroid (Brinton et al., 2008). Also like PROG, it has been shown to be effective in both males and females in eliminating neuronal loss and learning dysfunction after ischemia (Sudo et al., 1997; Wise, 2006). The key synthetic enzyme of EST, aromatase, has been shown to be a very important factor in neuroprotection, and injury in aromatase-deficient animals has shown increased damage to excitotoxic injury in sensitive areas such as the hippocampus (Garcia-Segura et al.,

2003). In addition to protecting neurons from excitotoxicity, EST has also shown effectiveness in a wide a variety of injury models ranging from amyloid beta (Aβ) to oxidative stress (Brinton et al., 2008). Although EST has received a significant amount of scientific attention and has shown a number of positive effects, the data are not unequivocal and it is possible that EST may exacerbate injury in some contexts (Emerson et al., 1993; Marriott et al., 2002; Smith and Woolley, 2004). In this respect PROG has proven to be a more likely single therapeutic agent.

As one would expect from the significant interaction between PROG and EST in reproductive functions, the two affect similar mechanisms, albeit not always in the same directions, in non-reproductive contexts as well (Brinton et al., 2008). In fact, "it is unlikely that the different steroids act independently, and they may have either similar, complementary, redundant or opposing actions" (Schumacher et al., 2007). PROG and EST both appear to increase anti-apoptotic and decrease pro-apoptotic signals (Garcia-Segura et al., 1998; Nilsen and Brinton, 2002; Yao et al., 2005), as well as increasing mitochondrial respiration (Irwin et al., 2008) and reducing free radical leakage and mitochondrial lipid peroxidation (Brinton et al., 2008). At the same time, the two steroids exert clearly divergent effects on neuronal excitability, with PROG and its metabolites generally being inhibitory while EST appears to be stimulatory (Smith and Woolley, 2004). In certain regions, however, PROG action appears to depend on EST priming, such as within the hypothalamus and some structures of the limbic system in which EST induces the expression of PR; this is not the case in cerebellum, cortex, putamen, midbrain, or septum (MacLusky and McEwen, 1978; Parsons et al., 1982; Romano et al., 1989). The reason for this localization is unknown (Kraus et al., 1994). What is

interesting is that PR expression is affected by PROG itself only in regions where it is upregulated by EST, but not in regions where it is not (Camacho-Arroyo et al., 1994; Guerra-Araiza et al., 2003).

3.2.4.2. Testosterone

Testosterone and other androgens also demonstrate neuroprotective effects, and while a number of these are due to their conversion to estrogens by aromatase, some of them are direct (Bialek et al., 2004; Veiga et al., 2003). For example, testosterone has been shown to promote spinal motoneuron survival and regrowth, and to exert antiseizure effects (Frye and Reed, 1998; Jones et al., 2001). Due to their important role in the differentiation of secondary sexual characteristics and other undesirable side effects, however, both EST and testosterone, while showing promise in the treatment of traumatic CNS injury, also have significant drawbacks not found with PROG.

3.2.4.3. Glucocorticoids

Although high-dose methylprednisolone continues to be the standard of care for brain and spinal cord injury in many cases, its use has become more controversial since the failure of the MRC CRASH trial (Medical Research Council of Great Britain "Corticosteroid Randomization After Significant Head injury"), a clinical study of 9673 adults with TBI that showed the drug to increase risk of death and disability over placebo (Roberts et al., 2004). The researchers' conclusion was that "corticosteroids should not be used routinely in the treatment of head injury" (Edwards et al., 2005). Thus, although glucocorticoids have shown promise as a treatment by reducing inflammatory activity, promoting neural cell survival, and upregulating the expression of trophic factors and the Na^+,K^+-ATPase pump (De Nicola et al., 1998; Herbert et al., 2006; Ogata et al., 1993), they have also

been shown to worsen damage in models of excitotoxic and ischemic injury (Antonawich et al., 1999; Kaufer et al., 2004). This dual effect may be due to the fact that glucocorticoid effects seem to be exquisitely sensitive to exposure time and concentration and therefore require strict control; in the short term after injury this can be beneficial, but with extended exposure, and very much like inflammation, glucocorticoids appear to become detrimental (McEwen, 2002; Sapolsky et al., 2000).

As one may expect, interactions between PROG and glucocorticoids exist as well, both at the levels of receptor cross-talk (Leo et al., 2004; Pedersen et al., 2003) and in the activation of equivalent and different sets of genes (Wan and Nordeen, 2002). This may be an effect of various coactivators and corepressors, which may determine the specific mode of action depending on the genetic and cellular context (Li and O'Malley, 2003; Song et al., 2001)

3.2.5. TBI, Aging, and Progesterone

Lower levels of circulating steroids have been observed in senescent subjects of both sexes and, as discussed above, this could significantly affect an individual's capacity to respond adaptively to TBI. There is evidence that there is a loss of "intrinsic" neuroprotection after ischemic injury with reproductive senescence in human patients (Bounds et al., 2003). This effect could be reversed in a rat model through replacement of EST and PROG, which significantly reduced infarct size (Alkayed et al., 2000). While this result shows promise for females, this study did not examine whether similar PROG treatments would be effective in senescent males. There is little else to report on the use of neurosteroids in aged, brain-damaged subjects, with the exception of a few studies

suggesting that treatment with PROG could be successful in other diseases/disorders related to TBI outcomes in aged subjects.

Gangula et al. (Gangula et al., 2002) reported that hypertension morbidity increases in postmenopausal females when hormones like PROG and EST are depleted. This can be reversed with neurosteroid administration. This study also found that PROG regulates the effects of calcitonin gene-related peptide (CGRP), a potent vasodilator. The hypotensive effects of CGRP were significantly enhanced in the presence of EST or PROG treatments in both aged and younger female rats. Gibbs also recently reported that aging, combined with loss of ovarian function, causes substantial reduction in ChAT and TrkA mRNA in the medial septum and nucleus basalis relative to younger animals (Gibbs, 2003). Given its neuroprotective effects, exogenous PROG could be beneficial in preventing some of these changes.

PROG's effects on the aging nervous system have also been reported by Azcoitia et al. and by Ibanez et al. (Azcoitia et al., 2003b; Ibanez et al., 2003) who found that supplementary PROG promotes the expression of myelin proteins in the damaged sciatic nerves of young adult rats and in 22-24-month-old males with nerve crush injuries. Ibanez et al. took this work further and studied whether treatment with PROG in young and aged rats enhances remyelination in the brain itself after damage to brain-stem white matter. While the process of repair took longer in aged rats, treatment with PROG doubled the expression of myelin seen in aged controls. Interesting in the context of our model of brain injury is the observation that, in aged old-world monkeys, one of the changes seen in myelin is splitting of the intraperiod line (where two membrane surfaces come together), which then becomes swollen with fluid, a situation characteristic of one

form of cerebral edema (Peters and Sethares, 2002; Peters et al., 2001). In mature animals, PROG substantially reduces injury-induced cytotoxic and vasogenic swelling and leads to enhanced morphological and behavioral recovery after TBI in young adult animals. Implications of previous work on PROG-induced remyelination in aged laboratory rats provide a compelling motive for studying the role of PROG in functional and morphological recovery in the aged subject.

CHAPTER 4

PROGESTERONE IMPROVES ACUTE RECOVERY AFTER TRAUMATIC BRAIN INJURY IN THE AGED RAT

4.1. ABSTRACT

Recent evidence has demonstrated that treatment with progesterone can attenuate many of the pathophysiological events following traumatic brain injury (TBI) in young adult rats, but this effect has not been investigated in aged animals. In this study, twenty-month-old male Fischer 344 rats with bilateral contusions of the frontal cortex (n = 4/grp) or sham operations received 8, 16, or 32mg/kg of progesterone or vehicle. Locomotor activity was measured at 72 hours to assess behavioral recovery. Brain tissue was harvested at 24, 48, and 72 hours and Western blotting performed for inflammatory and apoptotic factors. Edema was assessed at 48 hours by measuring brain water content. Injured animals treated with 8 and 16mg/kg progesterone showed decreased expression of COX-2, IL-6, and NFκB at all time-points, indicating a reduction in the acute inflammatory process compared to vehicle. The 16mg/kg group also showed reduced apoptosis at all time-points as well as decreased edema and improved locomotor outcomes. Thus, in aged male rats, treatment with 16mg/kg progesterone improves short-term motor recovery and attenuates edema, secondary inflammation, and cell death after TBI.

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4.2. INTRODUCTION

Progesterone administration after traumatic brain injury (TBI) improves short- and long-term behavioral recovery and reduces inflammation, apoptosis, lesion volume, and edema in laboratory animals (Asbury et al., 1998; Attella et al., 1987; Chang et al., 1999; Galani et al., 2001; Grossman et al., 2004; Kumon et al., 2000; Lowery, 2002; Roof, 1994, 1997). These results are not limited to experimental models. A recent Phase IIa clinical trial (Wright et al., 2007) reports that 4 days of post-TBI intravenous progesterone reduces mortality by more than 50% in moderately to severely injured human patients and enhances functional outcomes at 30 days for the moderately injured. This is the first clinical trial to show pharmacological protection in TBI patients, indicating that progesterone may be an effective clinical treatment.

Most studies attempting to develop treatments for TBI focus on otherwise healthy young adults despite the fact that the past decade has seen a 21% increase in TBI events in individuals over the age of 65 (Adekoya et al., 2002). Further, the mortality rate resulting from TBI and its complications in the elderly is more than twice that of young and adult victims (Mosenthal et al., 2002). Age has also been found to be an independent predictor of mortality and early outcome in the geriatric human population with TBI (Mosenthal et al., 2002), indicating the importance of the problem in this demographic. Following lateral fluid percussion brain injury, for example, aged animals are impaired in Morris water maze performance and show disruptions in the beam walk and beam balance, tasks in which younger animals display fewer or no deficits (Hamm et al., 1992). Since recovery parameters and system homeostasis are clearly altered in the aging organism, it is possible that treatments such as progesterone that have been shown to be

effective in younger counterparts may not work in this population or may require different dosing and treatment regimens. This is not a trivial issue as it has important consequences for the human population, and any potential TBI treatment must therefore be demonstrated to work across different age groups if it is to be considered successful.

Very little literature has specifically addressed the effects of progesterone treatment for CNS injury in aged animals, but the few studies that do show it to be beneficial. In a model of stroke, Alkayed et al. (Alkayed et al., 2000) demonstrated that the loss of intrinsic neuroprotection due to estropause in aged females, as measured by increased infarct size after ischemic injury, is attenuated by replacement of both estrogen and progesterone. Ibanez et al. (Ibanez et al., 2004) looked at remyelination in young and old rats with progesterone treatment after damage to brain-stem white matter and found that, while the latency of repair was increased in aged rats regardless of treatment, progesterone doubled the expression of myelin compared to elderly controls. Azcoitia et al. (Azcoitia et al., 2003a) and Ibanez et al. (Ibanez et al., 2003) also reported that supplementary progesterone promotes the expression of myelin proteins in the damaged sciatic nerves of young and 22- to 24-month-old male rats with peripheral nerve crush injuries. In addition to these nervous system-specific effects, neurosteroid administration also appears to confer general systemic benefits and has been shown to reverse the increased hypertension morbidity documented in postmenopausal female rats (Gangula et al., 2002).

Given the severity of the problem of TBI in the aged and the fact that progesterone has proven to be effective in a number of studies in young adult animals and human patients (Stein, 2005; Stein and Hoffman, 2003a), we asked whether progesterone

could have significant beneficial effects in senescent TBI subjects as well, especially in the acute phase of injury. Brain trauma is generally followed by a stereotyped molecular response that can be investigated through expression levels of proteins such as TNFα, IL-6, and the inflammation-associated transcription factor NFκB and its inhibitor, IκB. Since much of the damage that occurs after TBI is secondary to the initial insult (Royo et al., 2003), acute phase reactants play a very important role in the evolution of the injury, and the benefit of a treatment can be measured partly by its effect on these compounds (Bazan et al., 2005; Bramlett and Dietrich, 2004; Kovacs, 2005; Stamatovic et al., 2006). The development of edema is an important factor in the generation of secondary injury, and is frequently the proximal cause of death after TBI (Galani et al., 2001). The p-glycoprotein (P-gp) efflux pump, a molecular marker of blood-brain barrier (BBB) function, is able to reduce cytotoxic and vasogenic edema by integrating into membranes and helping to maintain cellular homeostasis (Dazert et al., 2006). An effective treatment for TBI would therefore be expected to reduce edema, as measured by brain water content, and to do so through molecular mechanisms such as the upregulation of P-gp expression in the lesion area.

To determine the neuroprotective effects of progesterone during the initial evolution of TBI in the aged rat, we examined levels of edema, inflammatory cytokines, and apoptotic cell death at several time points in the acute phase of damage. To confirm that reduced inflammation and cell death are associated with improved behavioral recovery, we also tested the spontaneous locomotor behavior of our old animals during the same period. Based on the beneficial effects of post-TBI progesterone administration in adult animals and the positive response of aged animals to progesterone after stroke

and nerve crush injuries, we hypothesized that old male rats would also exhibit decreased expression of inflammatory cytokines and improved behavioral outcomes following progesterone treatment.

4.3. MATERIALS AND METHODS

4.3.1. Subjects

Eighty-five aged (22-month-old) male Fischer 344 rats weighing 450-550g at the time of injury were used in this experiment. Food and water were provided *ad libitum* before and after surgery. The animal housing facility was maintained on a reverse 12:12 light-dark cycle, at 22°C (*SD* = +/-1) with appropriate humidity levels. The air within the colony was continually cycled via an air filtration system complying with government legal standards for animal research. Rats were handled for a minimum of two months following their arrival in the housing facility and prior to surgery. This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) in accordance with NIH guidelines. All experimental animal procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), Protocol #146-2005.

4.3.2. Surgery and Contusion Injury

Rats were anesthetized using isoflurane gas (5% induction, 1.5% maintenance, 700mmHg N_2O, 500mmHg O_2) and then mounted in a Kopf stereotaxic device. The incision area was shaved and sterilized with Betadine® antiseptic and 70% isopropanol. Core body temperature (~37°C) was maintained with a homeothermic heating blanket system (Harvard Apparatus, Holliston, MA). Physiological parameters were monitored with SurgiVet™ (model V3304) pulse oximetry: heart rate was maintained above ~300 beats per minute and SpO_2 kept above 90%. Using aseptic techniques, a midline incision was made into the skin and fascia covering the skull. After appropriate time under anesthesia, the animals assigned to the sham group had their incision sutured closed and

were placed into heated recovery boxes. In the experimental groups, medial, lateral, and dorsal stereotaxic coordinates were determined at bregma after incision, and a 6 mm diameter mid-sagittal bilateral craniotomy was performed 3mm anterior to bregma. Cortical contusion injury (CCI) to the medial frontal cortex (MFC) was induced with a pneumatic cortical contusion device (5mm diameter) to a depth of 2.5mm at a pressure of 1.7psi, impact time of 50ms, and velocity of 2.25m/s. Sutures were used to close the incision after bleeding stopped. Animals were then placed into heated recovery boxes and allowed to recover from the anesthetic before being returned to their home cages (Hoffman et al., 1994). Animals dehydrated due to blood loss were given 10mL of lactated Ringer's solution subcutaneously within 6 hours of injury.

4.3.3. Progesterone Administration

Lesion animals (n = 4 animals per group per experimental condition) were randomly assigned to one of four treatment groups, henceforth referred to with the following acronyms: progesterone (8mg/kg (P8), 16mg/kg (P16), or 32mg/kg) and vehicle (22.5% 2-hydroxypropyl-β-cyclodextrin (VH)). Treatments were administered intraperitoneally at 1 hour post-injury, and then subcutaneously at 6 hours post-injury and every 24 hours thereafter until the brains were harvested. Serving as intact, or normal baselines, sham groups (SHAM) received no injury or injections. Animals were killed at 24, 48, and 72 hours following injury with 1mL Nembutal, decapitated, and their brains prepared for protein analysis. Dose response was studied since a dosage appropriate in younger animals may not be appropriate in order animals due to various physiological changes due to aging.

4.3.4. Activity Testing

Testing for activity was done under red light in a quiet environment one day prior to injury, then again at 72 hours post-injury. The purpose of testing prior to injury was to obtain a baseline for each animal that could be used to calculate percent change at 72 hours post-injury. For each trial, up to four animals were tested simultaneously in individual boxes using the Digiscan Activity Monitoring System (AccuScan Instruments Inc., Columbus, OH), with a total of three trials per test day per squad. Rats were placed in the farthest left corner of the Digiscan Activity Box with the recording apparatus on. Exactly five minutes later the computer stopped recording movements, ensuring that all tests were the same length regardless of start time. Animals were returned to their home cages at the end of testing. The activity boxes were cleaned with 70% ethanol and dried between trials.

4.3.5. Tissue Preparation

Brains were processed for protein analysis by taking tissue samples from the penumbral region of the contusion and the corresponding area in sham brains. The tissue was snap-frozen in 2-methylbutane, chilled on dry ice, and homogenized in T-per (78510, Pierce, Rockford, IL) and 10μL/mL protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). Resulting homogenates were centrifuged for 20 minutes at 10,000g. A Coomassie plus protein assay (Pierce, 1856210) was performed to ensure that all samples contained equivalent amounts of protein. Reducing sample buffer was prepared as 0.625 M Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% Bromophenol Blue. Samples consisting of homogenate, dH2O, and sample buffer were prepared at a 2μg/μL protein concentration, incubated at 90°C for 10 minutes, and stored at -20°C.

4.3.6. Western Blot Analysis

Fifteen μl of each sample (30μg protein) was added to individual wells of 4-20% Tris-HCL acrylamide Criterion gel (BioRad, Hercules, CA). The gel was run at 200V for approximately 1 hour. Proteins were transferred to a polyvinylidene difluoride (PVDF) nitrocellulose membrane at 100V for 30 minutes, and then incubated overnight in KPL milk diluent blocker (50-82-00 KPL, Gaithersburg, MD) in a 1:5 dilution at 4°C.

Blots were incubated with a polyclonal goat or rabbit primary antibody for COX-2 (ab15191, Abcam, Cambridge, MA), NFκB p65 (#3034, Cell Signaling Inc., Danvers, MA,), IκB (#9248, Cell Signaling), IL-6 (AB1839, Chemicon Inc., Temecula, CA), TNFα (AB1441, Chemicon), P-gp (ab3364, Abcam), and cleaved caspase-3 (Asp175, #9661S, Cell Signaling) in KPL milk diluent: phosphate-buffered saline (PBS, pH 7.4) (1:20) and agitated overnight at 4°C. Membranes were rinsed in PBS/Tween and incubated with secondary antibody donkey anti-goat IgG-HRP or goat anti-rabbit IgG-HRP (1:1000) in KPL diluent for 2 hours at room temperature. The blots were then incubated in chemiluminescent SuperSignal West Dura substrate (Pierce, 34076) for 5 minutes. Bands were detected on a Kodak Image station 440CF scanner (Rochester, NY) and analyzed with the accompanying Kodak1D densitometry image analysis software.

Band relative optical density was quantified by manually selecting a rectangular area within the Kodak1D software that would entirely encompass any individual band on a particular blot. This area was positioned over each band and the optical density calculated by the software. The values for each experimental group were then averaged to obtain the values for statistical analysis (shown in the figures). The images shown below the bars in each graph are band images most representative of the group as a whole. Band

intensity was compared only between treatment groups run on the same blots. β-actin was used as a loading control on all samples.

4.3.7. Edema Analysis

At 48 hours post-injury, brains from animals assigned to the edema study (n = 3 per condition) were extracted and dissected into anterior and posterior sections; the anterior section contained the entire lesion area. Each section was placed in a pre-labeled and pre-weighed tube that was immediately capped. Each tube was reweighed to the nearest 0.01mg and then opened and placed in a 60°C oven with 15mmHg vacuum pressure for 48 hours. Samples were reweighed after drying, and the percent water content calculated by: [(wet wt - dry wt)/(wet wt)]*100. The percent difference in water content between the anterior peri-contusional and the posterior distal section was calculated for each sample by: [(anterior H_2O% - posterior H_2O%)/(posterior H_2O%)]*100.

4.3.8. Statistical Analysis

All results were expressed as the mean +/- the standard error of the mean (SEM). Statistical significance was set at $p < 0.05$ and data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post-hoc* tests.

4.4. RESULTS

4.4.1. Pre- and Post-surgical Complications in Aged Rats

Of a total 85 old rats that entered the facility, 9.2% died prior to surgery due to various causes including tumors, 4.6% died during the surgical procedure from anesthesia or blood loss, and 10.7% died after surgery was completed but before the scheduled euthanasia. In contrast, young adult animals undergoing the same procedure had an attrition rate of approximately 1-2%. We also observed more blood loss during surgery in the old animals, as well as increased sensitivity to anesthesia and more extensive cranial hematomas on brain extraction compared to young adults. Old sham animals also showed significant bleeding during fascial clearing, something not generally observed in younger animals.

4.4.2. Effects of 32mg/kg Progesterone

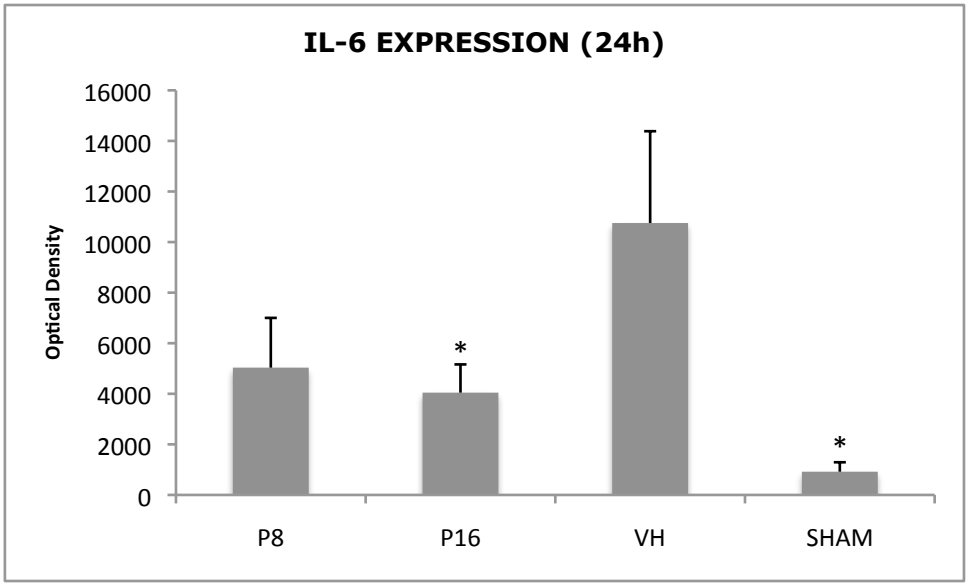
At 48 hours, the first time point investigated, we administered a 32mg/kg progesterone dose to our animals in addition to 8 and 16mg/kg. Consistent with the results of the dose response study conducted in young adult animals by Goss et al. (Goss et al., 2003), the 32mg/kg treatment exhibited fewer beneficial cellular/morphological and behavioral effects compared to the 8 and 16mg/kg doses at 48 hours (data not shown). Accordingly, we narrowed our dose response study at 24 hours and 72 hours to 8 and 16mg/kg progesterone and vehicle.

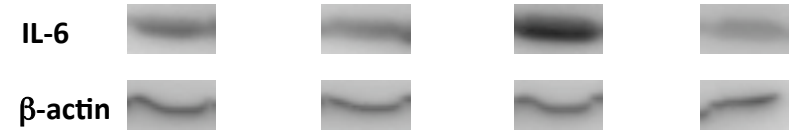
4.4.3. Inflammatory Cytokines at 24 Hours

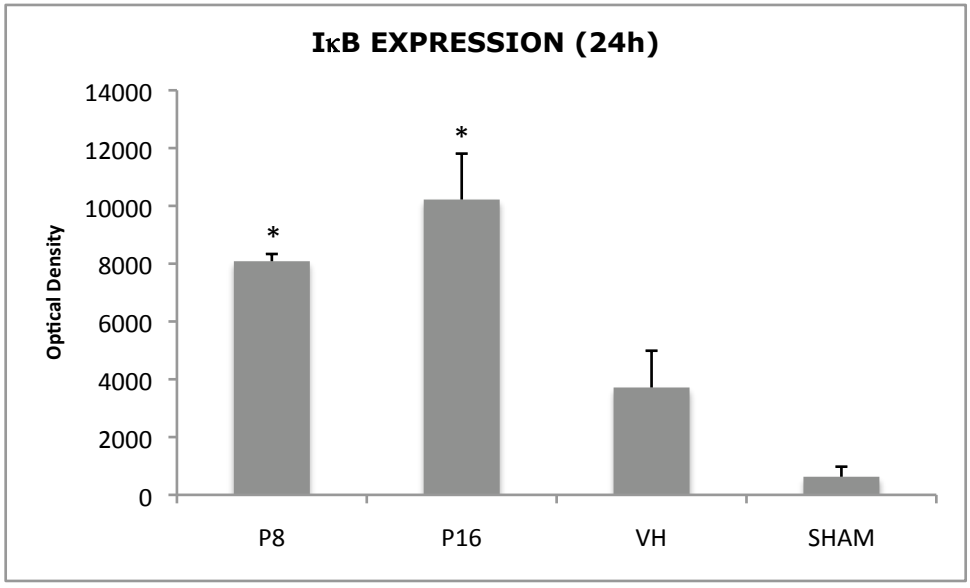
Figures 4.1 through 4.3 show the inflammatory cytokine response at 24 hours. One-way ANOVAs demonstrated significant group differences for IL-6 (Figure 4.1: $F_{3,12} = 4.75$, $p < 0.05$), IκB (Figure 4.2: $F_{3,12} = 10.97$, $p < 0.05$), and COX-2 (Figure 4.3: $F_{3,12} = 6.04$, p

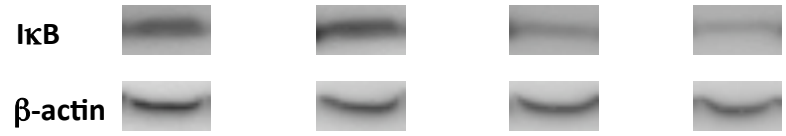
< 0.05). Post-hoc analysis showed that 16mg/kg (P16) progesterone produced a decrease in IL-6 expression ($p = 0.038$) and an increase in IκB expression ($p = 0.004$) over vehicle (VH). IκB prevents translocation of NFκB into the nucleus and therefore acts as an anti-inflammatory agent. Treatment with 8mg/kg (P8) progesterone significantly increased IκB levels as well ($p = 0.026$). 8mg/kg also decreased levels of COX-2 ($p = 0.032$) compared to vehicle, although 16mg/kg appeared to have no effect. Sham (S) expression levels were significantly lower for IL-6 ($p = 0.007$) and COX-2 ($p = 0.032$), indicating an injury effect. IκB levels were unaffected by the lesion. All Western blot results are shown in relative units of optical density.

Figure 4.4 shows Western blotting for cleaved caspase-3, the "gatekeeper" molecule in the extrinsic apoptosis pathway (Budihardjo et al., 1999) and a marker of apoptotic cell death. One-way ANOVA showed a significant treatment effect ($F_{3,12} = 9.05$, $p < 0.05$), with levels of cleaved caspase-3 comparable to sham levels with 16mg/kg treatment ($p = 0.019$ vs. vehicle), but not different from vehicle with 8mg/kg. As expected, sham group levels were also significantly lower than vehicle ($p = 0.044$).

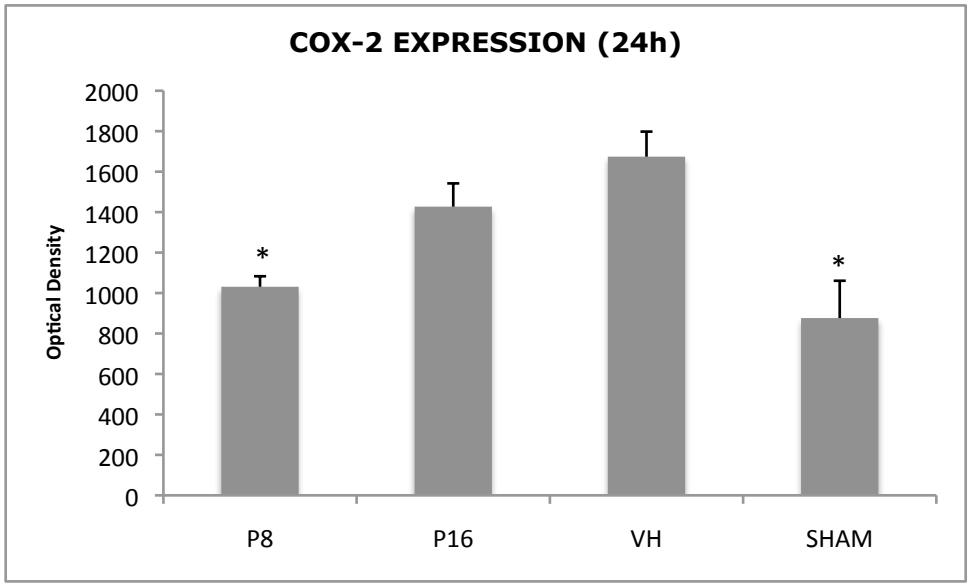


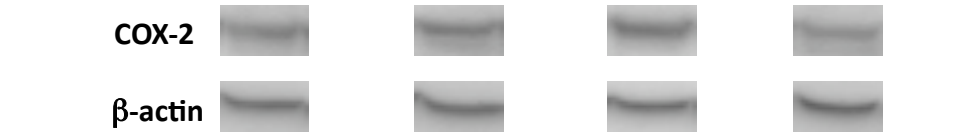


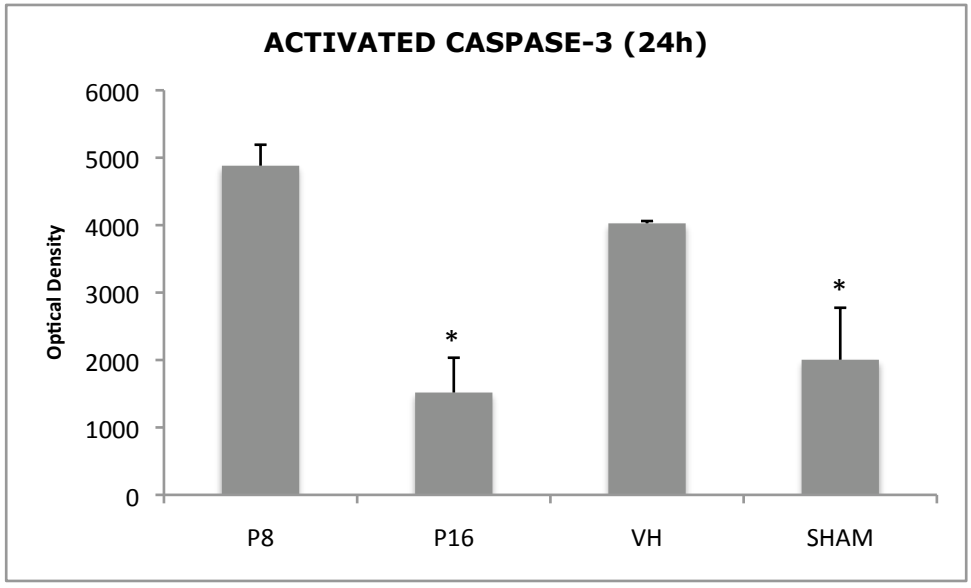


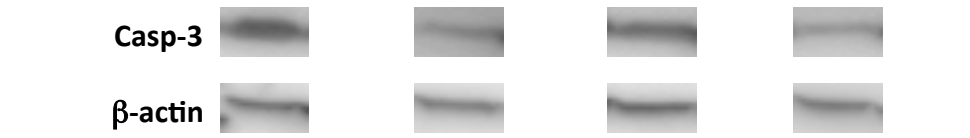


Figures 4.1 and 4.2. IL-6 and IκB levels in aged animals 24 hours after injury.
* $p < 0.05$ vs. VH.







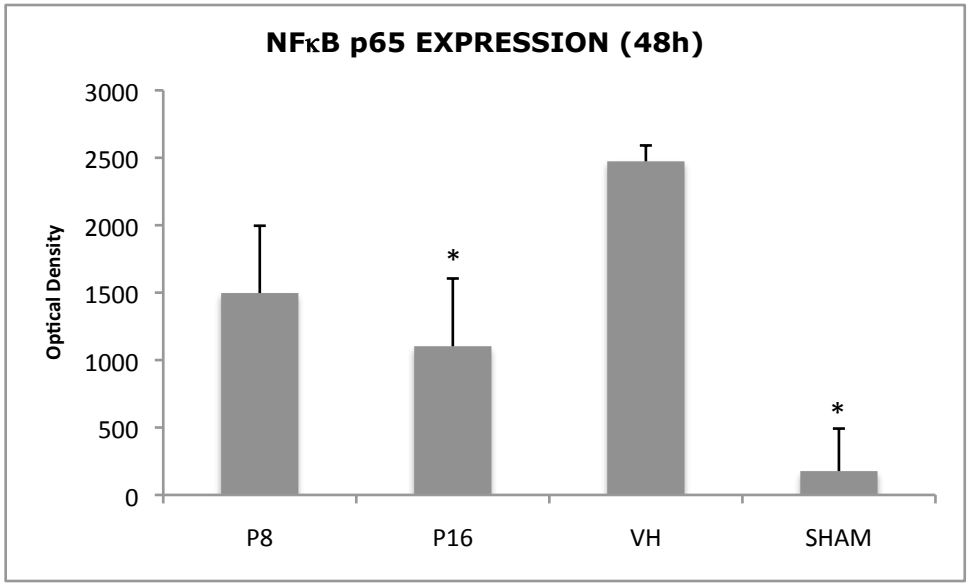


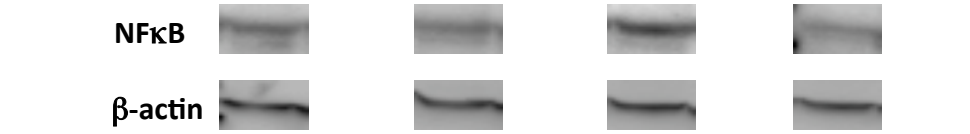
Figures 4.3 and 4.4. COX-2 and activated caspase-3 levels in aged animals 24 hours after injury. * $p < 0.05$ vs. VH.

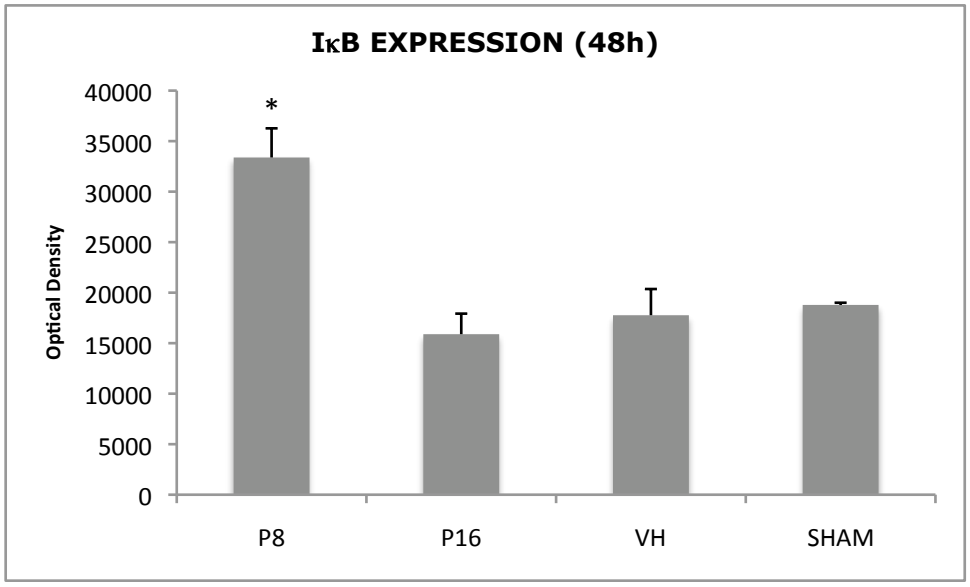
4.4.4. Inflammatory Cytokines at 48 Hours

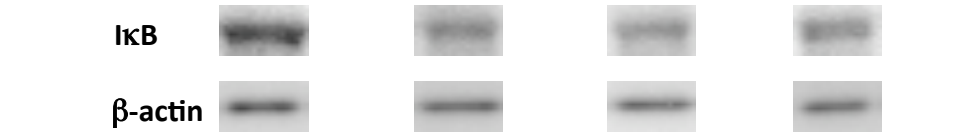
Figures 4.5 through 4.7 show inflammatory cytokine analysis for the four treatment groups at 48 hours. One-way ANOVA showed a significant effect of progesterone treatment on NFκB (Figure 4.5: $F_{3,12} = 9.59$, $p < 0.05$), IκB (Figure 4.6: $F_{3,12} = 15.17$, $p < 0.05$), and COX-2 (Figure 4.7: $F_{3,12} = 5.67$, $p < 0.05$). Both COX-2 ($p = 0.019$) and NFκB p65 ($p = 0.030$) were decreased by 16mg/kg progesterone, but not 8mg/kg, compared to the vehicle lesion group. Only 8mg/kg treatment increased IκB over lesion vehicle ($p = 0.002$). Sham animals showed significantly lower expression for NFκB ($p = 0.002$) and COX-2 ($p = 0.009$) than lesion animals. Once again, IκB did not show a lesion effect.

Figure 4.8 shows cleaved caspase-3 levels at 48 hours. Both 8mg/kg ($p = 0.011$) and 16mg/kg ($p = 0.010$) progesterone treatments provided a significant decrease in apoptotic cell death (one-way ANOVA, $F_{3,12} = 6.59$, $p < 0.05$) at this time-point. Sham levels were also significantly lower than vehicle ($p = 0.004$).

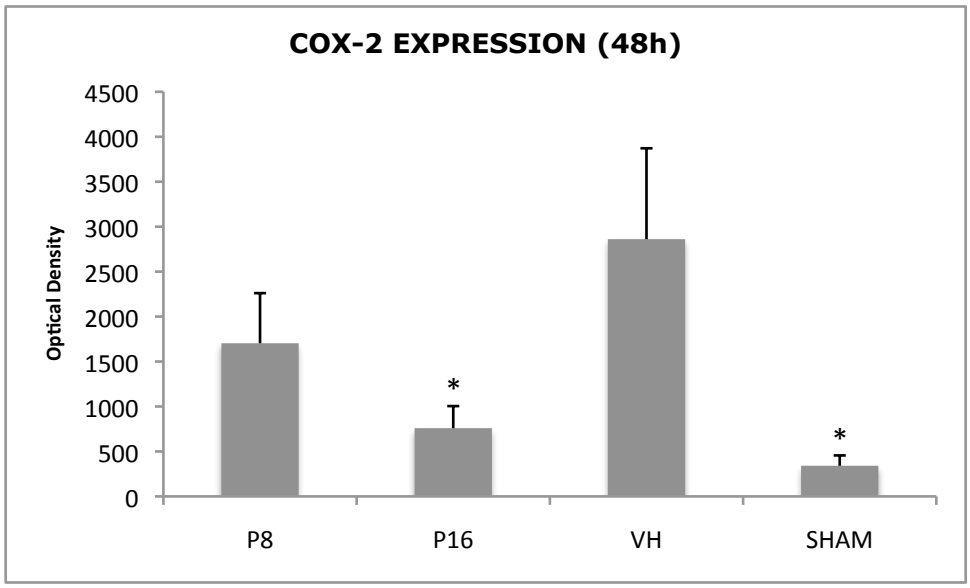


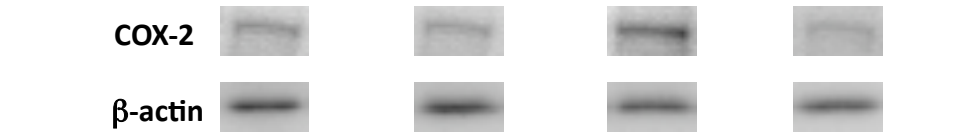


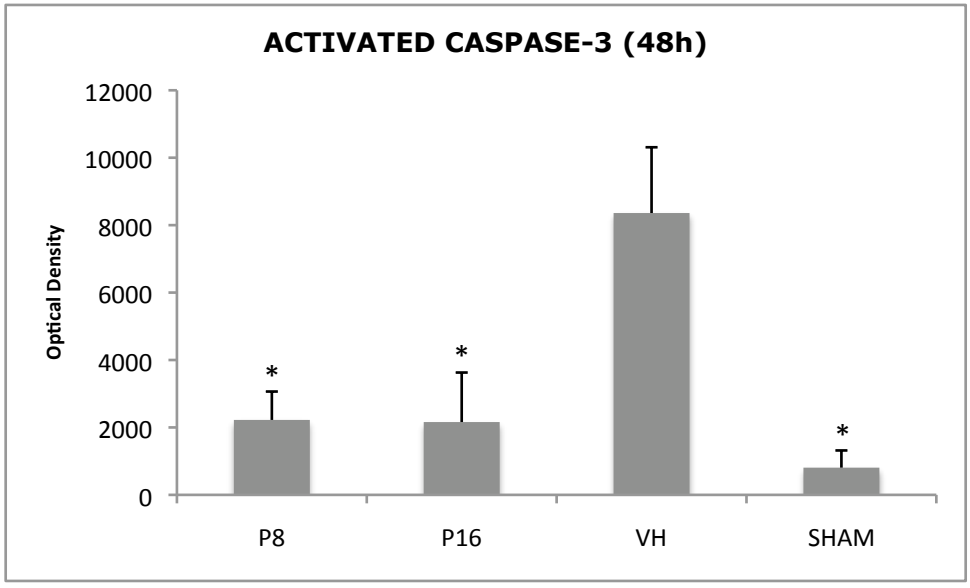




Figures 4.5 and 4.6. NFκB p65 and IκB levels in aged animals 48 hours after injury. * $p < 0.05$ vs. VH.





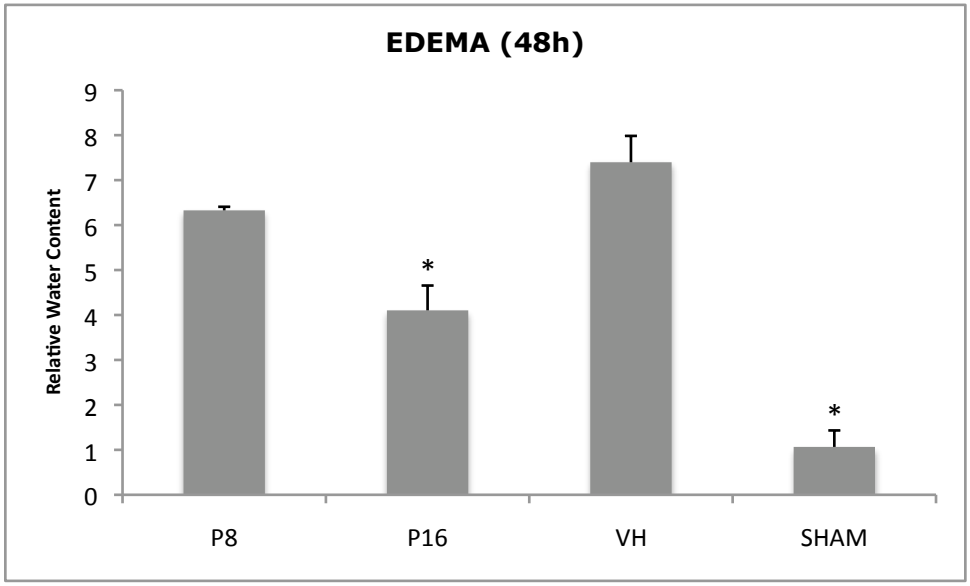


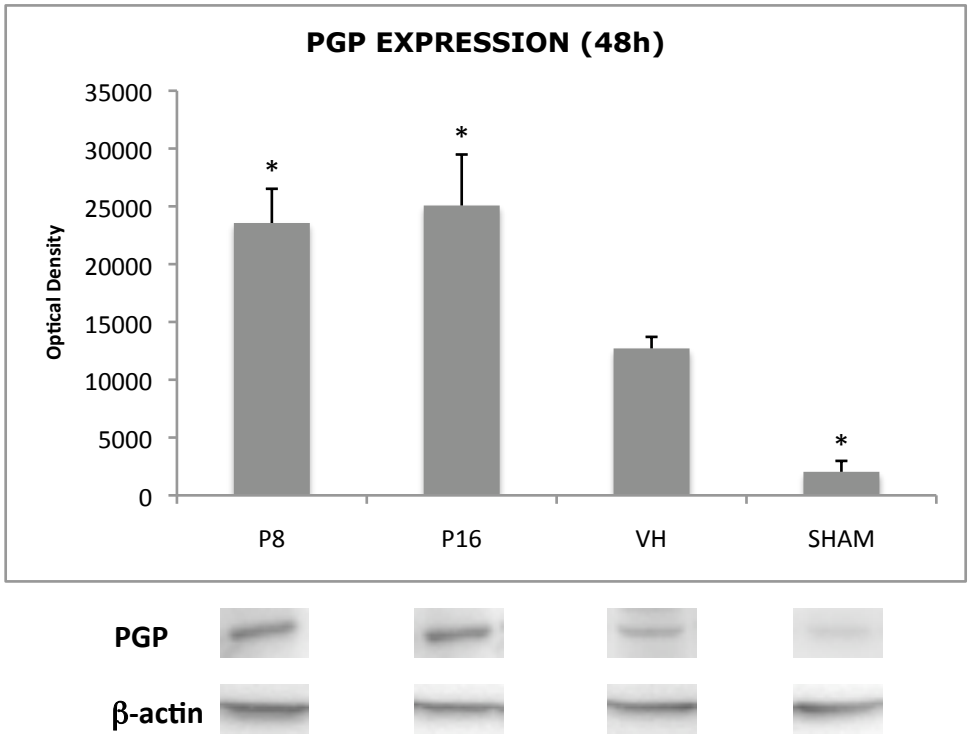
Figures 4.7 and 4.8. COX-2 and activated caspase-3 levels in aged animals 48 hours after injury. * $p < 0.05$ vs. VH.

4.4.5. Edema at 48 Hours

As seen in Figure 4.9, edema was significantly reduced at 48 hours by treatment ($F_{3,8}$ = 33.51, $p < 0.001$) with 16mg/kg ($p = 0.005$) but not with 8mg/kg progesterone. As expected, sham animals also demonstrated lower levels of edema than vehicle- ($p < 0.001$) or progesterone-treated (P8: $p = 0.001$; P16: $p = 0.011$) lesion animals.

Figure 4.10 is generally consistent with the data in Figure 4.9. One-way ANOVA shows that P-gp protein levels, hypothesized to be inversely proportional with edema, are increased for progesterone-treated rats ($F_{3,8}$ = 25.42, $p < 0.05$). Increased P-gp is seen with both 8mg/kg ($p = 0.012$) and 16mg/kg ($p = 0.007$) progesterone treatment compared to vehicle, indicating improved BBB integrity at this dose. The 8mg/kg dose shows significantly increased P-gp expression, but only a trend regarding edema (Figure 4.9), suggesting that there are additional factors involved in the attenuation of edema seen at 16mg/kg. There appears to be a clear lesion effect, since sham animals are also significantly different from all other groups, but in the other direction (P8 and P16: $p < 0.001$; V: $p = 0.008$).



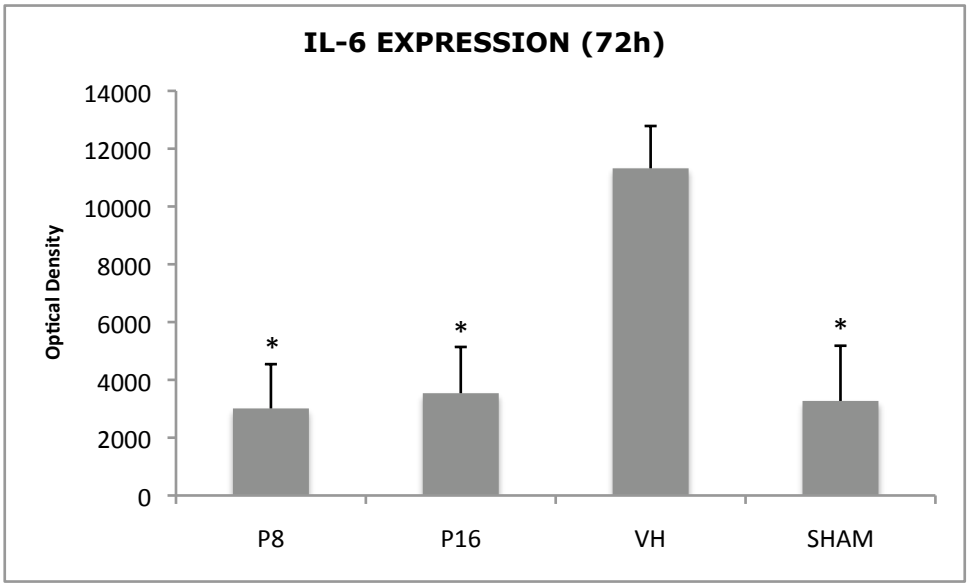


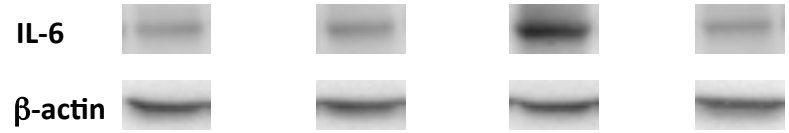
Figures 4.9 and 4.10. Edema and P-glycoprotein expression levels in aged animals 48 hours after injury. * $p < 0.05$ vs. VH.

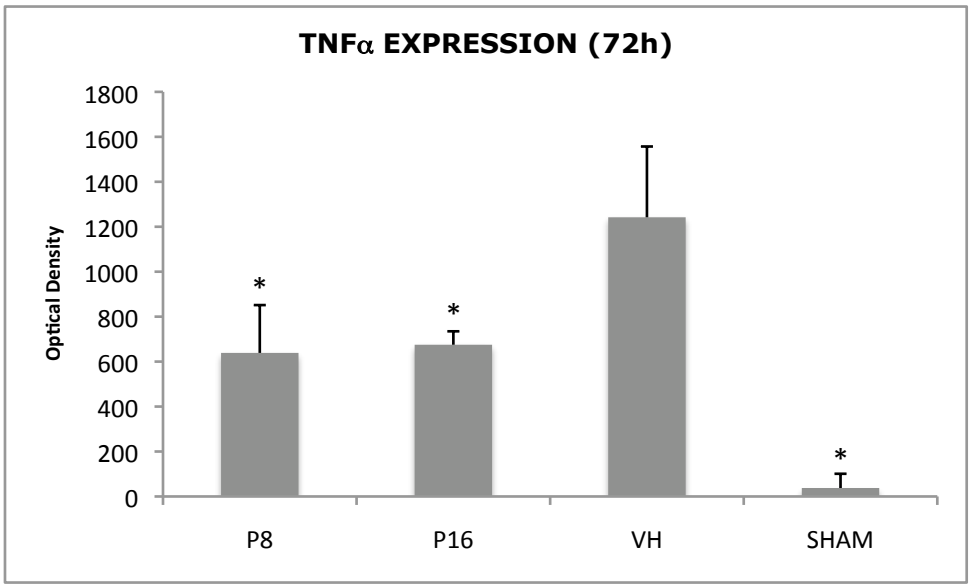
4.4.6. Inflammatory Cytokines at 72 Hours

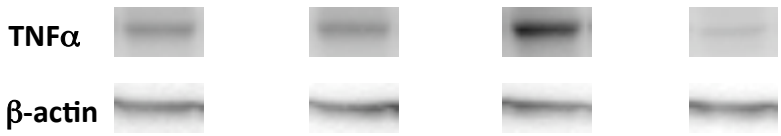
Treatment results at 72 hours are shown in Figures 4.11 through 4.14. Progesterone showed a reduction in IL-6 levels (Figure 4.11: $F_{3,12} = 5.61$, $p < 0.05$) at both the 8mg/kg ($p = 0.008$) and 16mg/kg ($p = 0.008$) doses compared to vehicle. The same was the case for TNFα (Figure 4.12: $F_{3,12} = 8.94$, $p < 0.05$), also at both 8mg/kg ($p = 0.034$) and 16mg/kg ($p = 0.035$) doses. No differences in COX-2 levels were seen with any treatment or shams (Figure 4.13: $F_{3,12} = 2.60$, $p = 0.125$). Sham responses were significantly lower for both IL-6 ($p = 0.009$) and TNFα ($p = 0.001$) compared to lesioned animals.

Figure 4.14 shows an overall treatment effect on active caspase-3 at 72 hours ($F_{3,12} = 4.45$, $p < 0.05$,). This was evident with 16mg/kg ($p = 0.014$), but not 8mg/kg progesterone compared to vehicle. Sham animals were, as expected, also significantly lower than vehicle ($p = 0.048$).

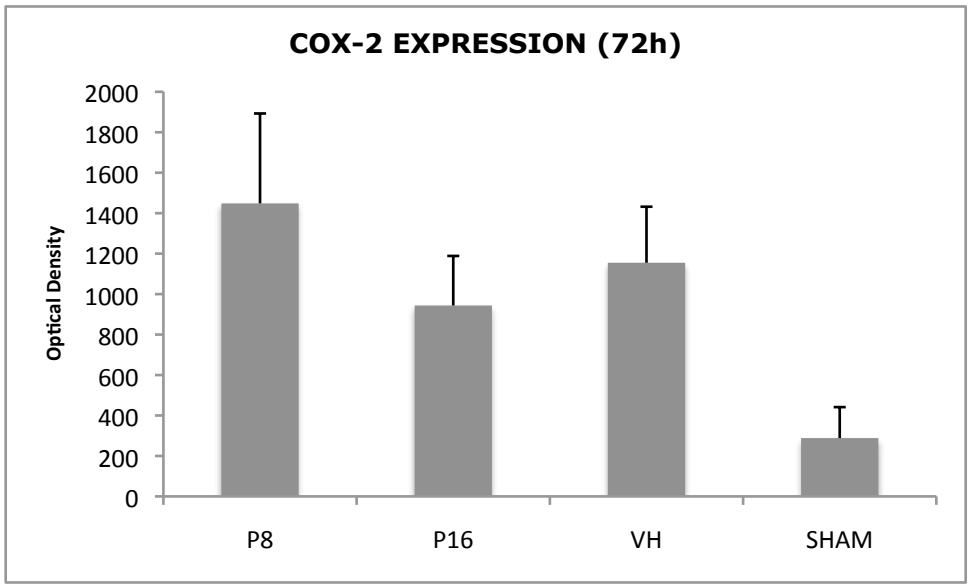


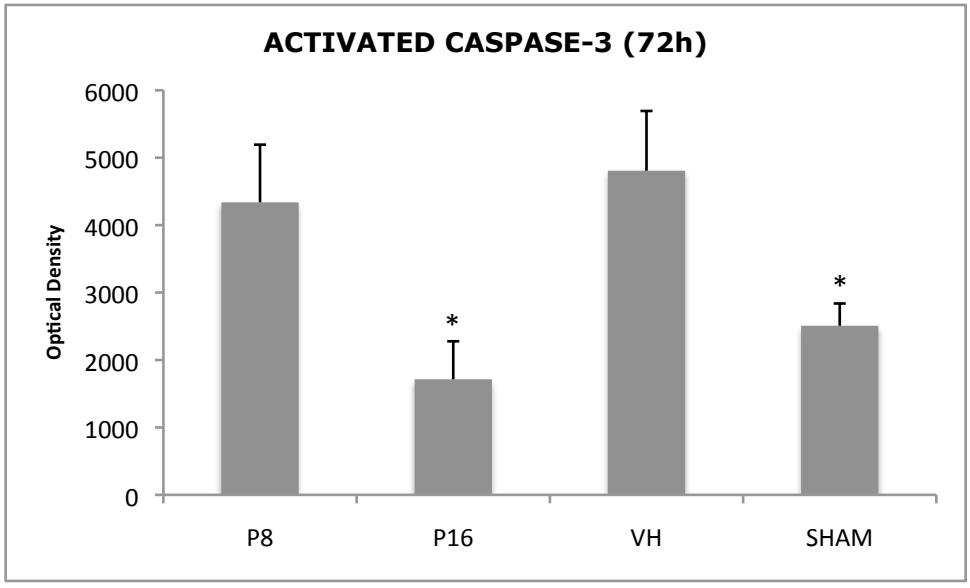






Figures 4.11 and 4.12. IL-6 and TNFα levels in aged animals 72 hours after injury. * $p < 0.05$ vs. VH.

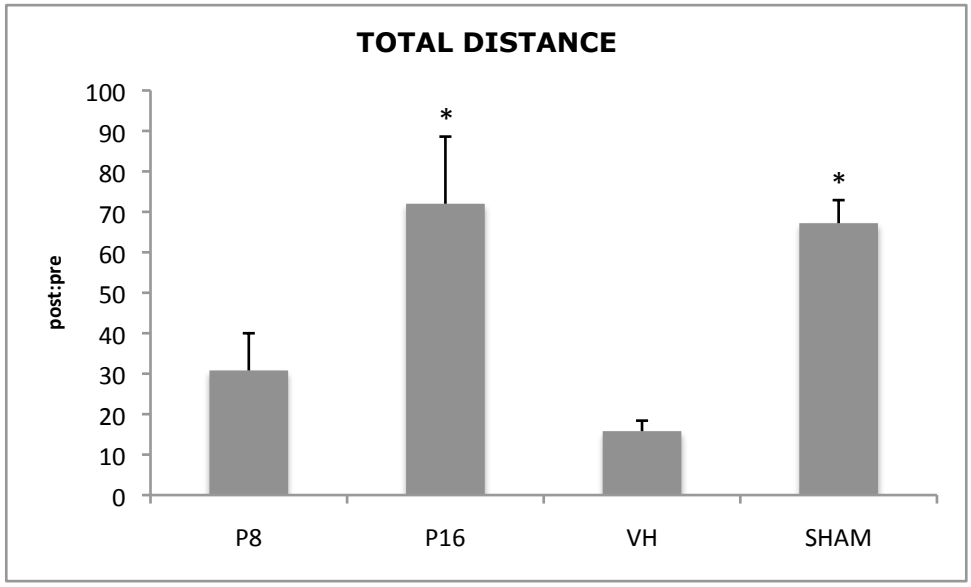


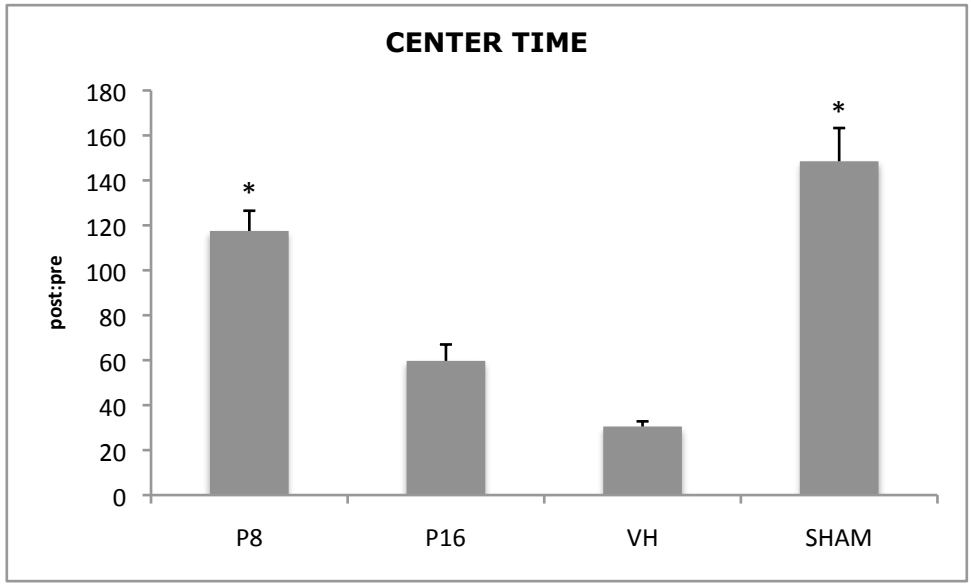


Figures 4.13 and 4.14. COX-2 and activated caspase-3 levels in aged animals 24 hours after injury. * $p < 0.05$ vs. VH.

4.4.7. Locomotor Activity at 72 Hours

Figures 4.15 and 4.16 show activity data as a ratio of testing performed 72 hours post-injury to that done before surgery. One-way ANOVA results show an overall treatment effect on locomotor behavior ($F_{3,12} = 5.66$, $p < 0.05$). The 16mg/kg treatment group displayed an increase in total distance traveled compared to both 8mg/kg ($p = 0.029$) and vehicle ($p = 0.012$), indicating increased spontaneous activity (Figure 4.15). The 8mg/kg dose did promote a significant anxiolytic effect, however, as evidenced by increased center time compared to vehicle in Figure 4.16 ($p = 0.005$; overall ANOVA, $F_{3,12} = 21.27$, $p < 0.05$). While the 16mg/kg dose showed an increasing trend over vehicle on this metric, this difference was not significant. Sham animals were different from vehicle on both distance ($p = 0.019$) and center time ($p = 0.001$).





Figures 4.15 and 4.16. Spontaneous locomotor behavior measurements of total distance traveled and center time in aged animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values. * $p < 0.05$ vs. VH.

4.5. DISCUSSION

In this study we show that our model of bilateral frontal cortical contusion injury can be successfully applied to old animals. In addition, progesterone treatment is shown to have beneficial molecular and behavioral effects similar to those seen in younger animals after TBI. Despite many similarities between the current results in the aged and our previously published data with young adults (Goss et al., 2003; Pettus et al., 2005), we observed a number of important differences. These include greater mortality in the old rats, both pre- and post-TBI, increased (3-4X) bleeding, and enhanced sensitivity to anesthesia. Because of these findings, our TBI model was slightly adjusted for the elderly animals: 1) they were handled for a much longer period prior to surgery (2 months vs. 1 week for young animals) in order to reduce stress through greater contact; 2) anesthesia was maintained at a higher O_2% with a lower overall isoflurane percentage during surgery; and 3) the aged animals were given subcutaneous Lactated Ringer's solution post-surgery to replace fluids lost through increased bleeding, a procedure not required in younger conspecifics.

We had initially planned a dose-response study with 3 progesterone doses (8, 16, and 32mg/kg) at 3 time-points (24, 48, and 72 hours). After analyzing inflammatory factor expression at 48 hours, however, we noted that 32mg/kg was consistently less beneficial than the low and median doses of 8 and 16mg/kg. These results were comparable to previous outcomes seen by Goss et al. in young animals (Goss et al., 2003), so only the 8 and 16mg/kg doses were used for the entirety of the study.

A number of well-established molecular markers associated with secondary injury and subsequent recovery after TBI were used as benchmarks in this study. NFκB is a dimeric inflammatory transcription factor that requires the p65-p50 complex for

translocation to the nucleus (Ghosh and Karin, 2002). Thus, an increase in the p65 subunit of NFκB can be taken to indicate inflammatory NFκB activity, as the p50-p50 dimer is transcriptionally inactive. The IκB inhibitor protein also acts to contain NFκB in the cytosol, rendering it inactive (Wissink et al., 1998). We found that 16mg/kg progesterone decreased levels of the p65 NFκB subunit and increased IκB at various time points after injury, suggesting a direct attenuation of inflammation. IL-6, a cytokine secreted by T-cells and macrophages that stimulates inflammation after trauma (Lenzlinger et al., 2001), was also reduced by both 8 and 16mg/kg progesterone at 24 and 72 hours. COX-2 levels, known to be elevated in activated macrophages at an injury site (Cernak et al., 2001), were reduced early after TBI showing suppression at 24 and 48 hours, and levels of TNFα, a ubiquitous acute phase inflammatory factor, were also reduced at 72 hours. Consistent with our previous results in younger animals (Grossman et al., 2004), these data suggest that progesterone reduces acute inflammation through its effects on various components of the inflammatory cascade in aged rats. The reduction in inflammation observed with progesterone treatment in the elderly is an especially interesting result, since increased levels of IL-6 and other inflammatory factors have been found to be associated with normal aging (Johnson, 2006) in addition to traumatic injury (Ruppel et al., 2002), and could therefore serve as confounding or exacerbating factors in determining treatment efficacy.

Reduced inflammation is associated with a reduction in secondary apoptotic cell death and is therefore an important aspect of neuroprotection in the early phase of the injury response (Verma, 2000). We assayed cell death by measuring levels of activated caspase-3, the final effector molecule in the extrinsic apoptotic pathway and an important

marker of tissue loss. Our data analysis showed decreased cleaved caspase-3 at all time points with 16mg/kg progesterone, implying significantly reduced cell death at this dose. Significantly, we also observed marked improvement in locomotor activity at 72 hours with the 16mg/kg dose, suggesting a relationship between better behavioral outcome and decreased levels of inflammatory cytokines and reduced cell loss. This effect was not observed with 8mg/kg treatment.

Another important factor in damage secondary to brain trauma is the rapid development of cytotoxic and vasogenic edema, both of which are known to be attenuated by the expression of P-gp. Present on neurons and on endothelial cells of capillaries throughout the brain, P-gp is a membrane-bound protein that works as an efflux pump to remove low molecular weight toxins from cells and as such is a key molecular marker of BBB integrity (Karssen et al., 2004). We found a reduction in edema 48 hours after TBI with 16mg/kg progesterone treatment that correlated with increased expression levels of P-gp. This observation can be taken to suggest that progesterone confers protection against TBI-induced edema, at least partially by increasing the expression of P-gp and maintaining BBB function (Mima et al., 1999). These results suggest the presence of additional factors. Thus, while P-gp levels were elevated in both the 8mg/kg and 16mg/kg-treated animals, edema was significantly reduced only by 16mg/kg progesterone, although the 8mg/kg dose did show a trend in the same direction. This suggests that the extent of edema is not wholly dependent on P-gp expression and that a reduction in inflammation also contributes to reduced swelling. The larger implication is that progesterone can affect a variety of mechanisms and its efficacy is likely due to pleiotropic action on multiple interacting systems.

Of the two dosages studied, 16mg/kg progesterone produced the most consistent beneficial effects on all measures over the time points we examined. These benefits, as compared to the 8mg/kg dose, are summarized in Table 3.1. A reduction in inflammatory response was also seen in the 8mg/kg group, and the outcomes trended in the same direction as those seen with 16mg/kg in many cases where they were not significant. However, overall the results were more variable and there was less observable behavioral improvement compared to those animals receiving a 16mg/kg dose of progesterone. The 16mg/kg dose also decreased brain swelling by more than 50% compared to vehicle- and 8mg/kg-treated groups, a result similar to that repeatedly observed in younger rats with TBI (Goss et al.). Given the complex nature of the injury and the potentially multivalent effects of administered progesterone, there may be other molecular modes of action that we have not assayed here. These data suggest that it is important to consider multiple effects and interactions when evaluating treatment efficacy and that behavioral and other measures such as edema are important in confirming the molecular results. Because 16mg/kg was found to be optimally protective in both young and old animals, both on a molecular and physiological scale, we suggest that this is the "best dose" for treating brain injury in mature rats.

Overall, our results indicate that progesterone treatment at 16mg/kg decreases inflammation, reduces cell death, and improves BBB integrity in the acute phase of injury in aged rats with TBI. These molecular events are correlated with reduced edema and improved measures of functional activity, and are consistent with data obtained in younger conspecifics, although further studies on recovery in the chronic post-injury

period still need to be performed. The optimal doses and duration of treatment were also similar to those found in younger animals, suggesting that progesterone and its metabolites may be effective as a treatment for TBI across the developmental spectrum. Given the rapidly increasing significance of brain injury in the aging human population, and the promising outcomes of progesterone treatment in early clinical trials (Wright et al., 2007), these results could have a significant impact on the clinical management of TBI.

Table 4.1: Summary of dosage outcomes compared to vehicle

	Time	16mg/kg PROG	8mg/kg PROG
IL-6	24 Hours	++	←
IκB		++	++
COX-2		→	++
Caspase-3		++	0
NFκB	48 Hours	++	←
IκB		0	++
COX-2		++	←
Caspase-3		++	++
P-gp		++	++
Edema		++	←
IL-6	72 Hours	++	++
TNFα		++	++
COX-2		→	0
Caspase-3		++	0
Tot Distance		++	0
Center Time		++	0
Total Positive Outcomes		13	7

++: Significant positive difference from vehicle

←, →: Positive trend from vehicle

0: No difference from vehicle

CHAPTER 5

VITAMIN D DEFICIENCY REDUCES THE BENEFITS OF PROGESTERONE TREATMENT AFTER BRAIN INJURY IN AGED RATS

5.1. 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 (D-deficiency) and increasing traumatic brain injuries (TBIs) in the elderly, we investigated the interaction of D-deficiency and PROG with cortical contusion injury in aged rats. D-deficient animals showed elevated inflammatory proteins (TNFα, IL-1β, IL-6, NFκB p65) in the brain even without injury. D-deficient rats with TBI, whether given PROG or vehicle, showed increased inflammation and greater open-field behavioral deficits compared to vitamin D sufficient animals. Although PROG was beneficial in injured vitamin D sufficient animals, in D-deficient subjects neurosteroid treatment conferred no improvement over vehicle. Our results suggest that D-deficiency can increase baseline brain inflammation, exacerbate the effects of TBI, and attenuate the benefits of PROG treatment; these results may have significant implications for the clinical management of TBI.

NOTE: Sections of this chapter were published previously as Cekic M, Cutler SM, VanLandingham JW, and Stein DG (2011) "Vitamin D deficiency reduces the benefits of progesterone treatment after brain injury in aged rats." *Neurobiol Aging* 32(5):864-74. Epub 2009 May 30.

5.2. INTRODUCTION

Traumatic brain injury (TBI) is a significant cause of death and mental and physical disability worldwide. Although the TBI mortality rate has decreased for most age groups, it has risen by over 21% in the elderly (CDC, 2004) and is now more than double the death rate of the younger population (Mosenthal et al., 2002). A host of recent studies have demonstrated that treatment with progesterone (PROG) significantly improves functional outcome after TBI in rats and humans. A neuroactive steroid, PROG has been shown to reduce neuronal cell death, oxidative damage, cerebral edema, and expression of inflammatory cytokines (Djebaili et al., 2005; Galani et al., 2001; Grossman et al., 2004; He et al., 2004a; He et al., 2004b; Pettus et al., 2005; Roof et al., 1994; Roof et al., 1997; Shear et al., 2002), although its specific modes of action remain unclear. Although two recent clinical trials were successful in demonstrating that PROG could reduce mortality and enhance functional outcomes in moderately to severely injured TBI patients (Wright et al., 2007; Xiao et al., 2008), the effectiveness of PROG treatment in the elderly has not been clearly established as almost all of the study subjects were between 18-40 years of age. The same is the case in laboratory studies since most model systems involve young adult animals.

In addition to advanced age, itself a predictor of mortality and morbidity (Mosenthal et al., 2002), the elderly are likely to suffer from a number of additional systemic exacerbating factors that may include hypertension, atherosclerosis, cardiovascular disease, diabetes, and cancer (Onyszchuk *et al.,* 2008). All of these systemic issues have recently been connected through their association with vitamin D deficiency (D-deficiency), a largely ignored problem from the clinical perspective (Grant,

2006; Holick and Chen, 2008; Peterlik and Cross, 2005). 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). D-deficiency is very common in industrialized countries and affects even healthy, young populations (Calvo and Whiting, 2003), but it is extremely widespread in the old and the ill (Chatfield *et al.,* 2007; Corino *et al.,* 2007), with reported prevalences ranging from 75% (Holick, 1994) to 87% (Larrosa et al., 2001) in institutionalized elderly people and even higher in hospitalized inpatients (Eyles et al., 2003; Eyles et al., 2005; Hewison et al., 2000; Holick, 2003b; McGrath et al., 2004; Townsend et al., 2005). It is thus rapidly becoming an important consideration in dealing with health issues affecting the elderly.

Although vitamin D has classically been associated with systemic calcium homeostasis and bone maintenance, there is now evidence that it is a potent modulator of the immune system and can significantly affect infectious, inflammatory, and autoimmune conditions (Cantorna et al., 2004; DeLuca and Zierold, 1998; Griffin et al., 2003; Hayes et al., 2003; Holick, 2003a, b; Mahon et al., 2003), cancer (Peterlik and Cross, 2005), cardiovascular disease risk (Martins et al., 2007; Melamed et al., 2008), neuromuscular function (Pfeifer *et al.,* 2002), cell cycle control (Banerjee and Chatterjee, 2003; Zhu et al., 1999), and functional outcome in the elderly (Dawson-Hughes, 2008).

Vitamin D levels are commonly determined by serum levels of 25-hydroxyvitamin D_3 ($25OHD_3$), with levels below 25nmol/L defined as deficiency in humans, levels between 25nmol/L and 50nmol/L defined as insufficiency, and higher than 50nmol/L as normal (Lips, 2004), although exact cutoff values are still being

debated (Holick, 2007; Holick and Chen, 2008). Levels below 20nmol/L are associated with rickets and adult osteomalacia, the hallmark of deficiency, but recently the value range considered necessary for optimal health has shifted to 100-120nmol/L (Prentice et al., 2008), and a daily intake of at least 2000 IU/day has been suggested (DeLuca, 2004).

In this study we extend our investigation of the effects of PROG treatment on injury and recovery of function in the aged rat begun in Chapter 4 by evaluating the role of D-deficiency 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 D-deficiency is virtually endemic in the elderly population, the obvious approach is to examine the efficacy of PROG treatment for TBI in an aged model combined with D-deficiency. We asked several questions: 1) Does D-deficiency cause increased baseline inflammation in the brain of uninjured aged rats, thereby establishing a potentially detrimental underlying condition? 2) Does D-deficiency exacerbate brain injury in animals treated with vehicle compared to vehicle-treated but nutritionally normal animals? 3) Does D-deficiency interact with PROG treatment, and does it affect the acute phase inflammatory response in treated D-deficient animals versus treated non-deficient animals? The hypothesis was that D-deficiency would elevate baseline inflammation in sham animals, increase the inflammatory response and exacerbate injury in animals with TBI, and attenuate the beneficial effects of PROG treatment.

5.3. MATERIALS AND METHODS

5.3.1. Subjects

Eighty-seven 22-month-old male Fischer 344 rats weighing 450-550g at the time of injury were used in this experiment. Animals were housed and handled as previously described (Chapter 4). This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All experimental animal procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), Protocol #146-2005.

5.3.2. Diet

The animals in this study were separated into two groups, vitamin D normal (D-normal) and vitamin D deficient (D-deficient). The D-normal group was given standard rat chow used in our animal care facility (Rodent Diet 5001, LabDiet®, St. Louis, MO). The D-deficient 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 D-deficient group were maintained on the diet for at least 21 days prior to surgery. Eight days has been shown to be sufficient time to induce a circulating 25-hydroxyvitamin D_3 level consistent with deficiency (Narayanan et al., 2004a), but we extended this time period to allow the sequelae of D-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 D-deficient group were maintained on it until they were killed for harvesting of brain tissue. Since vitamin D is activated by UVB light (280-315nm wavelength), we ensured that the overhead lights in our animal colony did not produce radiation in this range.

5.3.3. Surgery and Contusion Injury

Rats were anesthetized using isoflurane gas (5.0% induction, 1.0–1.5% maintenance, 700mmHg N_2O, 500mmHg O_2) and surgery was performed using aseptic techniques as previously described (Chapter 4). Briefly, a 6mm diameter mid-sagittal bilateral craniotomy was performed 3mm anterior to bregma and a cortical contusion injury (CCI) was produced in the medial frontal cortex (MFC) by a pneumatic cortical contusion device (5mm diameter) with impact velocity of 2.25m/s, impact time of 500ms, and depth of 3.5mm ventral to bregma. The incision was sutured closed after all bleeding had fully stopped. In the sham group, the incisions were sutured closed after comparable time under anesthesia. Animals dehydrated due to blood loss were given 3mL of lactated Ringer's solution subcutaneously within 6 hours of injury.

5.3.4. Treatment

Animals were assigned to D-normal or D-deficient groups, and each of these groups was subdivided into three treatment groups (n = 5/group): no injury (SHAM), vehicle (VH, 22.5% 2-hydroxypropyl-β-cyclodextrin), and progesterone (PROG, 16mg/kg progesterone; P0130, Sigma-Aldrich, St. Louis, MO). The same assignment was followed for both 24-hour and 72-hour survival groups. We used our previously discussed treatment protocol (Chapter 4) consisting of an intraperitoneal injection 1 hour post-injury followed by subcutaneous injections at 6 hours, 24 hours, and every 24 hours 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 16mg/kg PROG because previous research demonstrated it to be the most effective dosage in young and aged rats (Goss et al., 2003).

5.3.5. Activity Testing

Spontaneous locomotor activity was performed as previously described (Chapter 4). The spontaneous locomotor activity task has previously been shown to be sensitive to our model of TBI and to the effects of PROG treatment (Djebaili et al., 2005), as well as to potential behavioral and motor derangements due to D-deficiency in open-field testing (Kalueff et al., 2004a).

5.3.6. Tissue Preparation and Western Blot Analysis

Animals were killed 24 or 72 hours after surgery with a lethal dose of Nembutal (1mL) and decapitated. Their brains were prepared for protein analysis and Western blots were performed as previously described (Chapter 4). 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 μL of each sample (at a concentration of 2μg/μL, for 30μg 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), 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 determined as discussed above (Chapter 4) and compared only between treatment groups run on the same blots. When more than one blot was used due to the large number of samples and experimental groups, 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.

5.3.7. Serum Vitamin D and PROG Levels

Blood (0.5 – 1.0mL) was drawn directly from the right ventricle of the heart with a 21G needle after the rats were unconscious from the Nembutal but before death or decapitation (24 hours after the last treatment). The whole blood was allowed to coagulate for 30 minutes at RT, after which the clot was removed and the serum centrifuged for 5 minutes at 1000 x g. The serum was removed and stored at -80°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 also measured with a solid-phase RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assays were performed by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center at Emory University in Atlanta, GA.

5.3.8. 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). GLMs were used to examine interaction effects in the behavioral data between vitamin D status and experimental groups, using deficiency, injury, and treatment as fixed factors and molecular measures as covariates. Since deficiency is an underlying condition and is therefore logically prior to the other independent variables, a Type I SS model for fixed factors was used. The same fixed factor model was applied to molecular measures, but without any covariates. All analyses were calculated using SPSS^{TM} 17.0 statistical analysis software.

5.4. RESULTS

5.4.1. General Observations of Frailty in Vitamin D Deficient Aged Rats

The deficient animals were observed to be more "frail" in comparison with rats fed the 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.

5.4.2. Serum Values Demonstrate 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 D-deficient status in rats (Rojanasathit and Haddad, 1977). No differences were observed between individual groups within the different nutritional conditions at either 24 or 72 hours after injury. PROG data showed no difference between the groups, with average serum PROG levels of 15.87 ± 2.83 ng/mL (n = 16) in D-normal animals and 16.96 ± 1.43 ng/mL in D-deficient animals (n = 37). There were no significant differences between the individual groups within each nutritional condition at either 24 or 72 hours, confirming earlier data (Wright et al., 2001) that PROG levels stabilize by 24 hours after injury even with PROG administration. All data are expressed as average ± SEM.

5.4.3. Vitamin D Deficiency Elevates Baseline Levels of Inflammatory Cytokines in Uninjured Brain

We first asked whether D-deficiency would increase baseline inflammation in intact animals, as this would suggest a general systemic inflammatory state even *before* injury. Figure 5.1 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 D-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. The *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 D-deficient rats compared to intact D-normal animals. While the data shown were from brains extracted 72 hours after the sham surgery, similar results were obtained at 24 hours as well (not shown).

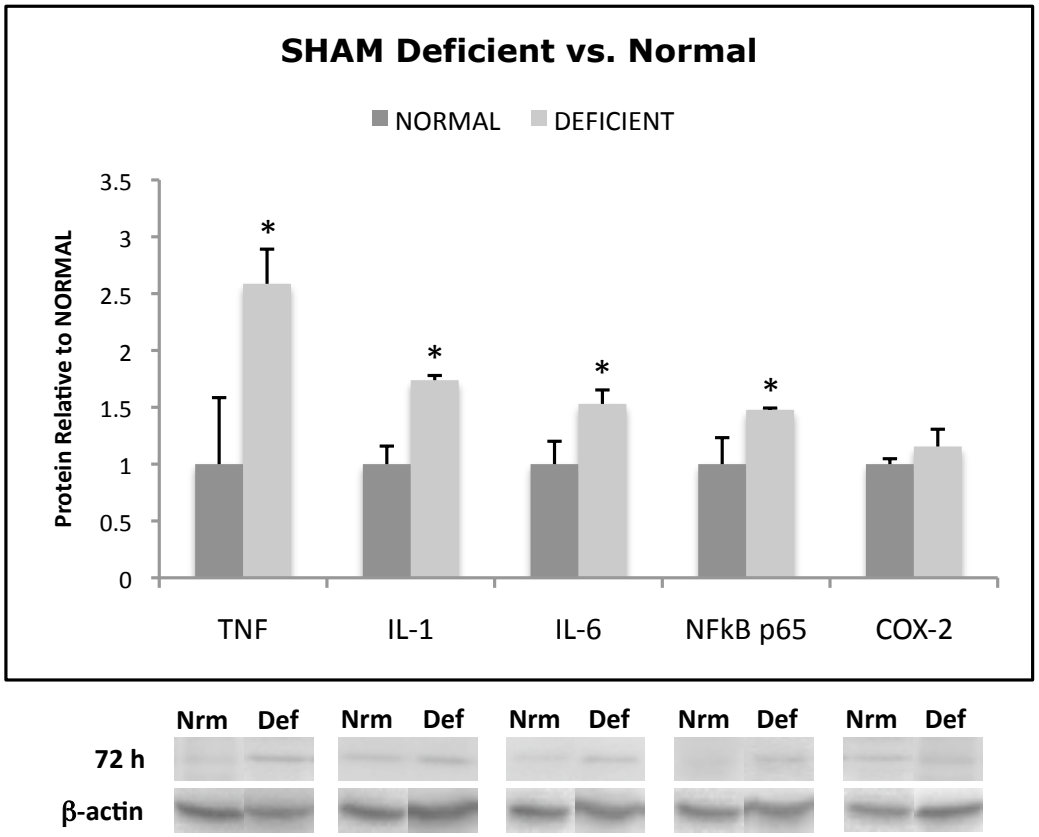


Figure 5.1. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal sham uninjured aged animals. All values are normalized to normal animal values (vertical axis = 1), * $p < 0.05$.

5.4.4. Vitamin D Deficiency Exacerbates Inflammation in Vehicle-treated Animals with TBI

Our second question was whether D-deficiency exacerbates inflammation in injured, vehicle-treated animals. Figure 5.2 shows the results for the same panel of inflammatory proteins identified above and activated caspase-3 24 and 72 hours after injury. The data were normalized to the respective cytokine at the same time-point in D-normal animals (vertical axis value = 1) and are shown as the ratio deficient:normal ± SEM. At 24 and 72 hours, respectively, the t-test *p*-values comparing normal and deficient animals were: TNFα ($p = 0.29$; $p = 0.039$), IL-1β ($p = 0.23$; $p = 0.078$), 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 hours after injury, only COX-2 was significantly elevated in D-deficient animals treated with vehicle compared to their D-normal counterparts. By 72 hours, however, *all* inflammatory markers with the exception of IL-1β and COX-2 were significantly higher in vehicle-treated D-deficient versus D-normal animals.

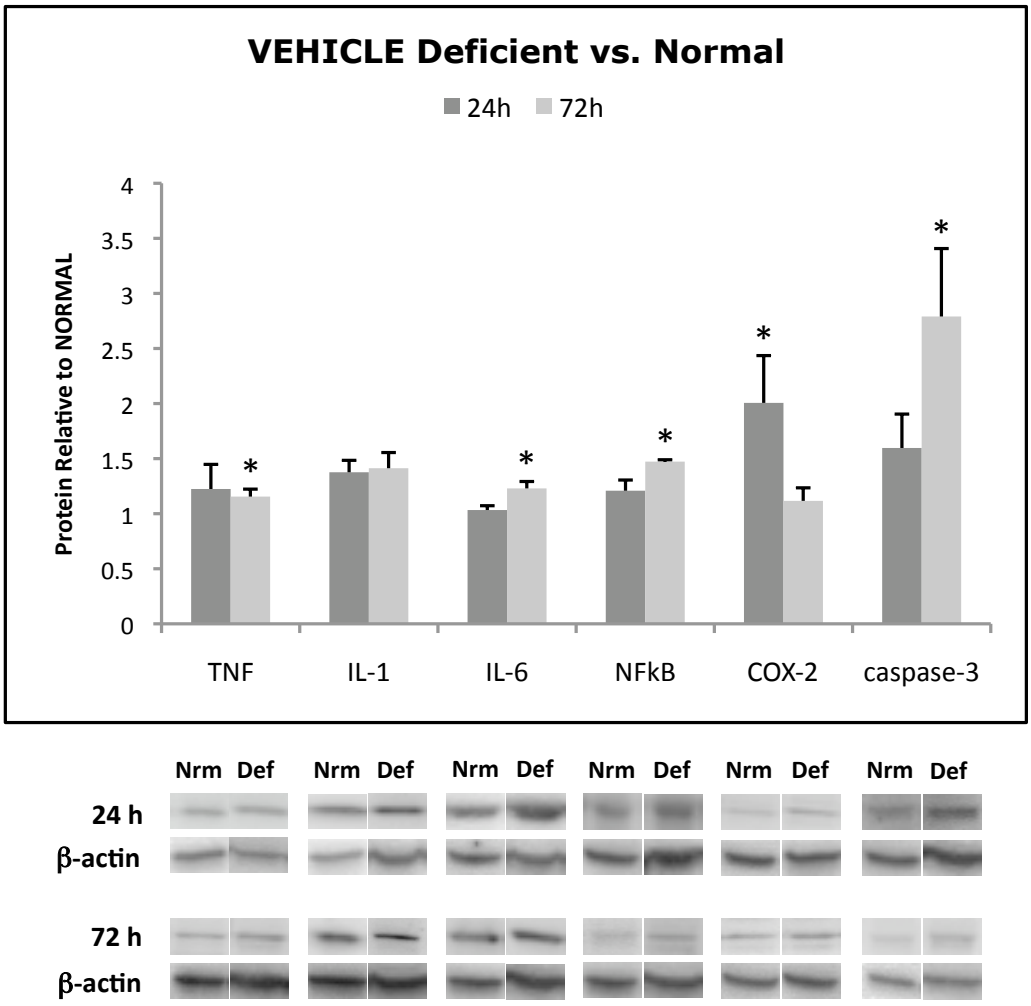


Figure 5.2. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal vehicle-treated injured aged animals 24 and 72 hours after injury. All values are normalized to normal animal values (vertical axis = 1), * $p < 0.05$.

5.4.5. Vitamin D Deficiency Exacerbates Inflammation in PROG-treated Animals After TBI

The third question was whether D-deficiency exacerbated injury-related inflammation in animals treated with PROG. Figure 5.3 shows the results for the same proteins in deficient versus normal PROG-treated animals 24 and 72 hours 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 hours 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 hours after injury in D-deficient versus D-normal animals treated with PROG, but by 72 hours all but IL-1β are significantly higher in the deficient group. This may suggest that effects of D-deficiency become more pronounced as the injury evolves over time.

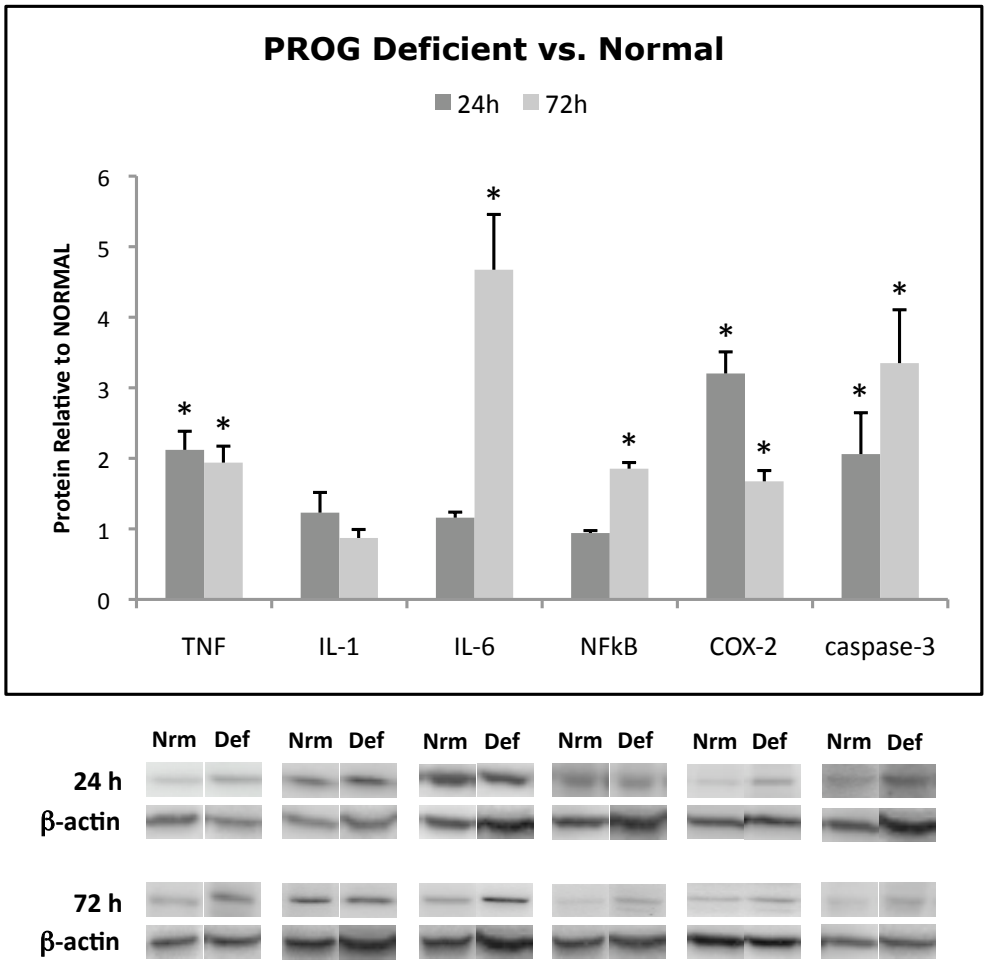
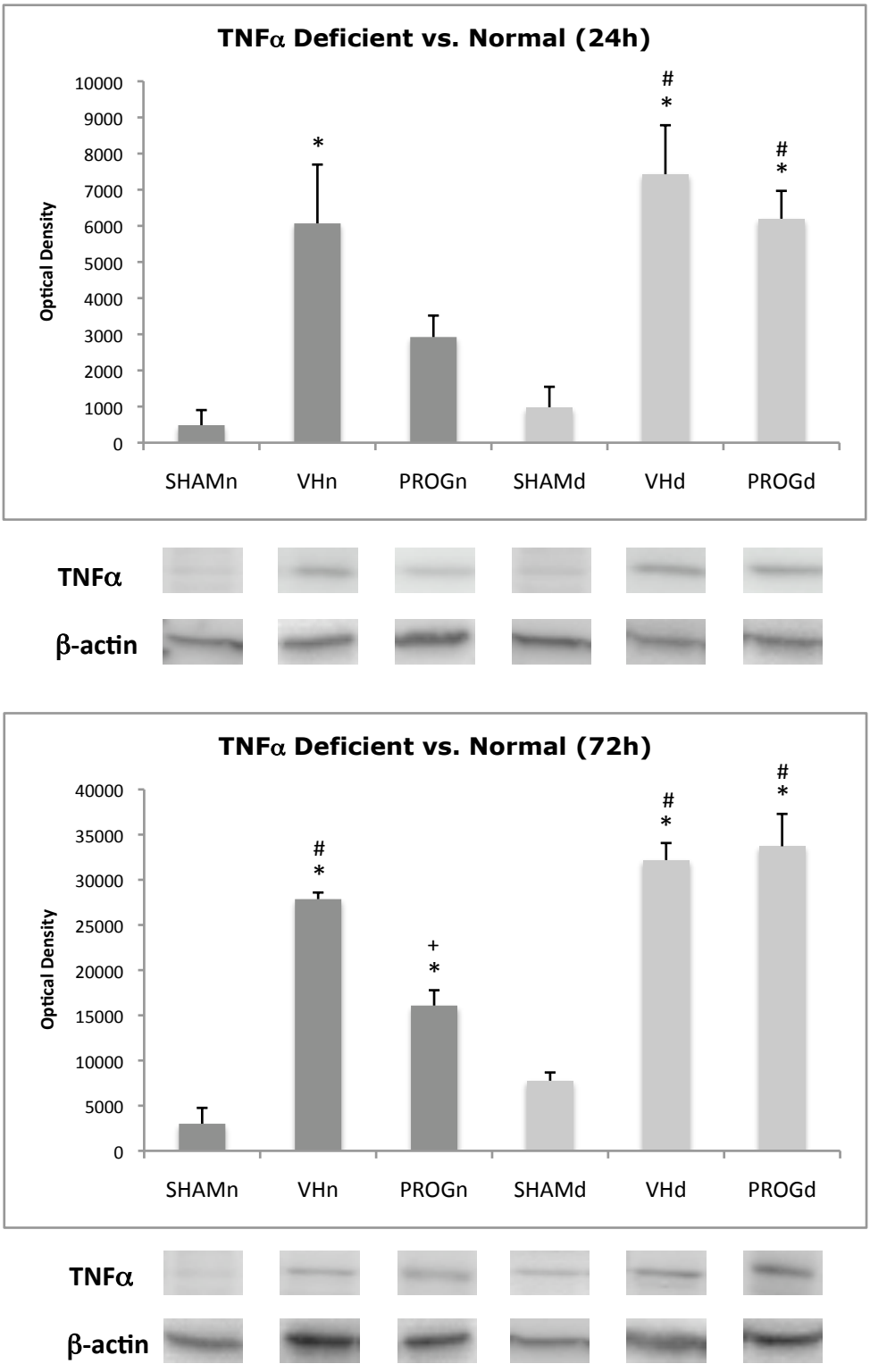


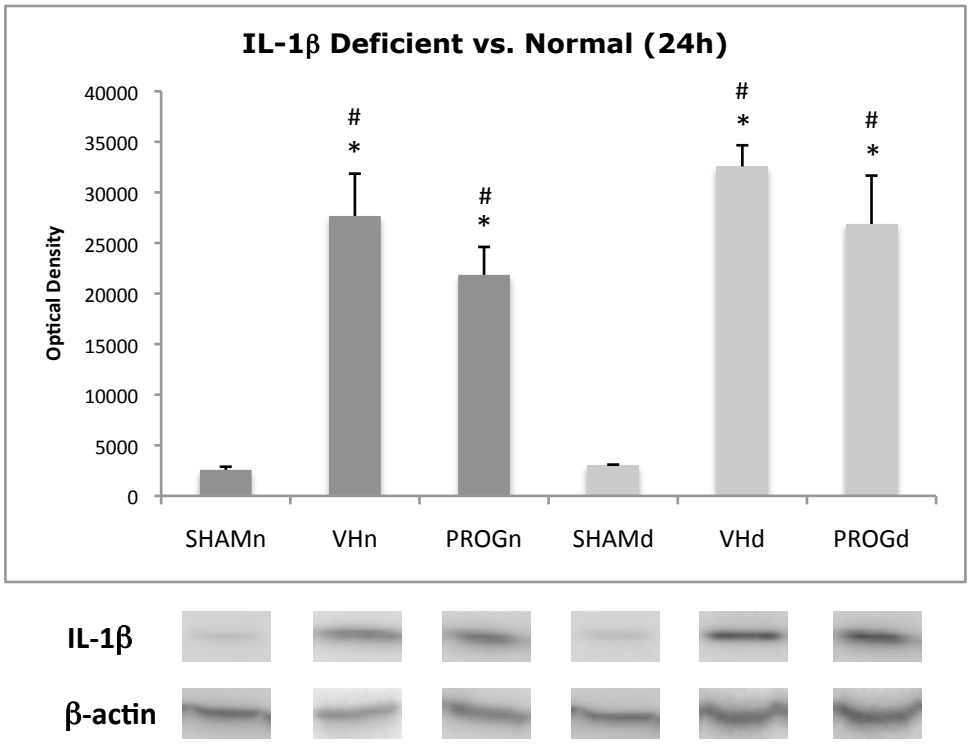
Figure 5.3. Levels of inflammatory cytokines in vitamin D deficient compared to vitamin D normal progesterone-treated injured aged animals 24 and 72 hours after injury. All values are normalized to normal animal values (vertical axis = 1), * $p < 0.05$.

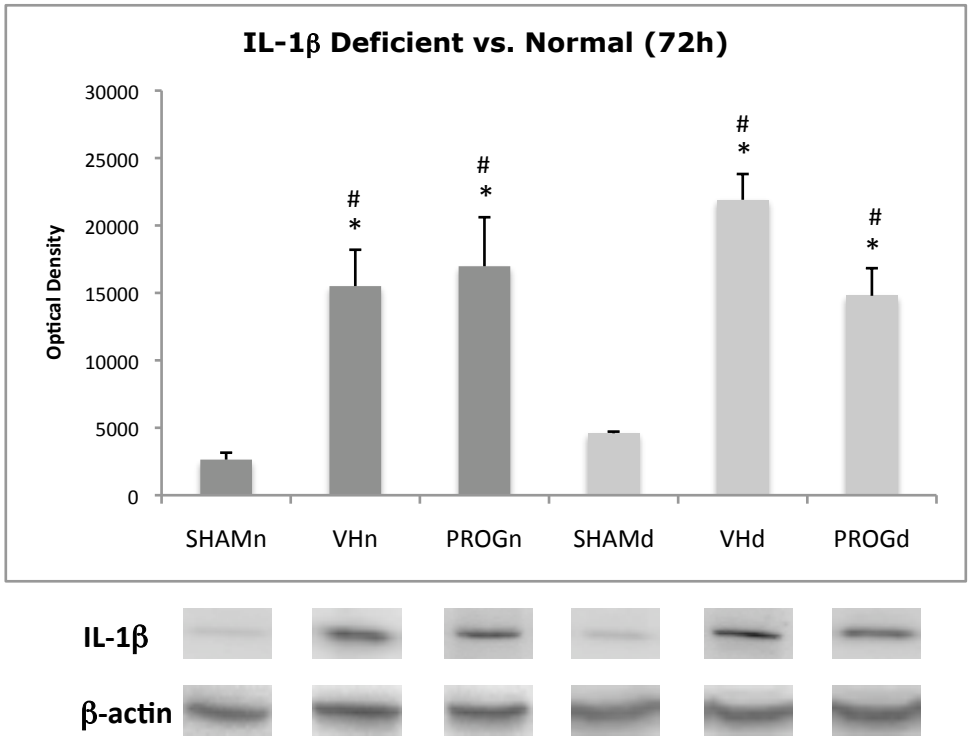
5.4.6. Vitamin D Deficiency Attenuates the Beneficial Effects of PROG on Acute Inflammatory Cytokines After TBI

Since cytokine profiles were in general significantly worse in deficient than in normal animals by 72 hours after injury, the next question we asked was whether PROG loses its therapeutic effectiveness under conditions of D-deficiency. In other words, although D-deficiency appears to increase inflammation over normal animals whether they are given PROG or vehicle, does PROG administration still reduce inflammation compared to vehicle in D-deficient animals as it does in those that are D-normal? Our results show that PROG treatment in D-deficient animals results in mild improvement compared to vehicle-treated D-deficient animals, but these effects are minimal compared to the significant improvements seen when it is given in D-normal animals. Figures 5.4 through 5.13 show levels for several cytokines and proteins 24 and 72 hours after TBI in D-deficient animals: TNFα (Figure 5.4: 24h: $F_{5,24} = 6.602$, $p = 0.002$; Figure 5.5: 72h: $F_{5,24} = 34.927$, $p < 0.001$), IL-1β (Figure 5.6: 24h: $F_{5,24} = 8.498$, $p = 0.002$; Figure 5.7: 72h: $F_{5,24} = 8.212$, $p < 0.001$), IL-6 (Figure 5.8: 24h: $F_{5,24} = 29.475$, $p < 0.001$; Figure 5.9: 72h: $F_{5,24} = 44.485$, $p < 0.001$), NFκB p65 (Figure 5.10: 24h: $F_{5,24} = 13.032$, $p < 0.001$; Figure 5.11: 72h: $F_{5,24} = 22.274$, $p < 0.001$), and activated caspase-3 (Figure 5.12: 24h: $F_{5,24} = 12.523$, $p < 0.001$; Figure 5.13: 72h: $F_{5,24} = 9.471$, $p < 0.001$). In most cases, PROG did not demonstrate a significant improvement over VH, and there was more extensive acute inflammation in PROG-treated D-deficient animals and in their D-normal counterparts, suggesting that vitamin D deficiency may interact with both the injury process and PROG treatment.

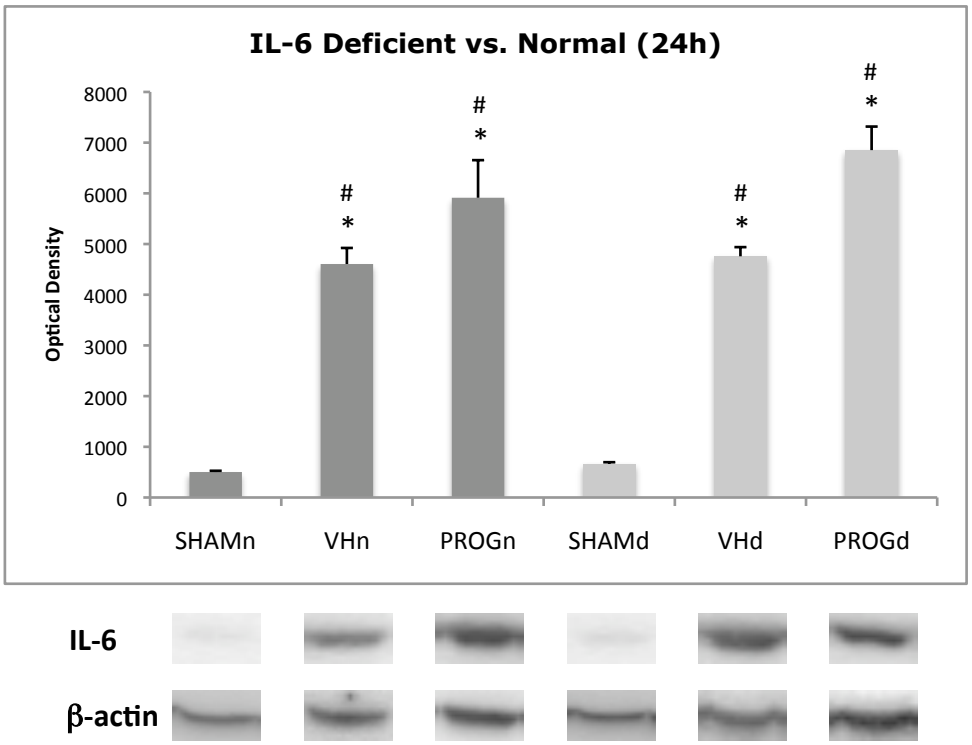


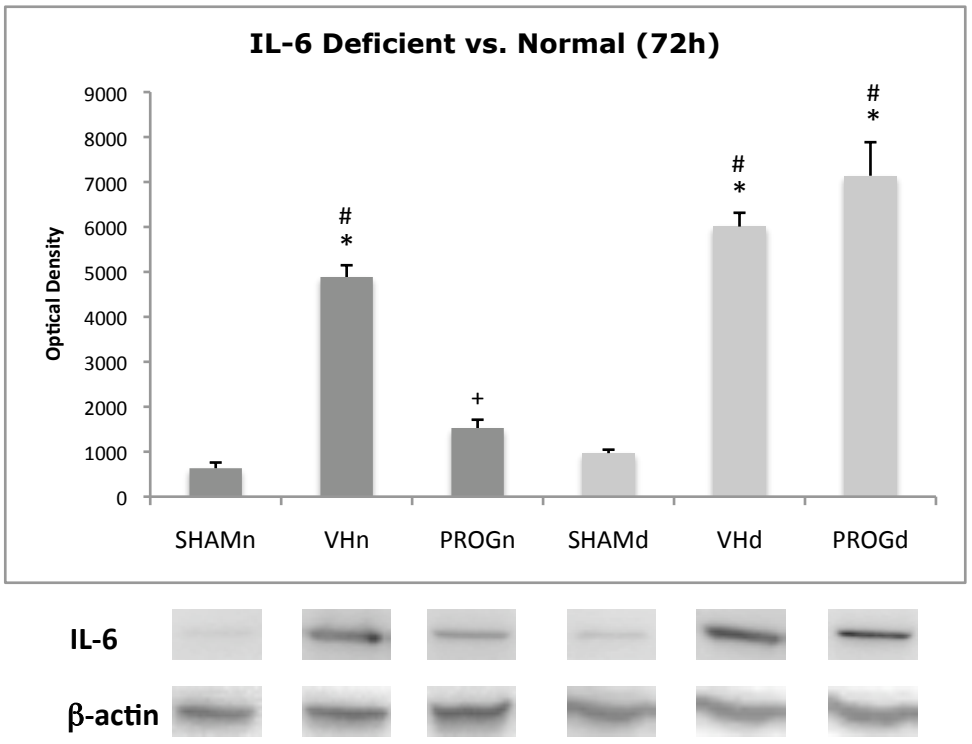
Figures 5.4 and 5.5. TNFα levels in vitamin D deficient (light grey) compared to vitamin D normal (dark grey) aged animals 24 and 72 hours after injury. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.



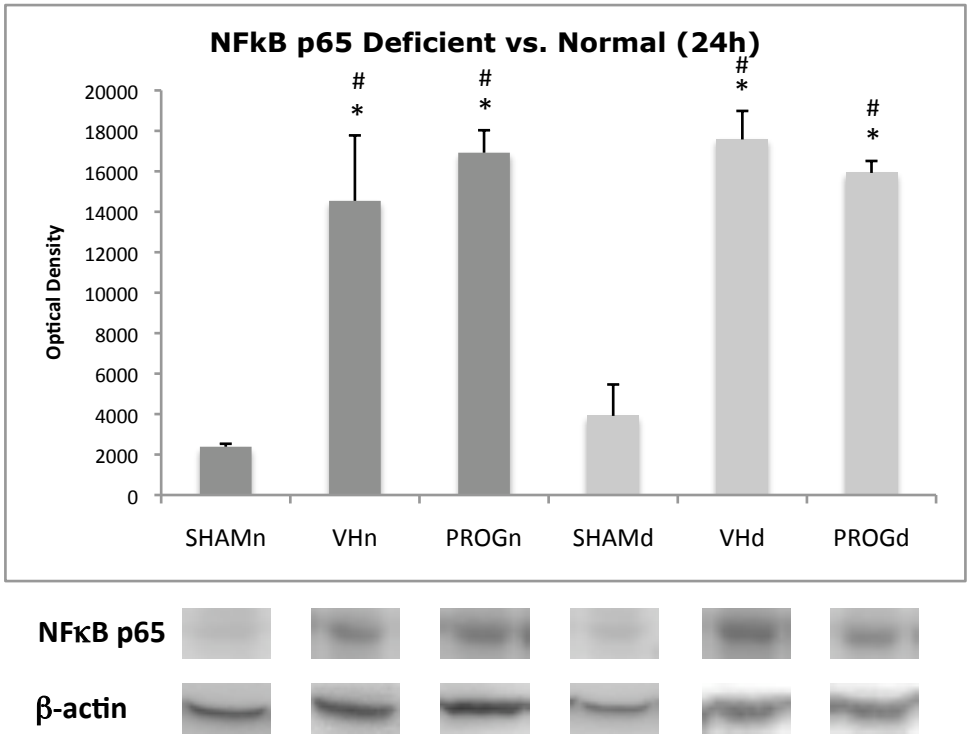


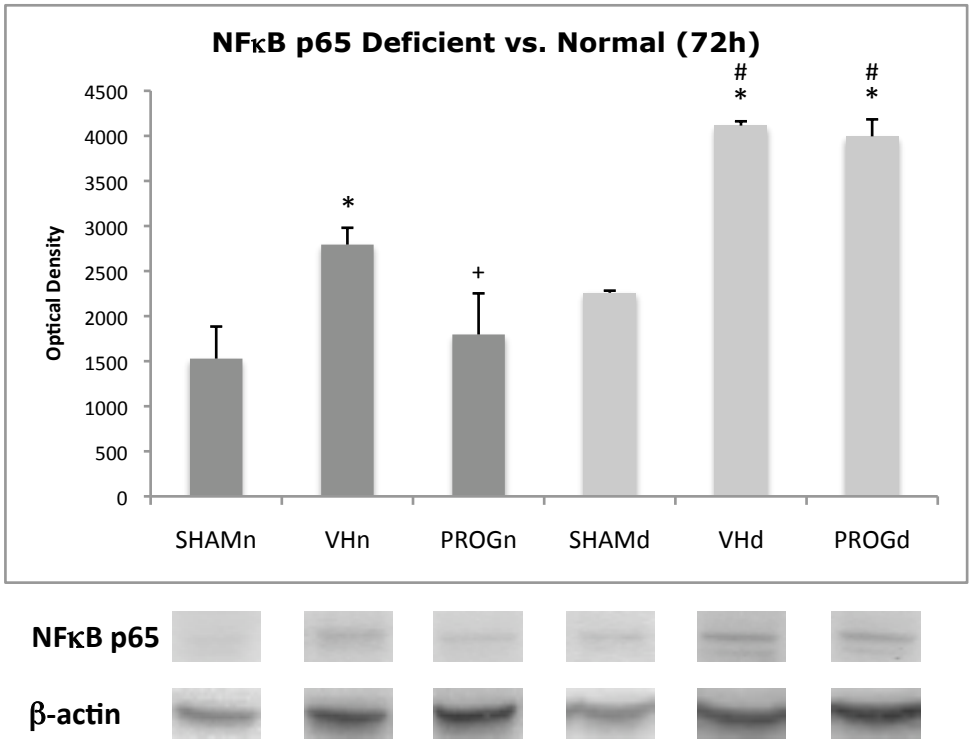
Figures 5.6 and 5.7. IL-1β levels in vitamin D deficient (light grey) compared to vitamin D normal (dark grey) aged animals 24 and 72 hours after injury. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.



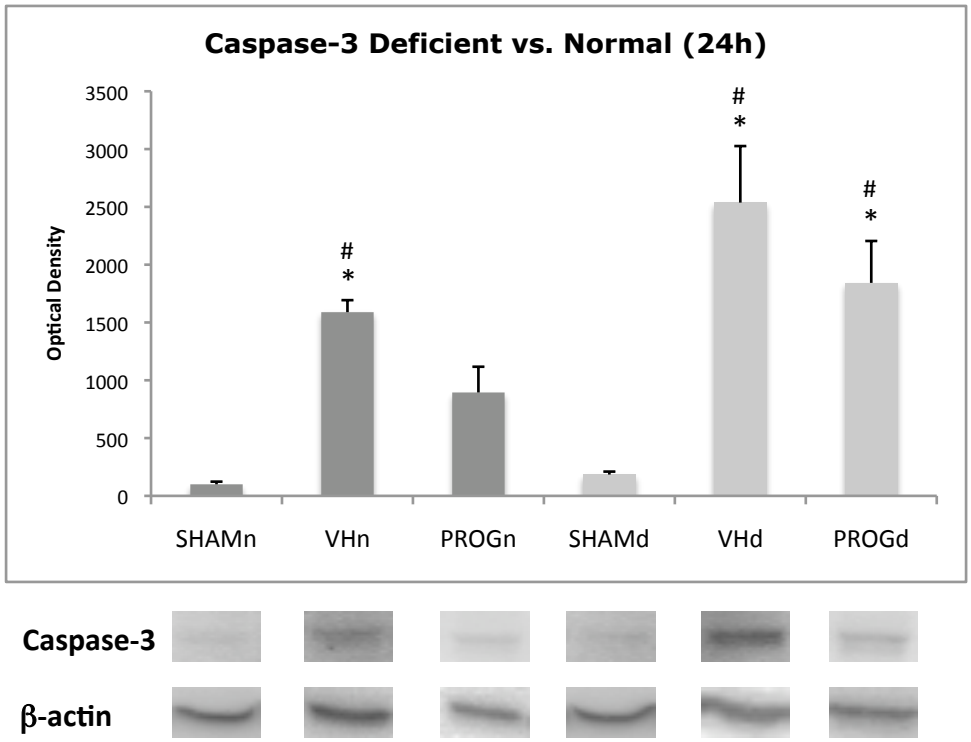


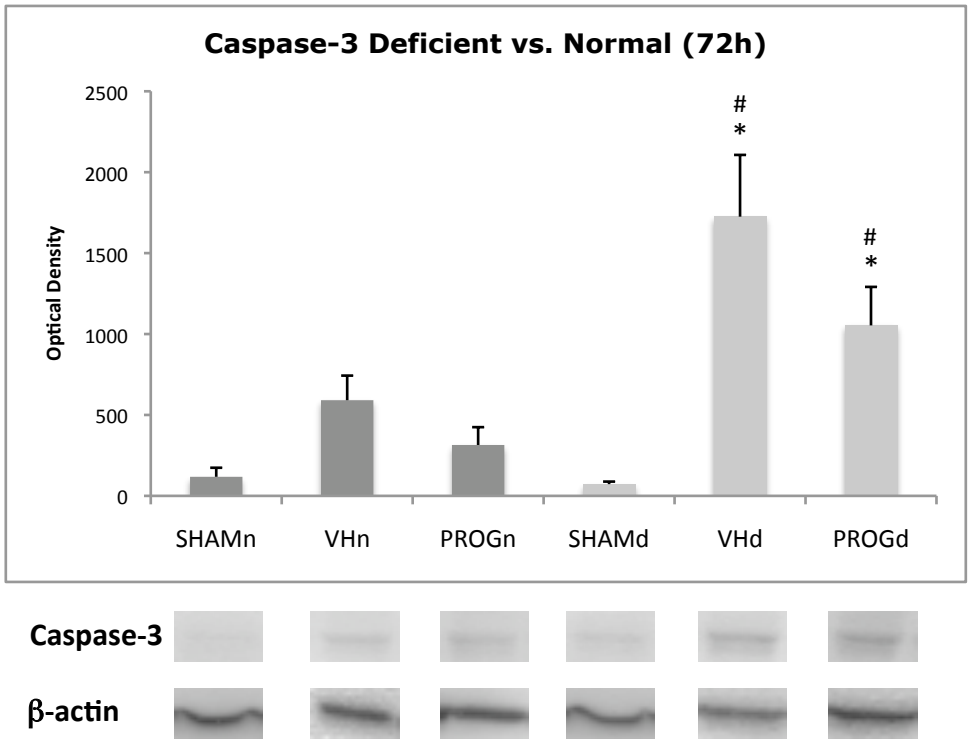
Figures 5.8 and 5.9. IL-6 levels in vitamin D deficient (light grey) compared to vitamin D normal (dark grey) aged animals 24 and 72 hours after injury. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.





Figures 5.10 and 5.11. NFκB p65 levels in vitamin D deficient (light grey) compared to vitamin D normal (dark grey) aged animals 24 and 72 hours after injury. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.

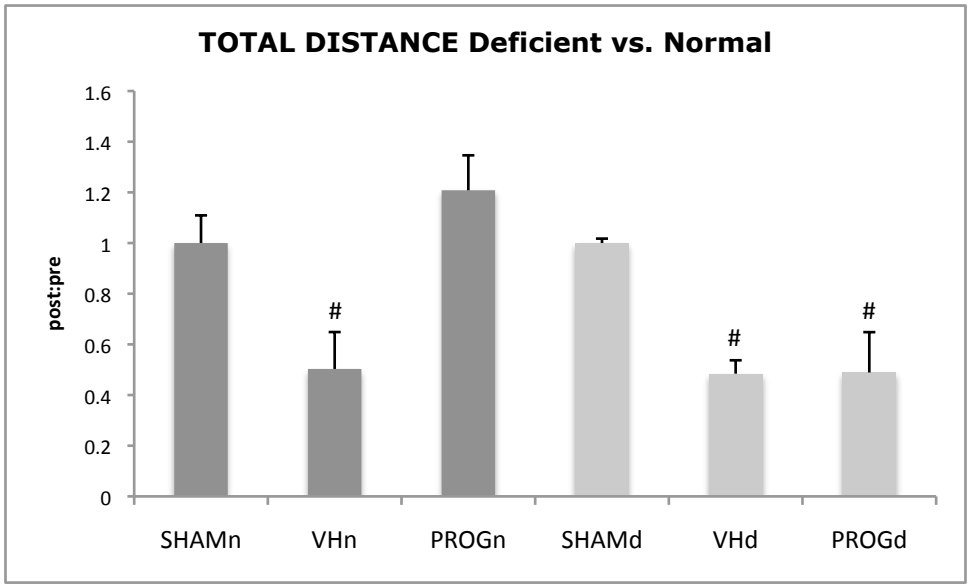


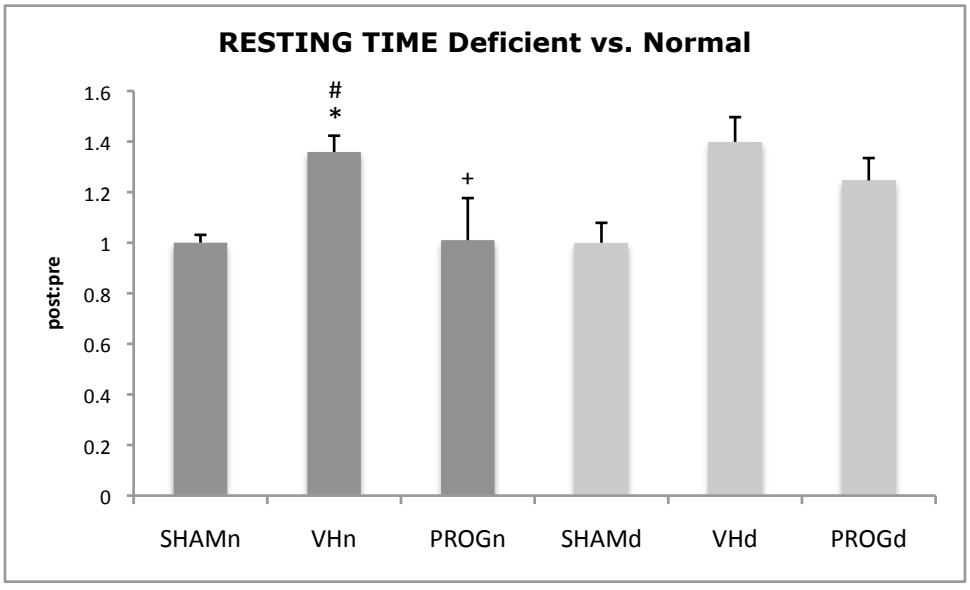


Figures 5.12 and 5.13. Levels of activated caspase-3 in vitamin D deficient (light grey) compared to vitamin D normal (dark grey) aged animals 24 and 72 hours after injury. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.

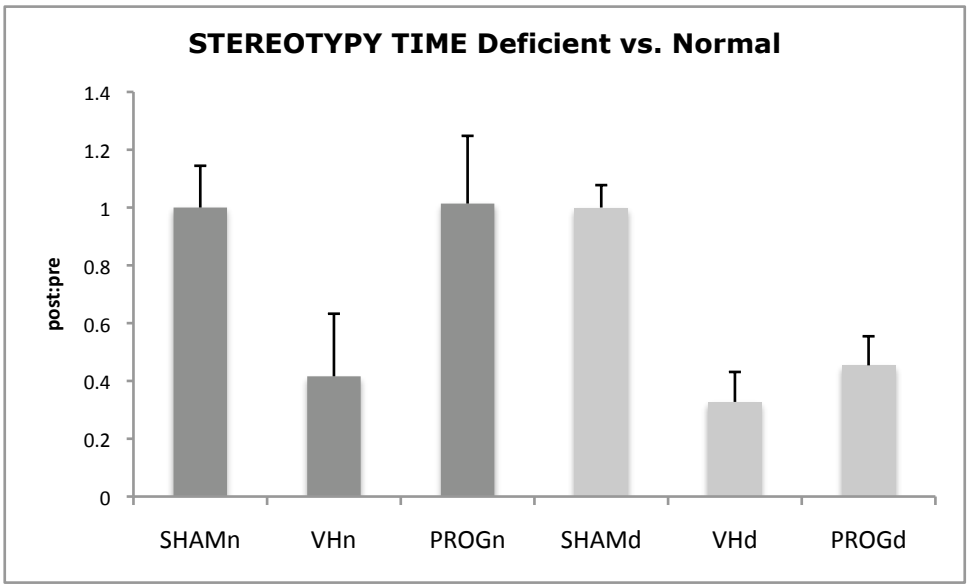
5.4.7. Vitamin D Deficiency Attenuates Short-term Behavioral Benefits of PROG Treatment After TBI

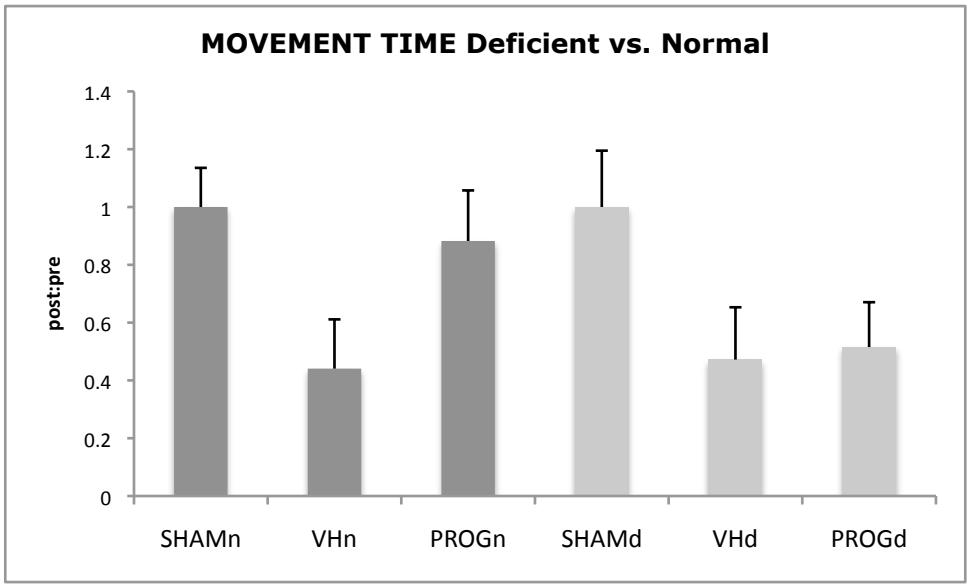
In addition to molecular measures of inflammatory cytokines, we examined the behavioral effects of D-deficiency. Since this study was limited to the short-term effects on inflammation, only short-term Spontaneous Locomotor Activity was used. The results are shown in Figures 5.14 through 5.17 as the ratios of post-injury:pre-injury measurements. The parameters examined were total distance traveled (Figure 5.14: $F_{5,24} = 4.387$, $p = 0.012$), resting time (Figure 5.15: $F_{5,24} = 6.886$, $p = 0.001$), stereotypy time (Figure 5.16: $F_{5,24} = 3.036$, $p = 0.035$), and movement time (Figure 5.17: $F_{5,24} = 2.578$, $p = 0.061$) 72 hours after injury. While PROG treatment showed functional benefits in most cases when the animals were D-normal, this effect was obliterated by D-deficiency, supporting the molecular data on a behavioral level.





Figures 5.14 and 5.15. Spontaneous locomotor behavioral measurements of total distance traveled and resting time 72 hours after injury expressed as the ratio of post-injury to pre-injury values. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.





Figures 5.16 and 5.17. Spontaneous locomotor behavioral measurements of stereotypy and movement times 72 hours after injury expressed as the ratio of post-injury to pre-injury values. * $p < 0.05$ vs. SHAMn, # $p < 0.05$ vs. SHAMd, + $p < 0.05$ vs. VHn.

5.4.8. Models and Correlations

A number of significant correlations were observed between our behavioral and molecular data. Total distance was negatively correlated with the expression of TNFα (r = -0.750, $p < 0.001$), IL-6 (r = -0.764, $p < 0.001$), NFκB p65 (r = -0.680, $p = 0.001$), and caspase-3 (r = -0.614, $p = 0.003$), but not IL-1β (r = -0.323, $p = 0.164$). Resting time was significantly correlated with TNFα (r = 0.782, $p < 0.001$), IL-1β (r = 0.554, $p = 0.007$), IL-6 (r = 0.623, $p = 0.003$), NFκB p65 (r = 0.650, $p = 0.001$), and caspase-3 (r = 0.577, $p = 0.004$). Stereotypy time was significantly negatively correlated with TNFα (r = -0.625, $p = 0.002$), IL-6 (r = -0.738, $p < 0.001$), NFκB p65 (r = 0.478, $p = 0.028$), and caspase-3, (r = -0.584, $p = 0.003$), but not with IL-1β (r = -0.276, $p = 0.202$). Movement time was also negatively correlated with TNFα (r = -0.709, $p < 0.001$), IL-1β (r = -0.449, $p = 0.032$), IL-6 (r = -0.685, $p = 0.001$), NFκB p65 (r = -0.567, $p = 0.007$), and caspase-3 (r = -0.534, $p = 0.007$). This is not surprising given the dependence of behavioral measurements and sickness behavior on molecular events, and confirmed that our behavioral data was a valid organism-level observation for acute phase inflammation. All the molecular measures were further significantly positively correlated with each other.

To further explore this interaction between inflammatory molecular events and behavior, we constructed a GLM using deficiency/injury/treatment as fixed factors (Type I SS due to logical priority within the factors) and normalized molecular measures as covariates for two behavioral outcomes, total distance traveled and resting time. The only outcome model that was significant was for stereotypy time (corrected model: F = 6.746, $p = 0.040$, $R^2 = 0.944$), with most of the variability accounted for by TNFα (F = 30.945, $p = 0.005$, $\eta_p^2 = 0.886$, where η_p^2 is partial eta-square, a measure of effect size) and IL-6

(corrected model: F = 9.360, $p = 0.038$, $\eta_p^2 = 0.701$), with trends for IL-1β (F = 6.328, $p = 0.066$, $\eta_p^2 = 0.613$), and deficiency (F = 5.985, $p = 0.071$, $\eta_p^2 = 0.599$). The models for the other behavioral outcomes were not significant.

We also constructed GLMs with each molecular measure (72 h) as outcome using only the fixed factors (deficiency/injury/treatment). All the models were significant, but we list here only he significant components of each ($p < 0.05$): TNFα (corrected model: F = 32.927, $p < 0.001$, $R^2 = 0.916$; main effects: deficiency, $\eta_p^2 = 0.554$; injury, $\eta_p^2 = 0.898$; treatment, $\eta_p^2 = 0.293$; interaction effects: deficiency*treatment, $\eta_p^2 = 0.411$); IL-1β (corrected model: F = 8.212, $p < 0.001$, $R^2 = 0.695$; main effects: injury, $\eta_p^2 = 0.671$); IL-6 (corrected model: F = 44.485, $p < 0.001$, $R^2 = 0.933$; main effects: deficiency, $\eta_p^2 = 0.725$; injury, $\eta_p^2 = 0.884$; treatment, $\eta_p^2 = 0.385$; interaction effects: deficiency*injury, $\eta_p^2 = 0.451$; deficiency*treatment, $\eta_p^2 = 0.687$); NFκB p65 (corrected model: F = 22.274, $p < 0.001$, $R^2 = 0.874$; main effects: deficiency, $\eta_p^2 = 0.774$; injury, $\eta_p^2 = 0.724$; treatment, $\eta_p^2 = 0.245$; interaction effects: deficiency*injury, $\eta_p^2 = 0.237$); cleaved caspase-3 (corrected model: F = 6.147, $p = 0.002$, $R^2 = 0.618$; main effects: deficiency, $\eta_p^2 = 0.289$; injury, $\eta_p^2 = 0.462$; interaction effects: deficiency*injury, $\eta_p^2 = 0.191$). These results are intriguing, as they suggest that D-deficiency primarily affects the levels of TNFα and IL-6 after injury, and that for these cytokines, the major interaction is between deficiency and treatment. In other words, while deficiency does interact with injury itself (it exacerbates it with respect to IL-6, NFκB p65, and caspase-3), it also interacts with treatment for the two most important acute phase cytokines, and therefore may significantly affect PROG metabolism and effectiveness.

5.5. DISCUSSION

In this study we examined the interaction of D-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) D-deficiency increases baseline inflammation in the brains of uninjured aged rats, potentially establishing a detrimental underlying condition; 2) D-deficiency increases a number of inflammatory markers after injury in aged rats treated with vehicle at both 24 and 72 hours; 3) D-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 hours after TBI.

The first general result is consistent with other data showing that D-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 D-deficient rats indicates. While only COX-2 is elevated in these animals at 24 hours, the acute response at 72 hours after injury is increased for most of the markers examined, suggesting an amplified extended

inflammatory response and a potentially amplified secondary injury cascade. This same effect is seen in PROG-treated D-deficient animals, which show significantly increased acute response at 72 hours post-TBI. Of note here is the especially deranged IL-6 system, as IL-6 is known to interact with D-deficiency (Gurlek et al., 2002; Thien et al., 2005) and hyperparathyroidism (McCarty, 2005), and is a key cytokine in the development of inflammation and acute phase response to injury (Keel and Trentz, 2005; McCarty, 2005).

Vitamin D affects a number of molecular pathways that, singly or in synergy, may provide some explanation for our results. For example, vitamin D 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., 2004b), 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). Vitamin D has also 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). It is likely that all these pathways contribute to the observed effects of vitamin D, making the elucidation of a single governing mechanism improbable. Since multiple factors contribute to the injury response, we suggest that the end result of D-deficiency is the sum effect of these

derangements in individual physiological functions and that extended D-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 (Chapter 4) that a higher dosage of PROG (16 mg/kg vs. 8 mg/kg) is necessary for maximum benefit in older animals.

Our results suggest that in aged rats, chronic D-deficiency can significantly exacerbate acute CNS inflammation and attenuate the benefits of PROG treatment after TBI. Increased inflammation is also observed in the brains of deficient uninjured animals, demonstrating that the previously reported effects of D-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.

CHAPTER 6

COMBINATION TREATMENT WITH PROGESTERONE AND VITAMIN D HORMONE MAY BE MORE EFFECTIVE THAN MONOTHERAPY FOR NERVOUS SYSTEM INJURY AND DISEASE

6.1. ABSTRACT

More than two decades of pre-clinical research and two recent clinical trials have shown that progesterone (PROG) and its metabolites exert beneficial effects after traumatic brain injury (TBI) through a number of metabolic and physiological pathways that can reduce damage in many different tissues and organ systems. Emerging data on 1,25-dihydroxyvitamin D_3 (VDH), itself a steroid hormone, have begun to provide evidence that, like PROG, it too is neuroprotective, although some of its actions may involve different pathways. Both agents have high safety profiles, act on many different injury and pathological mechanisms, and are clinically relevant, easy to administer, and inexpensive. Furthermore, vitamin D deficiency is prevalent in a large segment of the population, especially the elderly and institutionalized, and can significantly affect recovery after CNS injury. The combination of PROG and VDH in pre-clinical and clinical studies is a novel and compelling approach to TBI treatment.

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6.2. INTRODUCTION

In the past twenty years, dozens of phase II and III clinical trials for moderate and severe traumatic brain injury (TBI) have failed. This is in spite of the fact that over 130 drugs have shown some efficacy in animal models of injury (Margulies et al., 2008). One major reason cited for these disappointing outcomes is that the complex and varied mechanisms associated with different types of TBI are not being addressed by single drugs targeted towards only one or a few receptor sites. While pre-clinical experiments use mostly tightly controlled studies with well-circumscribed injuries and clearly defined outcomes, the pathophysiology of TBI in humans is often much more heterogeneous and systemic, affecting many different tissue systems and not just the brain itself. Treating patients suffering from a constellation of these injury-induced events may require a pleiotropic agent or a combination of drugs that can act simultaneously or even sequentially on the injury cascade without producing serious adverse events and complications.

Pre-clinical and clinical data accumulating over the last several years indicate that progesterone (PROG) may be highly effective in the treatment of TBI (Gibson et al., 2008; Singh et al., 2008; Stein, 2008b; Stein et al., 2008; Wright et al., 2007; Xiao et al., 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., 2004a; Wright et al., 2001). Although specific modes of action have yet to be completely defined, PROG has been shown to lead to improvements via a variety of molecular mechanisms (Pettus et al., 2005; Schumacher et al., 2007; VanLandingham et al., 2007), making it likely that interacting pleiotropic actions are responsible for its observed

benefits. PROG is therefore a hormone with multiple mechanisms of action and can even be considered a "combination therapy" in itself (Margulies et al., 2008). Given its demonstrated effectiveness and safety in human patients, it is reasonable to consider PROG as a basis for combinations with other potential therapies.

For such an inquiry, it is logical to ask first what contextual conditions might limit the hormone's beneficial effects in a clinical setting. In other words, what co-morbid conditions might affect TBI patients that could reduce the ability of PROG, or any other drug, to promote recovery? Recent research suggests, for example, that vitamin D deficiency (D-deficiency) can exacerbate injury and potentially reduce the beneficial effects of other treatments for TBI (Chatterjee, 2001; McCann and Ames, 2008). This is especially the case in older subjects and is no small problem, because it has been reported that well over half of older adults suffer from D-deficiency (Norman et al., 2007). There is also increasing evidence that about 30-35% of the general American public also suffers from D-deficiency, so patients of any age, including children, presenting with a TBI might be placed more at risk and have a less favorable outcome if they are D-deficient. In this context something as simple as providing vitamin D supplementation could improve recovery and potentially enhance the neuroprotective benefits of PROG (or any other) treatment. This could be important from a clinical perspective, given that most elderly patients who come to the hospital, with or without TBI, will be D-deficient.

Furthermore, it is becoming apparent in the wake of the failure of most TBI treatment clinical trials that multi-targeted pharmacotherapies hold more promise than drugs targeting specific pathobiological pathways (Narayan et al., 2002) and that treatment may be optimized by combinations of agents acting on different mechanisms or

the same mechanisms differently (Faden, 2001; Gingrich and Traynelis, 2000). The concept of multi-therapy has already become a standard approach for HIV/AIDS treatment, and patients are known to respond much more effectively to combinations of drugs, each targeted to different parts of the disease cycle, acting at different sites, and synergistically enhancing potencies and durations of action. The same approach has been suggested for TBI, especially due to its complex manifestation in human patients (Margulies et al., 2008), where the functions of multiple organ systems may be affected by a direct injury to the brain.

Based on the literature, we suggest that 1,25-hydroxyvitamin D_3 (or vitamin D hormone, VDH) is potentially a good candidate for a combination agent to be used in conjunction with PROG, since both hormones have high safety profiles, act on many different injury and pathological mechanisms, are readily available, easy to administer, and relatively inexpensive. In this article, we review the evidence for PROG neuroprotection after TBI and the emerging evidence for VDH as a neuroprotective agent, and discuss whether combining the two would be a good step to take in the development of a novel therapy for TBI.

6.3. VITAMIN D AND NEUROPROTECTION

6.3.1. Vitamin D Deficiency and its Consequences

According to the Third National Health and Nutrition Examination Survey, 61% of Caucasian- and 91% of African-Americans are D-deficient (Khazai et al., 2008). Similar figures have been cited for all segments of the population and in many countries (Fabian and Elmadfa, 2008; Holick and Chen, 2008; MacFarlane et al., 2004). Although D-deficiency is common in healthy young populations in industrialized nations (DeLuca, 2004; Garcion et al., 2002; Holick, 2003b; Ylikomi et al., 2002), it is especially frequent in the elderly, especially in resident (nursing) homes and patients with hip fracture (Lips, 2006), with reported prevalence 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). It is a significant problem with a number of potential consequences, many of which are still unknown (Holick, 1994).

Aside from its classical effects on bone density, D-deficiency has been associated with 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 events such as stroke and congestive heart failure (Michos and Melamed, 2008; Vieth and Kimball, 2006), all of which can significantly affect a patient's ability to recover from severe trauma. Several recent studies also suggest that inadequate vitamin D may predispose towards Parkinson's and other neurodegenerative diseases (Evatt et al., 2008), mood

disorders (Garcion et al., 2002; Kalueff et al., 2004b), and even tuberculosis infection (Zasloff, 2006).

The relationship of vitamin D to autoimmune disorders is especially relevant to diseases of the CNS, and deficiency has been associated with increased incidence of multiple sclerosis (MS) (Cantorna, 2008; Cantorna and Mahon, 2004; Grant, 2006), Sjögren's syndrome (Johnson et al., 2000), rheumatoid arthritis (Adorini and Penna, 2008), and Crohn's disease (Jahnsen et al., 2002; Pappa et al., 2006a; Pappa et al., 2006b). Systemic vitamin D levels have been suggested as a possible explanation for the latitudinal gradient in MS incidence (nearly zero at the equator and increasing with greater distance from it) (Niino et al., 2008), and correlations have been observed between circulating vitamin D status and the risk of developing MS (Cantorna, 2008; VanAmerongen et al., 2004), as well as a protective effect of vitamin D intake in both human disease (Munger et al., 2004; Smolders et al., 2008b) and animal models (Cantorna et al., 1996; Garcion et al., 2003a; Lemire and Archer, 1991). Vitamin D therapy for MS has been shown to be safe in humans (Kimball et al., 2007) and has recently been recommended for use in double blind controlled clinical trials (Niino et al., 2008; Smolders et al., 2008a).

A low level of vitamin D is also 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 psycho-social homeostasis and greater vulnerability to internal and environmental stressors such as trauma (Markle-Reid and Browne, 2003; Topinkova, 2008). This could be especially important in the elderly, who are already more vulnerable to TBI, and

studies have shown that advanced age is a major predictor of injury severity after TBI (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 independently 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). Vitamin D status has been specifically associated with functional outcomes in the elderly (Boxer et al., 2008; Dawson-Hughes, 2008), suggesting that supplementation could be especially helpful for this segment of the population (Dawson-Hughes, 2008).

6.3.2. Vitamin D Synthesis, Activation, and Metabolism

The first step in the synthesis of VDH is the activation in the epidermis of 7-dehydrocholesterol by sunlight, specifically ultraviolet B (UVB) radiation in the 290-320nm range (Gupta et al., 2007). Because DNA absorbs UVB in exactly the same spectrum, vitamin D is hypothesized to have evolved as a "sunscreen" for DNA (Holick, 2003a). This is supported by the fact that it is present in animals ranging from phytoplankton to humans (Holick, 2003a). The UVB radiation opens the B ring of the steroid precursor, resulting in the conjugated triene system that characterizes all D vitamins and defines them as secosteroids (Dusso et al., 2005; Norman and Henry, 2007). Although seven forms of vitamin D exist, D_3 is the naturally occurring form in animals and is present in the skin of all higher vertebrates (Norman and Henry, 2007).

After its activation by sunlight, vitamin D is activated to VDH in two enzymatic steps. The first of these occurs in the liver by the cytochrome P450 enzyme, vitamin D 25-hydroxylase (CYP2R1) (Holick, 2003b). This step is not tightly regulated and therefore the product, $25OHD_3$, is a good indicator of overall vitamin D status (Norman and Henry, 2007). The second step requires the 25-hydroxyvitamin D_3 1α-hydroxylase enzyme (CYP27 or 1α-OHase) and is a tightly controlled reaction regulated primarily by VDH itself, but also by levels of parathyroid hormone (PTH), calcium, and phosphate (Dusso et al., 2005). 1α-OHase is most abundant in the kidneys, although recent evidence has shown that it is present throughout the body, including the immune system (Hayes et al., 2003; Thien et al., 2005; van Etten and Mathieu, 2005) and the rodent and human brain (Baas et al., 2000; Eyles et al., 2003; Eyles et al., 2005; Glaser et al., 1999; Hewison et al., 2000; Langub et al., 2001; McGrath et al., 2004; Stumpf et al., 1988; Stumpf and O'Brien, 1987; Townsend et al., 2005; Walbert et al., 2001). The ubiquitous distribution of 1α-OHase suggests that VDH has both local and systemic effects (due to its synthesis by the kidneys and release into the bloodstream), and recent research suggests that a significant percentage of all VDH activity is autocrine or paracrine (Lips, 2006). VDH is inactivated by 25-dihydroxyvitamin D_3 24-hydroxylase (CYP24), which is present in almost all VDH target cells, is induced by VDH, and is regulated in a manner reciprocal to 1α-hydroxylase, allowing for very tight local and global control of VDH levels (Dusso et al., 2005). The fact that the CNS can locally catalyze both its activation and inactivation makes VDH by definition a neurosteroid (Garcion et al., 2002).

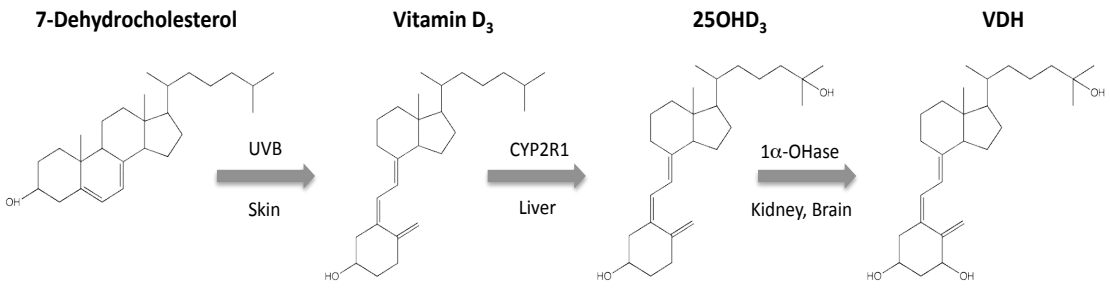

Figure 6.1. Vitamin D metabolism

Vitamin D and its metabolites are largely bound in the blood by vitamin D binding protein (DBP), also known as group-specific component of serum or Gc-globulin. DBP serves as the main reservoir and transporter of the vitamin D endocrine system, and binds about 88% of the total $25OHD_3$ and 85% of the total VDH in serum (Norman and Henry, 2007). This is an important fact in the pharmacokinetics of VDH, since only the free concentration of the hormone is considered to have biological activity (White and Cooke, 2000). Only about 5% of DBP is bound to vitamin D metabolites, and its serum concentration is about 20-fold that of the various vitamin D species (White and Cooke, 2000). DBP is an acute phase protein produced by the liver, and is upregulated by estrogen and during pregnancy when PROG is also very elevated (Norman and Henry, 2007).

In addition to its functions in regulating serum vitamin D metabolites, DBP, in concert with gelsolin, performs a crucial function in the response to massive injury by scavenging and clearing monomeric G-actin, which is released by damaged cells and aggregates in the bloodstream, potentially causing multi-organ system failure (Dahl et al., 1998; Dahl et al., 2001; Gomme and Bertolini, 2004; Meier et al., 2006; Raymond et al., 2005; Speeckaert et al., 2006; White and Cooke, 2000). Some studies show, in fact, that the level of DBP is the only biomarker positively associated with survival in human

patients after severe trauma (Dahl et al., 1998; Meier et al., 2006). DBP also acts in concert with complement factor C5a as a powerful activator of monocyte and neutrophil chemotaxis (Meier et al., 2006), an effect that is inhibited by the binding of VDH (Shah et al., 2006). T and B cells can convert DBP into Gc-macrophage activating factor (Gc-MAF), which stimulates neutrophil binding and macrophage activity at the tissue site (Gomme and Bertolini, 2004), and may be a reason that it can also be released in a paracrine manner by stressed endothelial cells (Raymond et al., 2005). DBP can stimulate both beneficial and damaging immune activity, offering another potential mechanism by which VDH may function to modulate immunological function.

6.3.3. Vitamin D Signaling Mechanisms

Most action of VDH is mediated by the VDR, a ligand-inducible transcription factor that regulates gene expression by binding to specific vitamin D response elements (VDREs) in DNA (Chatterjee, 2001; Christakos et al., 2003; DeLuca and Zierold, 1998; Hannah and Norman, 1994; Sutton and MacDonald, 2003; Wang et al., 2005). The specificity of the receptor for VDH is some 100 - 1000 times higher than for its precursor $25OHD_3$ (Norman and Henry, 2007). Like other nuclear steroid receptors, the ligand-receptor complex effects gene transcription after undergoing heterodimerization with the retinoid X receptor (RXR) and recruitment of nuclear receptor coactivation proteins (Brown et al., 1999; Christakos et al., 2003; DeLuca and Zierold, 1998; Matilainen et al., 2005; Narayanan et al., 2004b; Pascussi et al., 2003; Sutton and MacDonald, 2003; Thummel et al., 2001). The VDR belongs to the protein superfamily that includes receptors for PROG, estrogen, glucocorticoids, androgens, thyroid hormone, and peroxisome proliferator-activator receptor (PPAR) (Norman and Henry, 2007) and more specifically

to the NR1I subfamily of orphan nuclear receptors, which also includes the PXR and the constitutive androstane receptor (CAR) (Reschly and Krasowski, 2006). It is interesting to highlight here that the VDR is closely related (60% homology in the DNA-binding domain) to the PXR, a xenobiotic sensor through the activation of which PROG may exert some of its neuroprotective effects (Krasowski et al., 2005; Langmade et al., 2006; Pascussi et al., 2003). This suggests potential interactions and cross-talk between the two systems.

VDRs are widely distributed throughout the embryonic and adult brain, and appear most prominently in the neuroepithelium and proliferating zones in both rats (Langub et al., 2001; Stumpf et al., 1982; Sutton and MacDonald, 2003; Veenstra et al., 1998; Walbert et al., 2001) and humans (Eyles et al., 2005). Their presence has also been noted in neurons and glia of the human prefrontal and cingulate cortices, thalamus, hypothalamus, cerebellum, substantia nigra, caudate, putamen, amygdala, and hippocampus (Buell and Dawson-Hughes, 2008; Eyles et al., 2005), although notably not in the macrocellular cells within the NBM and the septum (Eyles et al., 2005). VDR distribution is mostly coextensive with the presence of 1α-OHase, except in the NBM, where 1α-OHase was present but VDR was not (Eyles et al., 2005). VDR and 1α-OHase expression in humans largely coincides with their distribution in the rodent brain (Buell and Dawson-Hughes, 2008; Eyles et al., 2005), and also strongly overlaps with the known distributions of receptors for androgens, glucocorticoids, estradiol, and PROG in rodents (Clancy et al., 1992; Fuxe et al., 1987; Kawata, 1995; Prufer et al., 1999). There is also significant overlap between VDH and 1α-OHase expression in the brain, and VDH synthetic and degradative pathways have been described in neurons and glia

(Clemens et al., 1988; Neveu et al., 1994a; Neveu et al., 1994b; Neveu et al., 1994c; Zehnder et al., 2001). This implies that VDRs in the brain are very likely activated by locally synthesized VDH and suggests a functional role for the hormone in the CNS (Buell and Dawson-Hughes, 2008).

Like most steroid hormones, VDH is also capable of rapid, non-genomic signaling (Dusso et al., 2005). These responses are likely mediated by receptors located on the cell surface, and although it has been suggested that these rapid events modulate genomic activity of VDH, the exact function of this signaling pathway has not yet been determined. Although previously thought to be a different receptor protein, the VDH receptor involved in non-genomic signaling now appears to be the VDR, but in this case it is located not in the nucleus or cytosol but rather in membrane caveolae (Norman and Henry, 2007). These caveolae, or lipid rafts, are invaginations in the plasma membrane and believed to be involved in the signal transduction of a number of signaling systems (Anderson, 1998). These rapid effects include activation of phosphoinositide metabolism (Bourdeau et al., 1990; Morelli et al., 1993), cyclic guanosine monophosphate (GMP) (Guillemant and Guillemant, 1980; Vesely and Juan, 1984), protein kinase C (PKC) (Sylvia et al., 1996), MAPKs (Beno et al., 1995; Song et al., 1998), opening of Cl^- channels (Zanello and Norman, 1996), and stimulation of cellular Ca^{2+} levels (Lieberherr, 1987; Lucas et al., 1989; Morelli et al., 1993; Sugimoto et al., 1988).

6.3.4. Vitamin D as a Neuroprotective Agent

6.3.4.1. In vivo *models*

VDH treatment has shown promising results in a variety of *in vivo* and *in vitro* CNS injury paradigms. In a model of stroke, Wang and colleagues showed that VDH pre-

treatment for 8 days can significantly increase levels of glial-derived neurotrophic factor (GDNF) and attenuate cortical infarction induced by middle cerebral artery (MCA) ligation in rats (Wang et al., 2000). In various modes of Parkinson's disease, a number of researchers have shown that 7 – 8 day pretreatment with VDH can restore levels of dopamine in the substantia nigra of 6-hydroxydopamine lesioned rats (Wang et al., 2001) and prevent lipid peroxidation and cytosolic cytochrome c in zinc chloride-infused rat substantia nigra (Lin et al., 2003). VDH pretreatment also prevented iron-induced oxidative injuries in the locus coeruleus (LC) of the rat (Chen et al., 2003a). Although these studies used a preventive paradigm by administering VDH for up to 8 days prior to injury, other studies have shown post-injury treatment benefits of VDH as well. Oermann and colleagues found that treatment with VDH after a photothrombotic lesion to the cerebral cortex of rats reduces the expression of glial fibrillary acid protein (GFAP), a key marker of reactive gliosis, in remote areas of secondary damage (Oermann et al., 2004). One recent report by Chabas and colleagues (Chabas et al., 2008a) examined axon regeneration after peripheral (peroneal) nerve injury in rats followed by chronic treatment with vitamin D2. The authors reported that the treatments enhanced the formation of new axons, increased axon diameter, and improved sensory responses to metabolic stimulation.

Further research has shown that concurrent administration of VDH with lipopolysaccharide (LPS) significantly inhibited inducible nitric oxide synthase (iNOS) expression in monocytes in the rat brain, suggesting that VDH can also help attenuate immune-induced oxidative damage in the CNS (Garcion et al., 1998). Lin et al. found a similar effect on zinc-induced toxicity in the CNS, where concurrent administration of

VDH reduced apoptosis and oxidative damage (Lin et al., 2005). VDH was also found to perform a direct anti-convulsant role in the brains of mice with chemically induced seizures (Kalueff et al., 2005).

The majority of *in vivo* studies with VDH, however, have focused on its effect on MS and its animal model, chronic relapsing experimental autoimmune encephalomyelitis (EAE). The VDH effect in this model has been known for a long time (Garcion et al., 2002; Lemire and Archer, 1991). VDH has been reported to be able to block the development of disease after onset in both rats (Nataf et al., 1996) and mice (Cantorna et al., 1996), an improvement correlated with inhibition of iNOS (Garcion et al., 1997; Garcion et al., 2003a), CD4 antigen expression (Nataf et al., 1996), and IL-12-dependent T_H1 cell development in the CNS (Mattner et al., 2000). VDH also increased levels of transforming growth factor β (TGFβ) and IL-4, which were increased in a mouse model and are anti-inflammatory T_H2 immune response cytokines (Cantorna et al., 1998). In another EAE system, VDH significantly reduced acute inflammation and levels of GFAP by inducing inflammatory cell apoptosis (Spach et al., 2004). Since a significant component of secondary damage after many types of brain injury including TBI is related to excessive and prolonged inflammation, these data suggest that VDH might be an effective adjunct to treatments for immune disorders of the CNS.

6.3.4.2. In vitro *models*

There is also significant *in vitro* evidence for VDH neuroprotection. Two studies using mesencephalic dopaminergic neuron culture have shown that VDH protects these neurons from glutamate and dopaminergic toxins by increasing neuronal functions that serve to reduce oxidative stress (Ibi et al., 2001; Shinpo et al., 2000). A similar anti-oxidant effect

was described by Garcion et al., who found that VDH treatment increased γ-glutamyl transpeptidase (γ-GT) expression and activity, enhanced glutathione pools, and reduced nitrite production in LPS-stimulated primary rat astrocyte culture (Garcion et al., 1999). VDH has also been observed to reduce the production of inflammatory cytokines TNFα, IL-6, and nitric oxide (NO) in stimulated microglia (Lefebvre d'Hellencourt et al., 2003). In addition to neurons, astrocytes, and microglia, VDH has an effect on oligodendrocytes (Baas et al., 2000) and Schwann cells (Cornet et al., 1998). VDH also appears to regulate the expression of N-myc, c-myc, PKC, and TGFβ in neuroblastoma cells (Veenstra et al., 1997), suggesting that it may affect neural cell growth in ways other than the well-established induction of NGF and its receptors (Brown et al., 2003; Cornet et al., 1998; Neveu et al., 1994b; Samina Riaz and Tomlinson, 2000; Saporito et al., 1994; Wion et al., 1991).

In addition to NGF, VDH can directly affect the expression of other factors involved in regeneration and recovery after CNS injury, including GDNF (Naveilhan et al., 1996), neurotrophin 4 (NT-4) (Neveu et al., 1994a), and insulin-like growth factor binding proteins (IGFBPs) (Matilainen et al., 2005). The results from these studies suggest that not only does VDH affect oxidative stress, neurotoxicity, oxidative stress, and growth factor expression, but it also works on all cell types involved in the development of and recovery from CNS injury including neurons, astrocytes, oligodendrocytes, and immune cells such as monocytes and microglia.

6.3.5. Vitamin D Mechanisms of Action

The primary non-calcemic effect of VDH appears to be inhibition of cell proliferation and stimulation of cell differentiation, especially in the immune system, where it acts as a

powerful modulator (Cantorna et al., 2004; Christakos et al., 2003; DeLuca and Zierold, 1998; Griffin et al., 2003; Hayes et al., 1997; Holick, 2003a, b; Mahon et al., 2003; White and Cooke, 2000). VDH has been shown to skew all aspects of immune function (T-cell differentiation, macrophage and dendritic cell maturation and antigen-presenting ability, cytokine profiles) towards a type 2 (T_H2) immune response, which is generally anti-inflammatory, both directly and through inhibition of T_H1 differentiation.

Like PROG, VDH has been shown to decrease levels of pro-inflammatory T_H1 cytokines such as TNFα, IL-1β, IL-12, IL-6, IFN-γ (Cohen-Lahav et al., 2007; Imazeki et al., 2006; Lyakh et al., 2005; Mahon et al., 2003; Thien et al., 2005; Zhu et al., 2005), and the downstream reactive oxygen species generated by activated macrophages (Jung and Sung, 2004). Long-term D-deficiency has been shown to lead to generalized inflammatory conditions that compromise the cardiovascular system and glucose metabolism (Holick, 2004, 2005b; Levin, 2006; Norman and Powell, 2005; Wang et al., 2002), the health of which is essential to survival post-TBI. Related to macrophage/microglial activity and T_H1 response is the production of reactive species that cause oxidative stress and contribute to secondary injury (Bramlett and Dietrich, 2004). By modulating the development of a hyperactive and prolonged inflammatory response through adjusting T_H1/T_H2 balance and inducing macrophage apoptosis, VDH may limit the secondary injury cascade after TBI. This could be especially important under conditions of D-deficiency, where the underlying physiological state is already skewed towards a type 1 response (Imazeki et al., 2006; Mahon et al., 2003; Matsuzaki et al., 2005; Mattner et al., 2000; Topilski et al., 2004).

Considerable evidence also exists for a direct modulatory effect of VDH on inflammation. VDH is known to down-regulate NFκB (D'Ambrosio et al., 1998), the central mediator of inflammation that has also been linked with stress-response in humans (Bierhaus et al., 2003) and stress-induced neuronal loss in rats (Madrigal et al., 2002). VDH has also been shown to decrease inflammatory cytokine production in a variety of cell types, including endothelial cells (Equils et al., 2006), keratinocytes (Gurlek et al., 2002), monocytes (Stio et al., 2005), and microglia (Lefebvre d'Hellencourt et al., 2003). Systemic VDH administration has also been noted to lead to lower serum concentrations of TNFα and increased levels of anti-inflammatory IL-10 in heart failure patients (Schleithoff et al., 2006), as well as lower TNFα and symptom manifestation in a rat model of inflammatory bowel disease (IBD) (Zhu et al., 2005). Finally, higher pro-inflammatory cytokine levels were found in VDR-KO (knock-out) mice (Froicu and Cantorna, 2007), and an inverse correlation was seen between systemic inflammatory markers and $25OHD_3$ levels (Timms et al., 2002).

Since increased cellular Ca^{2+} concentration is the final common step in the initiation of cell death after injury, maintenance of adequate intracellular levels of Ca^{2+} is important for cell health and survival, not just in neurons but also in astrocytes and oligodendrocytes. VDH helps to regulate these levels and the cellular response through several mechanisms: 1) maintenance of adequate systemic parathyroid hormone (PTH) levels, 2) regulation of L-type voltage-sensitive Ca^{2+} channel (L-VSCC) expression, and 3) control of intracellular Ca^{2+} buffering systems. Control of systemic Ca^{2+} metabolism, along with regulation of parathyroid activity and PTH levels, belongs to the classical set of vitamin D functions. In addition, and very importantly for amelioration of secondary

injury after trauma, VDH has been observed to be neuroprotective in primary rat hippocampal cultures through the inhibition of L-VSCCs, which are strongly implicated in the development of glutamate-induced excitotoxic injury (Brewer et al., 2001). Finally, VDH upregulates proteins of the intracellular Ca^{2+} buffering system such as calbindin-D28k and parvalbumin (Kutuzova and Deluca, 2004; Xu et al., 2006b), thereby improving the ability of cells to cope with increased intracellular Ca^{2+} levels without entering the irreversible path towards cell death. The general effect of these mechanisms is enhanced resistance to perturbations and improved cellular adaptation. A D-deficient state, however, can lead to increased susceptibility to Ca^{2+}-induced damage (Choi and Jeung, 2008; de Viragh et al., 1989; Peterlik and Cross, 2005).

VDH is also a powerful regulator of the cell cycle: it inhibits cell proliferation and stimulates cell differentiation, and it is most likely this ability to control the cell cycle that makes it effective as an anti-inflammatory and an anti-neoplastic agent (Banerjee and Chatterjee, 2003; Gurlek et al., 2002; Kalueff and Tuohimaa, 2007). On a molecular level, several different microarray analyses indicate that VDH has effects on cell cycle regulating genes such as p53, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, which are involved in apoptosis and control of the G_1/S phase transition (Jensen et al., 2001; Johnson et al., 2006), and growth arrest and DNA-damage-inducible, alpha (GADD45), which is involved in the G_2/M phase transition (Eelen et al., 2004a; Eelen et al., 2004b; Griffin et al., 2004; Wang et al., 2005; White, 2004). VDH may control other aspects of the cellular reproductive machinery such as various cyclins and cyclin-dependent kinases (Chatterjee, 2001; Jian et al., 2005). Since terminally differentiated neurons undergoing severe stress are known to re-enter the cell cycle, only to be forced to undergo apoptosis because they have lost their

ability to proliferate (Cernak et al., 2005; Di Giovanni et al., 2003; Freeman et al., 1994; Kuan et al., 2004; McPherson et al., 2003; Zhu et al., 1999), the ability of VDH to induce cell cycle arrest and DNA repair might also be neuroprotective after TBI. Several studies suggest that this may be the case, and inhibition of cell cycle reentry has been neuroprotective in both experimental TBI (Di Giovanni et al., 2005) and Alzheimer's disease (Neve and McPhie, 2006) models. Since a significant amount of the damage in TBI is caused by a secondary cascade of injury mechanisms (Stein et al., 1993; Stein and Hoffman, 2003b), maintaining G_0 phase neurons in that state and inducing p53-mediated DNA repair could be a way to reduce post-TBI cell death and improve long-term neuronal survival (Kuan et al., 2004).

Vitamin D is critical for the early development of the brain. Young animals exposed to early or prenatal D-deficiency demonstrate significant changes in the gross morphology and circuitry of the brain (McGrath et al., 2004), neurogenesis (Cui et al., 2007), locomotion (Eyles et al., 2006; Kesby et al., 2006; O'Loan et al., 2007), and learning and memory (Becker et al., 2005). Developmental D-deficiency has been shown to cause derangement of a number of proteins essential to glucose and calcium metabolism, oxidative stress, cytoskeletal maintenance, and synaptic plasticity (Almeras et al., 2007; Eyles et al., 2007).

VDH has been shown to have protective properties in the context of aging as well, an important fact for this series of studies given its overarching context. One of the key markers of brain aging is an increased presence of IL-1β and activated microglia in the hippocampus of aged animals; this has been associated with impaired synaptic plasticity and deficits in long-term potentiation (Griffin et al., 2006; Maher et al., 2005). VDH

treatment has been shown to reduce these effects, as well the age-increased changes in IFN-γ function and activation of the stress-related c-Jun N-terminal kinase (JNK) and caspase-3 (Moore et al., 2007; Moore et al., 2005). VDH has also been shown to reduce the age-related derangement of neuronal Ca^{2+} metabolism (Brewer et al., 2006), increased vulnerability of hippocampal neurons (Brewer et al., 2007; Brewer et al., 2001), and decline in hippocampal CA1 neuronal density (Landfield and Cadwallader-Neal, 1998). These effects have also been observed with regard to VDR, as gene polymorphisms in this receptor protein were observed to influence cognitive function and susceptibility to depressive symptoms in old age irrelevant of effects on calcium levels (Kuningas et al., 2007). A reduction in VDR mRNA levels was also observed in the hippocampal CA1 region of Alzheimer's as compared to Huntington's disease patients, and this correlated significantly with calbindin D-28k message levels, indicating potential interactions with cognitive deficits and neuronal loss (Sutherland et al., 1992), and suggesting that vitamin D neuroendocrine function is critical for brain functioning across the life-cycle.

6.4. WHY COMBINE VITAMIN D AND PROGESTERONE?

6.4.1. Potential interactions with other neurosteroids, especially PROG

There is growing evidence that vitamin D may interact with other neurosteroids such as PROG and estradiol in a variety of tissues. For example, 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 single compound operates may modulate the end effect. Especially intriguing is the finding that xenobiotic activation of the 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., 2006a), the chief metabolizing enzyme of VDH. Furthermore, we have also observed that TBI induces lower serum levels of $25OHD_3$ (unpublished observation), suggesting that injury itself may cause a vitamin D-insufficient state. Given that PROG is a promising treatment for TBI that has been shown to work in a number of model systems and in human patients (Stein and Hurn, in press; Stein et al., 2008), the possibility that it may interact with vitamin D could have important consequences for treatment outcomes, and opens the possibility of developing a combined TBI treatment that may not only overcome the effects of vitamin D deficiency in the human population but may also enhance the effects of PROG treatment in normal patients with TBI.

From our review of the literature it is growing more apparent that vitamin D and PROG affect many of the same as well as a number of divergent processes involved in the repair of secondary injury following TBI. The similarities may be explained by the fact that VDRs have been found in rodent (and human) microglia, astrocytes, oligodendrocytes and Schwann cells (McCann and Ames, 2008), which are known to play a role in inflammation and CNS repair and which are also directly affected by PROG treatment after CNS injuries. It is certainly possible that, if PROG and VDH each work through different pathways to reduce cellular injury and enhance the metabolic processes of repair, then a combination of these agents might lead to more rapid neuronal repair and functional recovery, perhaps even with less dosing and duration of treatment.

A reason to attack the same injury pathways with different compounds lies in the fact that the same repair mechanisms may be modulated through different signals. An example of this would be intracellular Ca^{2+} levels, which can be independently affected by the reduction of glutamate excitation and by intracellular buffering systems. Here, a combination of PROG's actions through the GABAergic system to inhibit extracellular activity and the action of VDH to increase intracellular Ca^{2+}-binding proteins would both have an effect on calcium metabolism, but via different sub-pathways (Sayeed et al., In press). Another example would be apoptosis, which may be reduced by a number of different mechanisms: effects on Ca^{2+} metabolism, induction of trophic factors, inhibition of inflammation and pro-apoptotic signaling, and/or a reduction in lipid peroxidation by reactive species. Two different compounds that affect the same mechanism in this case would be synergistic and presumably enhance the protective effect after a CNS injury.

Another rationale for using a drug combination that affects the same mechanisms is the well-known inverted U-shaped dose response (or hormesis) of steroid action (Calabrese and Baldwin, 2001; Conolly and Lutz, 2004), in which the optimal result is obtained with a medium-range dosage while increasing dosages decrease the effectiveness. Why hormesis occurs is not clear, although recent mathematical modeling studies suggest that the artifact may be due to the second-order steroid receptor kinetics that produce a parabolic dose response curve (Li et al., 2007a). If the kinetics of the receptor mechanism indeed impose a limit on steroid action, it may be beneficial to attempt to overcome individual system saturation and activate similar protective end mechanisms through different steroid pathways (Wang et al., 2004; Webb et al., 1992). As an example, PROG and VDH may increase the activity of γ-GT by different mechanisms (PR-PROG activity and VDR-VDH activity), resulting in an increased overall antioxidant capacity. Although this is still not fully confirmed, the suggestion that it may be possible to amplify a neuroprotective effect simply through drug combination is intriguing and worth further exploration.

The concept of attacking different pathways can be extended to fully divergent mechanisms if one assumes that fewer damaging processes are ultimately better for protection and recovery. A complication may arise here if certain processes, such as inflammation (Chan, 2008), are potentially beneficial in the short term but end up being detrimental in the longer term, in which case an optimal treatment would only be achieved through the use of multiple agents given at different time points in the injury cascade. Another complication may involve non-linear interactions between PROG and VDH such that what may be best doses for treatment with each individually may not

work optimally in combination. Some of our initial data (unpublished observations) indeed suggest that this may be the case. This means that dosing parameters may have to be specifically reconfigured for novel combination therapies. Regardless of such considerations, a recent NINDS Workshop on Combination Therapies specifically recommended that, "With its [PROG's] pleiotropic characteristics, it would be advantageous to consider combination therapies for TBI that combine PROG with other agents that 1) protect the intracerebral vasculature, 2) diminish the effects of glutamate release and calcium influx, 3) more directly protect the mitochondria, 4) protect against the toxic effects of heme breakdown products, 5) enhance free radical scavenging, 6) enhance cerebral blood flow, 7) modulate the kalkrein-kinin system, 8) protect the axonal and cytoskeleton infrastructure, and 9) protect against diffuse axonal injury" (Margulies et al., 2008). VDH meets several of these recommendations for a combinatorial agent:

1. *Diminish the effects of glutamate release and calcium influx:*

   VDH maintains intracellular Ca^{2+} through downregulating L-VSCCs and upregulating intracellular Ca^{2+} buffering capacity (de Viragh et al., 1989; Kutuzova and Deluca, 2004; Xu et al., 2006b).

2. *Protect against the toxic effects of heme breakdown products:*

   VDH has been reported to upregulate glial heme oxygenase-1 (HO-1) concomitantly with a reduction in GFAP following focal cortical ischemia (Oermann et al., 2004). HO-1 is one of the rapidly induced heat shock proteins which metabolizes and thus detoxifies free heme to the powerful endogenous antixodants biliverdin, CO and Fe2+ (Maines, 1997; McCoubrey et al., 1997).

These studies suggest that HO-1 induction by VDH protects cells from the oxidative toxicity of free heme.

3. *Enhance free radical scavenging:*

   VDH induces the expression of γ-GT and significantly increases intracellular glutathione in response to LPS-induced oxidative stress in astrocytes (Garcion et al., 1999) and protects neurons from chemical toxicity (Shinpo et al., 2000).

4. *Modulate the renin-angiotensin system:*

   VDH plays an important role in the regulation of renin biosynthesis and blood pressure homeostasis (Kong and Li, 2003). It also functions as an endocrine suppressor of renin biosynthesis and genetic disruption of the VDR results in overstimulation of the renin-angiotensin system (RAS), leading to high blood pressure and cardiac hypertrophy (Xiang et al., 2004).

5. *Protect the axonal and cytoskeleton infrastructure:*

   VDH potentiates axon regeneration in a rat model of peripheral nerve injury (Chabas et al., 2008b). Following nerve injury, treatment with vitamin D_2 (100 IU/kg/day) significantly increased axogenesis and axon diameter, improved the response of sensory neurons to metabolites such as KCl and lactic acid, and induced a fast-to-slow fiber type transmission of the *Tibialis anterior* muscle.

It therefore seems clear that VDH not only shares many CNS repair mechanisms with PROG, but also adds to the mechanisms of action that compensate for missing mechanisms in PROG's arsenal.

Finally, in the context of aging and D-deficiency, it makes sense to assume that any damage or exacerbation caused by D-deficiency can be at least partially overcome

with supplementation to correct the deficiency. To this end, and since we are primarily interested in developing and improving treatment modalities, we recommend that treatment be combined with VDH to correct the potential loss of efficacy of PROG treatment in the D-deficient aged population. If this is effective, it could have significant implications for the treatment of elderly people with TBI.

6.5. CONCLUSION

Insults to the CNS, including TBI, induce neuroinflammatory and oxidative stress reactions, which then induce the secondary cascade of brain damage. Both PROG and VDH are pleiotropic hormones acting on several common, as well as on independent, CNS pathway mechanisms to reduce CNS damage and enhance CNS repair after TBI. Many studies now show that treatment with PROG significantly improves functional outcome after TBI in rats and humans (Gibson et al., 2008; Singh et al., 2008; Stein, 2008a). PROG has been shown to reduce inflammatory responses (He et al., 2004b; Pan et al., 2007) and oxidative stress. In addition PROG can activate protective pathways and increase the expression of genes and proteins associated with neuroprotection after brain damage. VDH has also 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 (Lin et al., 2003), EAE (Garcion et al., 2003b), LPS-induced oxidative stress (Garcion et al., 1999) and animal models of Parkinson's disease (Shinpo et al., 2000; Wang et al., 2001). VDH has an immunomodulatory effect and regulates the differentiation, growth and function of a broad range of immune system cells (Adorini, 2002). A growing literature demonstrates that VDH restriction impairs a number of physiologic processes associated with healthy CNS functions such as mitosis, mitogenesis, neurite outgrowth, possibly adult neurogenesis in hippocampal cells, and mitochondrial dysfunction (Almeras et al., 2007). Treatment with VDH induces the expression of NGF, GDNF, pro-apoptotic proteins (Kiraly et al., 2006) and upregulation of OH-1 and reduction in GFAP immunoreactivity in injured brain (Oermann et al., 2004). Given the wide spectrum of action by the two hormones it is likely that a combination of the two, operating through

unique and slightly different but compatible molecular mechanisms, might be synergistic in reducing the cytotoxic events associated with the injury cascade and increasing the neuroprotective events related to anti-apoptotic signaling and brain repair.

CHAPTER 7

PROGESTERONE AND 1,25-DIHYDROXYVITAMIN D_3 IMPROVE OUTCOME IN YOUNG AND VITAMIN D DEFICIENT OLD RATS WITH BRAIN INJURY

7.1. ABSTRACT

Progesterone (PROG) has been shown to be an effective treatment in various experimental models of brain injury and in two clinical trials. We have previously shown that PROG is also effective in aged rats, and that vitamin D deficiency in these rats significantly reduces the effectiveness of PROG in alleviating acute phase inflammation. In this chapter we provide a "proof of concept" demonstration of the effectiveness of 1,25-dihydroxyvitamin D_3 (VDH) as a neuroprotective agent in young, vitamin D sufficient rats with traumatic brain injury (TBI) and its potential interaction with PROG in decreasing the inflammatory response. We then extend this result to a more detailed examination of the effects of co-administration of VDH with PROG in vitamin D deficient aged rats in acutely counteracting deficiency and improving outcome post-TBI. Our results in vitamin D sufficient young rats show that VDH acts similarly to PROG and reduces the expression of inflammatory proteins, DNA damage, and cell death (TNFα, IL-1β, NFκB p65, p53, activated caspase-3) when given alone and also acts in synergy with PROG to further reduce TNFα and p53. In aged vitamin D deficient rats, VDH co-administraton with PROG improved outcome and reduced inflammation, cell death, and DNA damage more than either treatment given alone. It also restored the PROG benefits that were attenuated by vitamin D deficiency. Together these results suggest that VDH is an effective supplement to PROG in both young, vitamin D

sufficient and old, vitamin D deficient rats. Further work should be done to developt a potential combination treatment and to correct vitamin D deficiency in treatment for TBI.

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7.2. INTRODUCTION

There is extensive evidence that progesterone (PROG) significantly attenuates damage and improves functional outcome after traumatic brain injury (TBI) (Djebaili et al., 2005; He et al., 2004b; Roof et al., 1994; Shear et al., 2002). PROG administration to both males and females acutely post-TBI: 1) reduces cerebral edema (Galani et al., 2001; Roof et al., 1996); 2) decreases molecular expression of inflammatory cytokines (Grossman et al., 2004; He et al., 2004a; Pettus et al., 2005); 3) decreases the inflammatory NFκB p65 fragment as well as the inflammatory metabolites of complement factor C3 (Pettus et al., 2005); 4) decreases the pro-apoptotic proteins caspase-3 and Bax and apoptotic DNA fragmentation (Djebaili et al., 2005); 5) decreases the expression of the pro-apoptotic proteins Bad and Bax, and increases the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x (Djebaili et al., 2005; Yao et al., 2005); 6) decreases lipid peroxidation (Roof et al., 1997); 7) decreases reactive astrocyte infiltration (Grossman et al., 2004); 8) reduces retrograde neuronal degeneration (He et al., 2004b); and 9) maintains integrity of the blood brain barrier (BBB) (Duvdevani et al., 1995).

PROG has also been demonstrated to be protective in other models of neural injury such as middle cerebral artery (MCA) occlusion in rats (Ishrat et al., 2009; Jiang et al., 1996; Sayeed et al., 2007), cortical aspiration (Asbury et al., 1998), penetrating injury (Garcia-Estrada et al., 1993; Garcia-Estrada et al., 1999), in vitro injury (Goodman et al., 1996), spinal cord injury (Thomas et al., 1999), peripheral nerve crush injury (Baulieu, 2000), myelin and oligodendrocyte damage (Baulieu and Schumacher, 2000; Melcangi et al., 2003), and epilepsy (Frye and Scalise, 2000; Herzog and Frye, 2003). This research

has culminated in two clinical trials showing PROG to be the first treatment to show promise in a clinical setting with human patients (Wright et al., 2007; Xiao et al., 2008).

In Chapter 4 we showed that PROG is also beneficial in older subjects in reducing inflammation, cell death, BBB dysfunction, and short-term behavioral deficits in the acute phase after TBI. These benefits were significantly reduced or entirely eliminated by underlying vitamin D deficiency (D-deficiency), as demonstrated in Chapter 5. This naturally begs several questions: 1) since D-deficiency interacts with PROG treatment, is vitamin D in its active form (1,25-dihydroxyvitamin D_3 or vitamin D hormone, VDH) also neuroprotective in young, vitamin D adequate animals? 2) If this is the case, does VDH act in synergy with PROG in reducing acute inflammation in young, vitamin D adequate (D-normal) animals? And, 3) does acute administration of active VDH with PROG improve outcome in older D-deficient rats? The first two questions are important in terms of developing alternative or combination treatments that optimize neuroprotection and recovery after TBI; the conceptual basis for VDH neuroprotection and combination with PROG was discussed in Chapter 6. The last question is even more pertinent from a clinical perspective. Given that most elderly patients who come to the hospital, with or without TBI, will be D-deficient, it is very important for optimizing standard of care to know if is it possible and desirable to correct the effects of deficiency with VDH and potentially improve treatment outcome.

The experiments described in this chapter attempt to answer these questions. The first set of experiments in young D-normal animals is essentially a “proof of concept” that VDH can have a beneficial effect on acute inflammation after TBI, and we demonstrate that a combination of VDH and PROG is more effective than either

compound alone. This preliminary study examines the molecular effects on inflammatory cytokines, cell death, and DNA damage 72 hours post-injury. The main experiments then extend the results discussed in Chapter 5 using VDH as supplemental to PROG in aged D-deficient aged animals at both 24 and 72 hours after injury and correlate the results with behavioral outcomes. We show that a combination of PROG and VDH is the optimal treatment in aged, vitamin D deficient animals after TBI, a result that could be clinically useful in the management of the elderly human population with brain injury.

7.3. MATERIALS AND METHODS

7.3.1. Preliminary Experiments in Young D-Normal Animals

Prior to testing VDH effects in D-deficient aged animals, we performed a proof of concept experiment to determine 1) if VDH is neuroprotective after TBI, and 2) if VDH interacts synergistically with PROG on markers of inflammation after TBI. As a preliminary study, this experiment used a smaller N (n = 4/group), and we performed the protein analysis only at 72 hours after injury (versus 24 and 72 for the aged animals). We did not perform behavioral testing. Otherwise, the experimental protocols were similar. Thirty-five 8-12 week-old Sprague-Dawley rats weighing 250-300g at the time of injury were used. The animals were fed the standard diet from our animal care facility (Diet 5001, LabDiet®, Richmond, IN), and were handled for two weeks prior to any experimental or surgical procedures (Djebaili et al., 2005).

Surgical procedures were identical to those performed in the aged animals (detailed below), except that the depth of impact was 4.0mm ventral to bregma instead of 3.5mm for the aged. These animals were assigned to one of five experimental groups (n = 4/group): no injury (SHAM), vehicle (VH, 22.5% 2-hydroxypropyl-β-cyclodextrin), progesterone alone (PROG, 16mg/kg progesterone), VDH combined with progesterone (D+PROG, 16mg/kg PROG and 1μg/kg 1,25-dihydroxyvitamin D_3), and VDH alone (D, 1μg/kg 1,25-dihydroxyvitamin D_3). The VDH dose of 1μg/kg was used in the young animals instead of 5μg/kg for two reasons: 1) 1μg/kg is the standard dose used in the literature on VDH neuroprotection *in vivo* (Chen et al., 2003a; Wang et al., 2001; Wang et al., 2000); and 2) we were not contending with D-deficiency in this population and did

not wish to use a "megadose." The dosing schedule was identical to that performed in the aged animals, as were all protein analysis procedures (detailed below).

7.3.2. Aged Animal Subjects

Sixty 22-month-old male Fischer 344 rats weighing 450-550g were used to examine the effect of VDH treatment in combination with PROG in aged, D-deficient animals. All animals were housed and handled as previously described (Djebaili et al., 2005). This study was conducted in a facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). All experimental animal procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), Protocol #146-2005.

7.3.3. Diet

The aged animals in this study were fed a vitamin D-null version of the standard diet used in our animal care facility (Diet 5A4Y, modified 5001 with no D_3, TestDiet®, Richmond, IN); all rats were weighed daily to ensure consistent energy intake. Animals in the D-deficient group were maintained on the diet for at least 21 days prior to surgery, for reasons discussed in Chapter 5. All precautions in terms of light exposure were taken, also as detailed in Chapter 5.

7.3.4. Surgery and Contusion Injury

Rats were anesthetized using isoflurane gas (5.0% induction, 1.0–1.5% maintenance, 700mmHg N_2O, 500mmHg O_2) and surgery was performed using aseptic techniques as previously described (Chapter 4). Briefly, a 6mm diameter mid-sagittal bilateral craniotomy was performed 3mm anterior to bregma and a cortical contusion injury (CCI) was produced in the medial frontal cortex (MFC) by a pneumatic cortical contusion

device (5mm diameter) with impact velocity of 2.25m/s, impact time of 500ms, and depth of 3.5mm 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 3mL of lactated Ringer's solution subcutaneously within 6 hours of injury.

7.3.5. Treatment

All aged D-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-hour and 72-hour survival groups. The treatments were: VH: 22.5% 2-hydroxypropyl-β-cyclodextrin; PROG: 16mg/kg PROG (P0130, Sigma-Aldrich, St. Louis, MO); D+PROG: 16mg/kg PROG combined with 5μg/kg VDH (D1530, Sigma-Aldrich) for the first injection and 16mg/kg PROG with equivalent volume VH for the rest; D: 5μg/kg VDH for the first injection and vehicle for the rest. We used our previously discussed treatment protocol (Chapter 4) consisting of an intraperitoneal injection 1 hour post-injury followed by subcutaneous injections at 6 hours, 24 hours, and every 24 hours 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 16mg/kg PROG because previous research demonstrated it to be the most effective dosage in young (Goss et al., 2003) and aged (Chapter 4) rats. Animals receiving VDH treatment were given only a single 5μg/kg VDH injection 1 hour post-injury based on the evidence that a single megadose of VDH can reverse deficiency (Diamond et al., 2005).

7.3.6. Activity Testing

Spontaneous locomotor activity was performed as previously described (Chapter 4). The spontaneous locomotor activity task has previously been shown to be sensitive to our model of TBI and to the effects of PROG treatment (Djebaili et al., 2005), as well as to potential behavioral and motor derangements due to D-deficiency in open-field testing (Kalueff et al., 2004a).

7.3.7. Tissue Preparation and Western Blot Analysis

Animals were killed 24 or 72 hours after surgery with a lethal dose of Nembutal (1mL) and decapitated. Their brains were prepared for protein analysis and Western blots were performed as previously described (Chapter 4), using 15μL of each sample (30μg protein) per well in 18-well 4-20% Tris-HCL acrylamide Criterion Gels (BioRad, Hercules, CA). 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).

7.3.8. Serum Vitamin D and PROG Levels

Blood (0.5 – 1.0mL) was drawn directly from the right ventricle of the heart with a 21G needle after the rats were unconscious from the Nembutal but before death or decapitation. The whole blood was allowed to coagulate for 30 minutes at RT, after which the clot was removed and the serum centrifuged for 5 minutes at 1000 x g. The serum was removed and stored at -80°C. Vitamin D levels were determined with a 25-hydroxyvitamin D_3 ($25OHD_3$) 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 also measured with a solid-phase RIA kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assays were performed by the Biomarkers Core Laboratory at the Yerkes National Primate Research Center at Emory University in Atlanta, GA.

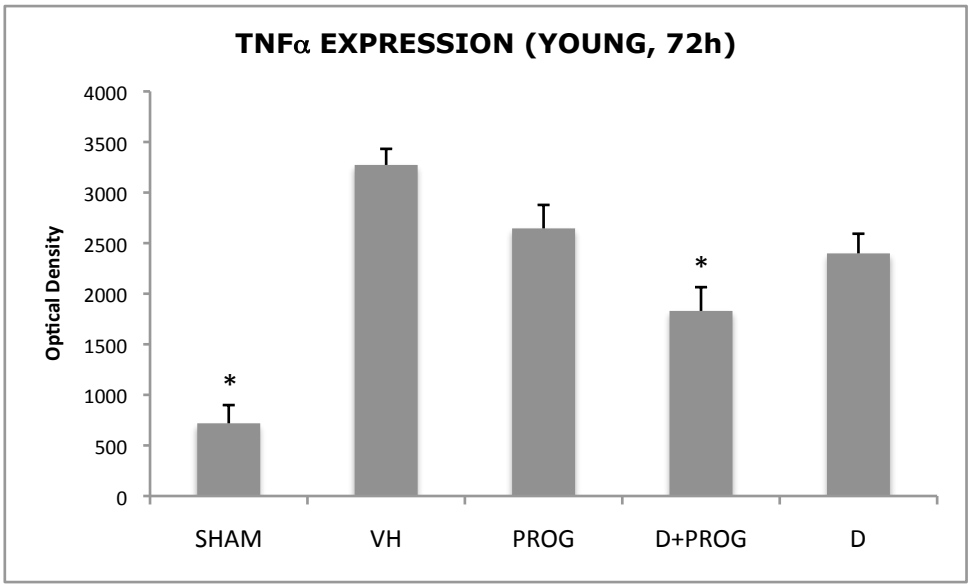
7.3.9. Statistical Analysis

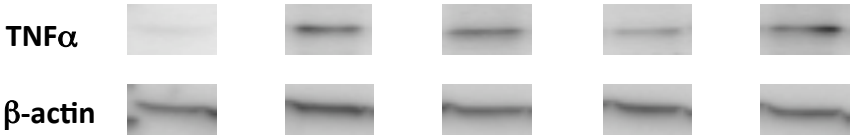
All results were expressed as the mean +/- the standard error of the mean (SEM). Statistical significance was set *a priori* at $p < 0.05$ and data were analyzed using t-tests, Pearson correlations, one-way analysis of variance (ANOVA) with Tukey-HSD *post hoc* tests, and general linear models (GLMs). GLMs were used to examine interaction effects in the behavioral data between vitamin D status and experimental groups, using deficiency, injury, and treatment as fixed factors and molecular measures as covariates; since deficiency is an underlying condition and is therefore logically prior to the other independent variables, a Type I SS model for fixed factors was used. The same fixed factor model was applied to molecular measures, but without any covariates. All analyses were calculated using SPSS™ 17.0 statistical analysis software.

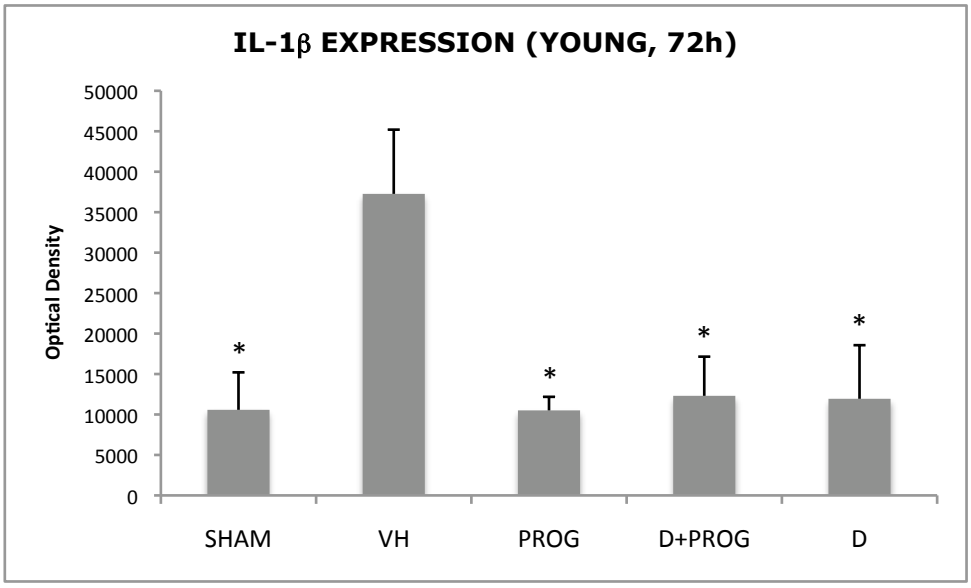
7.4. RESULTS

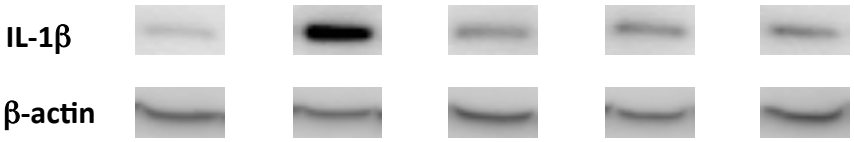
7.4.1. Vitamin D in Combination with PROG Reduces Inflammatory Cytokines, Cell Death, and DNA Damage in Young, Vitamin D Adequate Rats with TBI

Figures 7.1 through 7.5 show the results of our proof-of-concept study with young injured animals treated with vehicle, PROG, VDH, or a combination of PROG and VDH. All young animal data are at 72 hours after TBI only. The cytokines examined and the ANOVA results: TNFα (Figure 7.1: $F_{4,15} = 19.988$, $p < 0.001$), IL-1β (Figure 7.2: $F_{4,15} = 4.369$, $p = 0.027$), NFκB p65 (Figure 7.3: $F_{4,15} = 2.335$, $p = 0.154$; the significance shown in the figure is a t-test with $p < 0.05$ vs. VH). At the doses studied, VDH had effects similar to PROG in reducing inflammatory cytokine levels after TBI, *although combined treatment was superior to either compound alone in reducing TNFα and NFkB p65 to SHAM levels*. Similar effects were observed on attenuation of cell death as measured by activated caspase-3 levels (Figure 7.4: $F_{4,15} = 6.184$, $p = 0.009$), with combination treatment again showing synergy in reducing DNA damage, as measured by p53 levels (Figure 7.5: $F_{4,15} = 15.882$, $p = 0.001$). Generally, PROG and VDH alone showed similar effects, although in some cases the combination demonstrated synergy, suggesting potential interactions between the two hormones and the possibility of improved outcome with combined treatment.

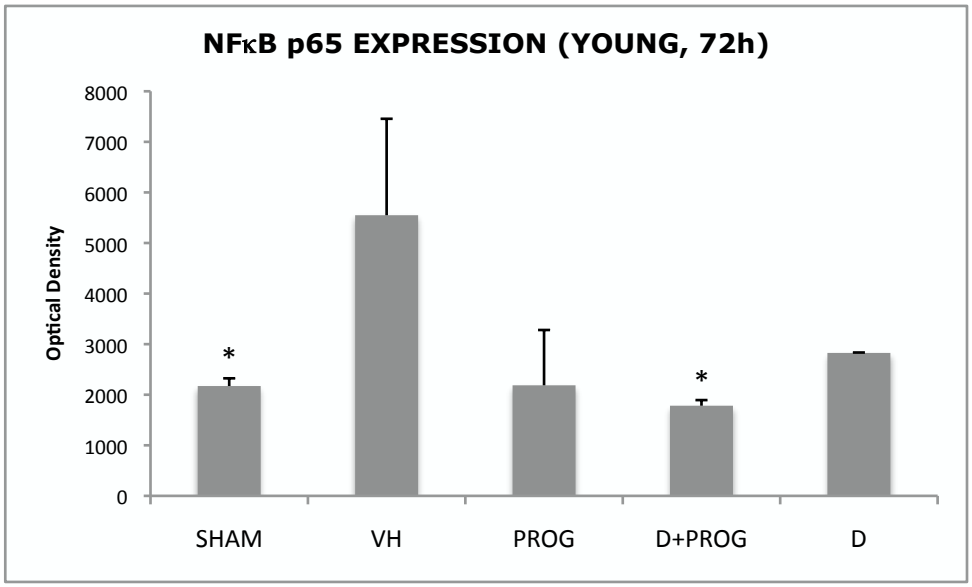


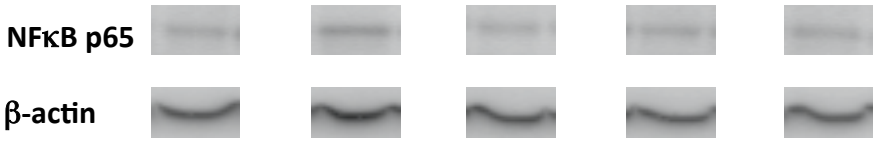


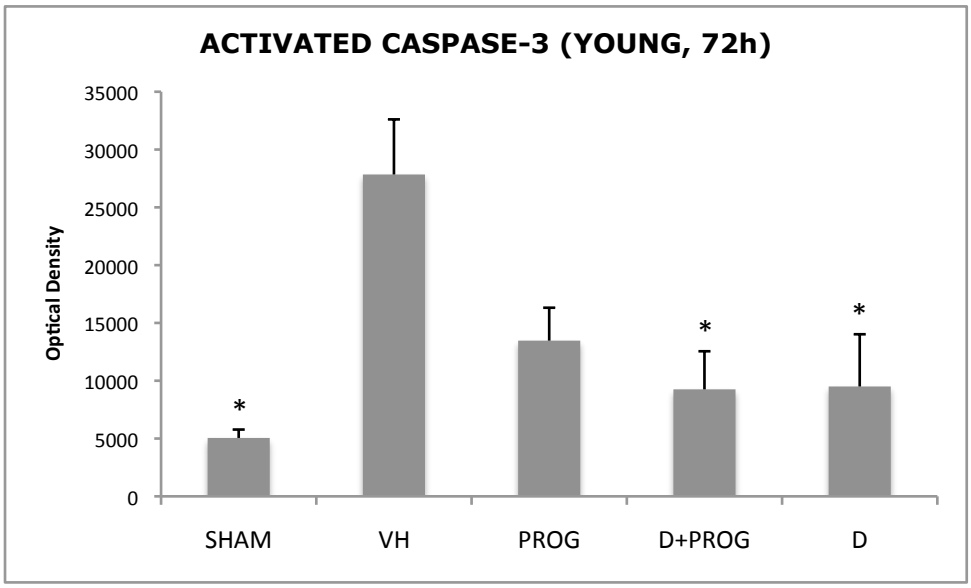


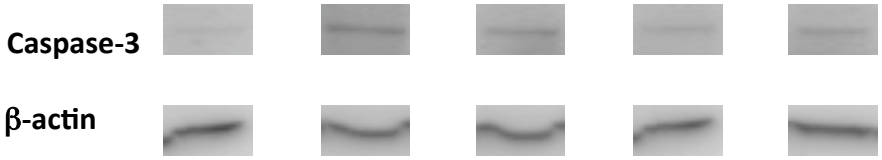


Figures 7.1 and 7.2. TNFα and IL-1β levels in normal young animals 72 hours after injury. * $p < 0.05$ vs. VH.









Figures 7.3 and 7.4. NFκB p65 and activated caspase-3 levels in normal young animals 72 hours after injury. * $p < 0.05$ vs. VH.

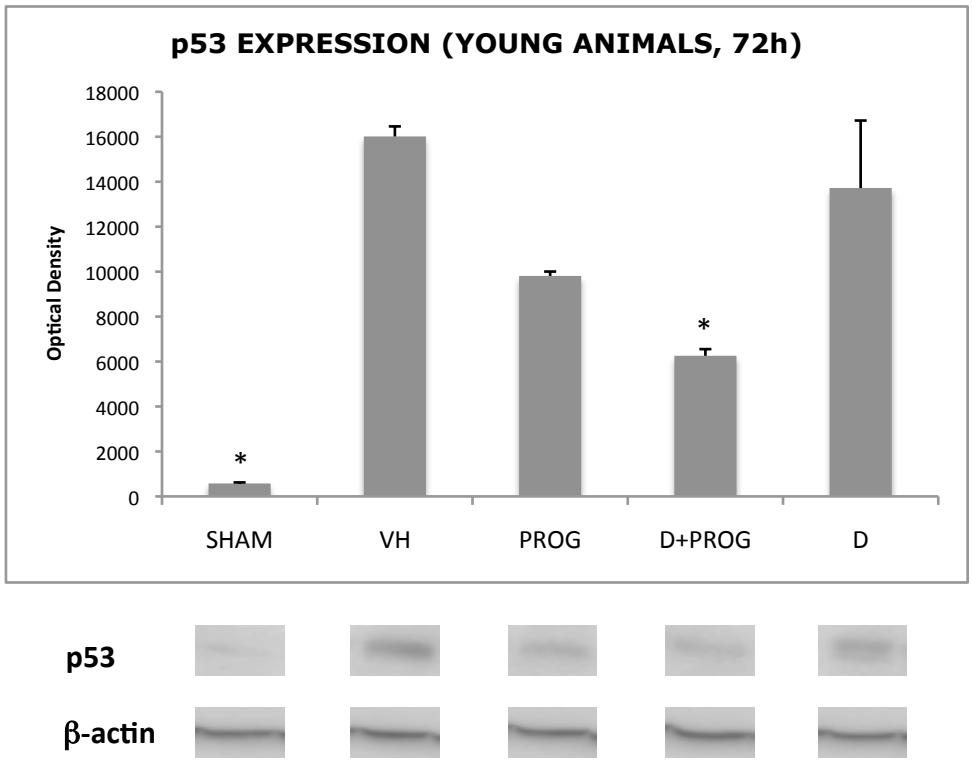
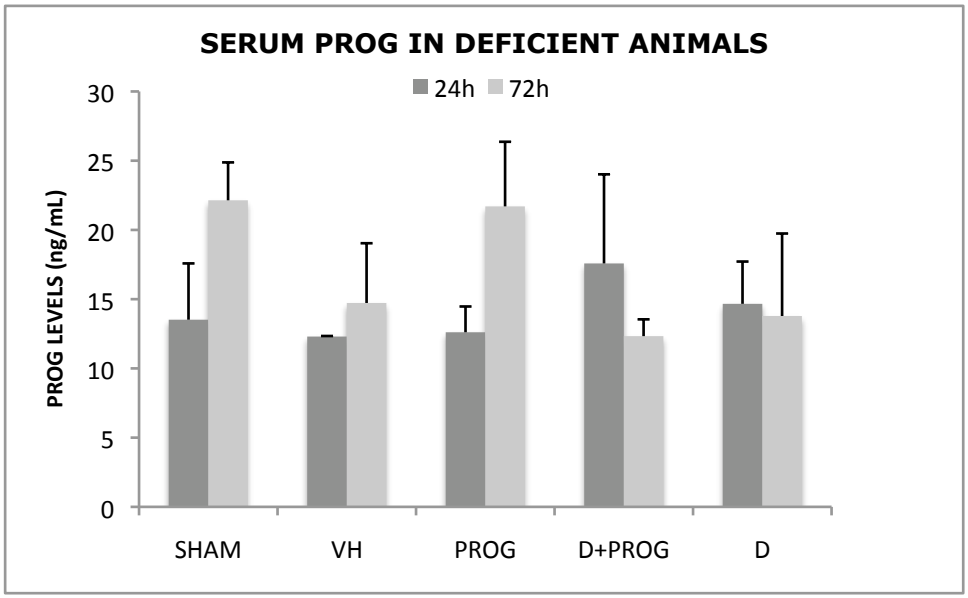
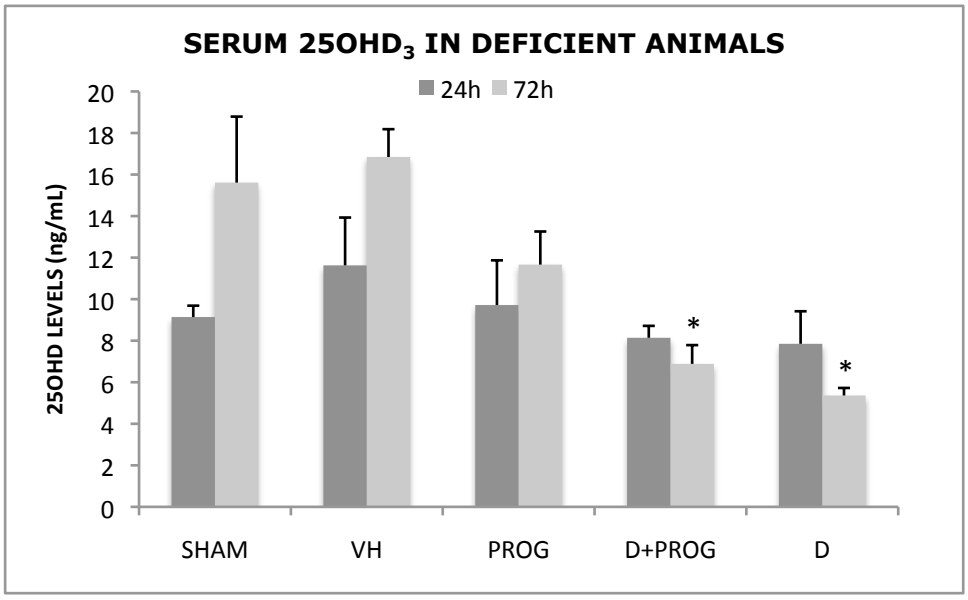


Figure 7.5. p53 levels in normal young animals 72 hours after injury. * $p < 0.05$ vs. VH.

7.4.2. Serum Values in Deficient Animals Show No Change in PROG Levels but a Paradoxical Effect on 25OHD$_3$ Levels 72 Hours After Injury

Our serum level analysis shows no effect of the different treatments on PROG levels in aged D-deficient animals at 24 and 72 hours after injury (Figure 7.6: 24h: $F_{4,20} = 0.238$, $p = 0.910$; 72h: $F_{4,20} = 1.369$, $p = 0.288$). There was also no effect on 25OHD$_3$ levels 24 hours after injury (Figure 7.7: 24h: $F_{4,20} = 0.870$, $p = 0.515$). *At 72 hours after injury, however, 25OHD$_3$ levels were significantly lower* (Figure 7.7: 72h: $F_{4,20} = 7.182$, $p = 0.001$) *in animals treated with VDH alone* ($p = 0.008$) *and with the PROG/VDH combination* ($p = 0.023$) compared to those treated with vehicle. Comparisons of the levels at 24 and 72 hours within each treatment group (t-test) showed no difference however.





Figures 7.6 and 7.7. PROG and $25OHD_3$ levels in aged deficient animals. * $p <$ 0.05 vs. VH.

7.4.3. Vitamin D Deficiency Attenuates the Beneficial Effects of PROG After TBI, but Co-treatment with VDH Improves Outcome in Deficient Animals

As discussed in Chapter 5, PROG appeared to lose its therapeutic effectiveness under conditions of D-deficiency. The data from the young animals suggested that VDH was neuroprotective and that it could interact with PROG to improve outcome more than either treatment alone. We then asked whether PROG would be more effective in conferring neuroprotection if it were given in combination with VDH in aged, D-deficient animals. Figures 7.8 through 7.11 show the relative levels for several cytokines 24 h and 72 h after TBI in D-deficient animals. All values are normalized to the vehicle-treated group average for each time-point: TNFα (Figure 7.8: 24h: $F_{4,20} = 8.530$, $p = 0.001$; 72h: $F_{4,20} = 26.931$, $p < 0.001$), IL-1β (Figure 7.9: 24h: $F_{4,20} = 5.911$, $p = 0.010$; 72h: $F_{4,20} = 15.393$, $p < 0.001$), IL-6 (Figure 7.10: 24h: $F_{4,20} = 16.481$, $p < 0.001$; 72h: $F_{4,20} = 23.538$, $p < 0.001$), NFκB p65 (Figure 7.11: 24h: $F_{4,20} = 9.960$, $p = 0.001$; 72h: $F_{4,20} = 6.847$, $p = 0.003$). In most cases, only the D+PROG treatment resulted in significant reduction of inflammation by 72 hours after injury.

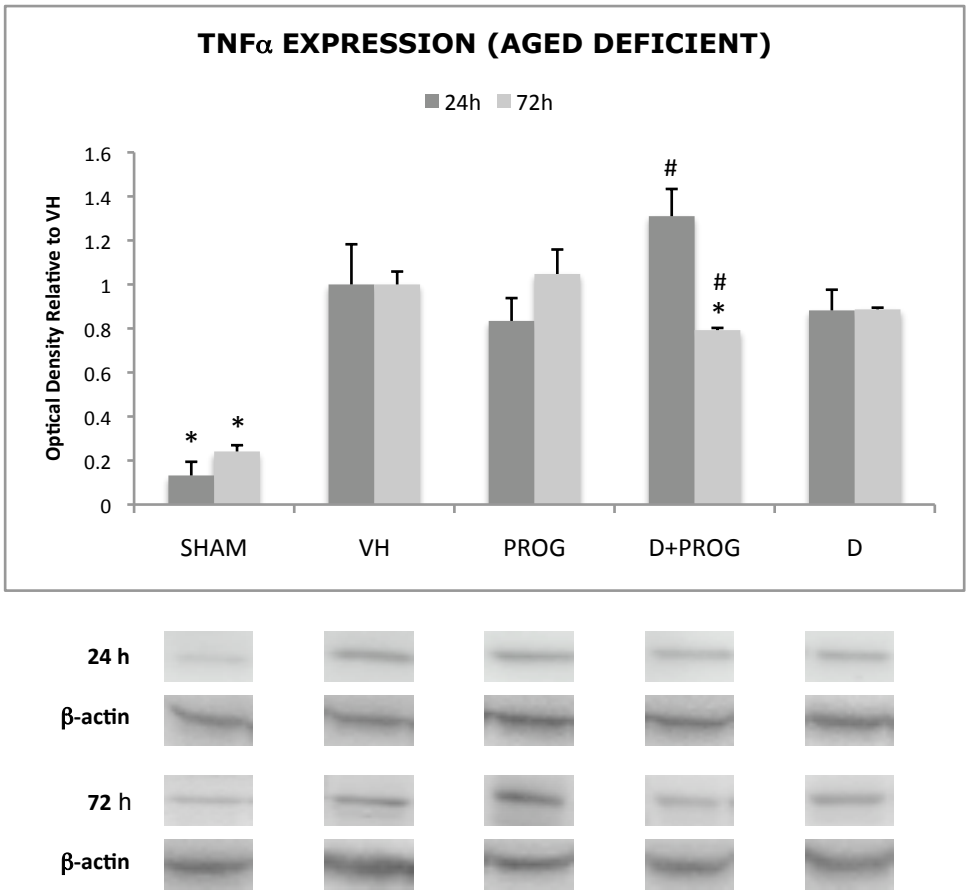


Figure 7.8. TNFα levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

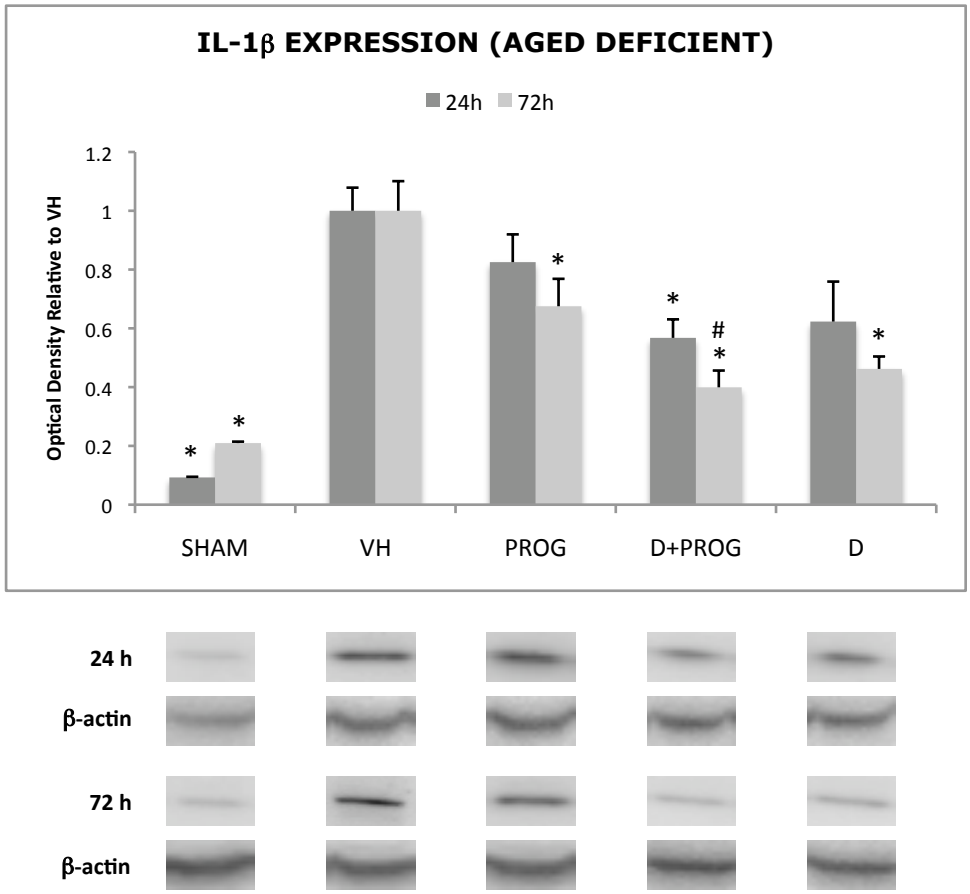


Figure 7.9. IL-1β levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

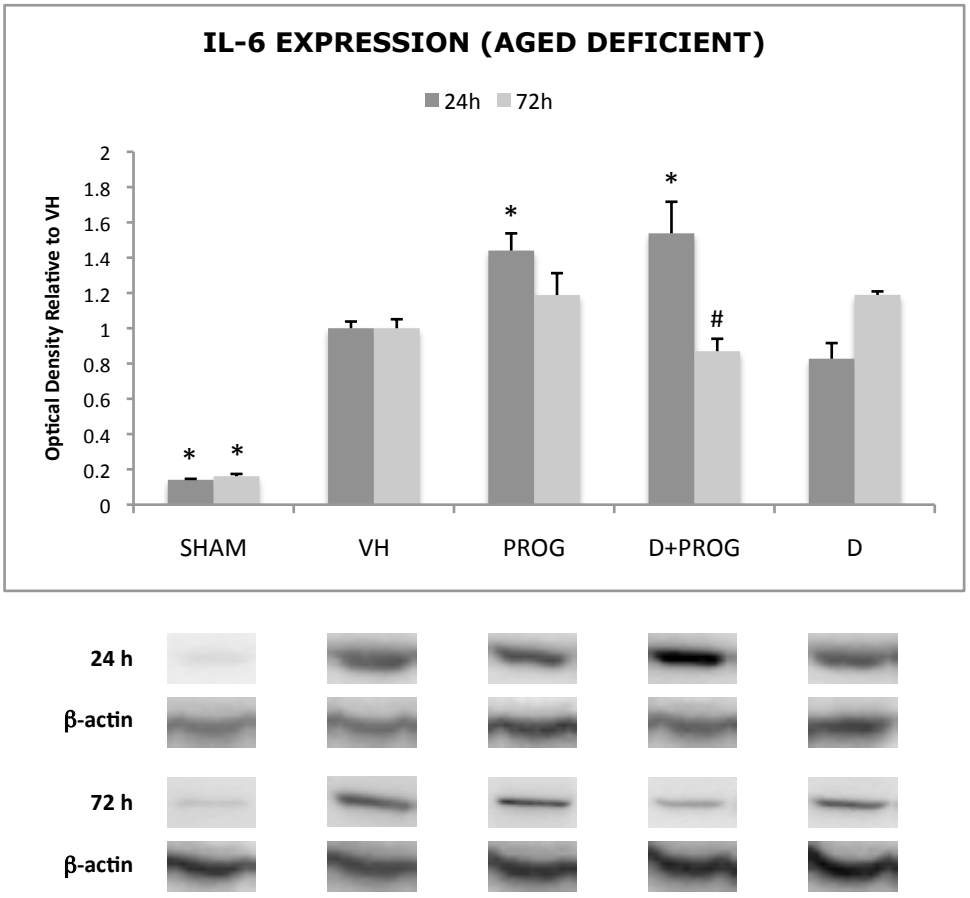


Figure 7.10. IL-6 levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

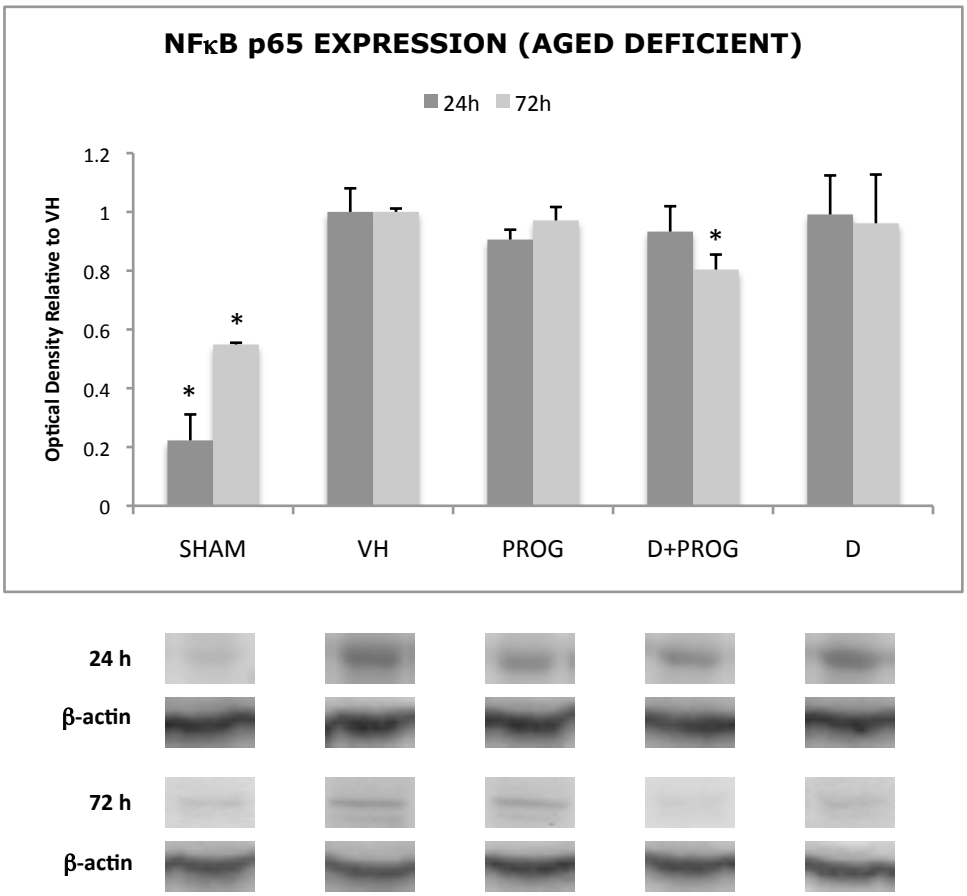


Figure 7.11. NFκB p65 levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

7.4.4. Administration of VDH with PROG in Vitamin D Deficient Animals Reduces Cell Death and DNA Damage Compared to Vehicle, VDH, or PROG 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 decision pathway 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 (Figure 7.12) show a significant decrease in activated caspase-3 (24h, $F_{4,20} = 6.332$, $p = 0.008$; 72h, $F_{4,20} = 11.634$, $p < 0.001$) and a bidirectional effect (Figure 7.13) on p53-DNA interaction (24h, $F_{4,20} = 6.563$, $p = 0.003$; 72h, $F_{4,20} = 6.181$, $p < 0.001$) only in animals treated with D+PROG. We interpret our findings to suggest that combined D+PROG is the most effective form of treatment in reducing cell death and DNA damage after TBI in D-deficient animals.

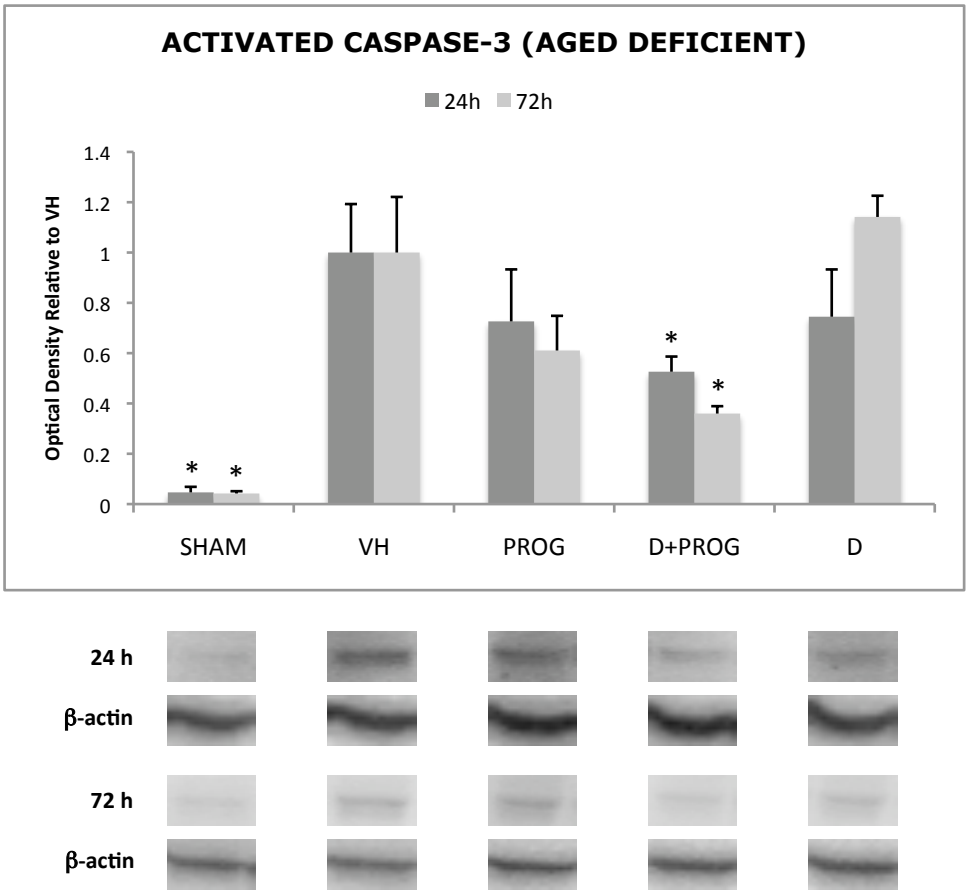


Figure 7.12. Activated caspase-3 levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

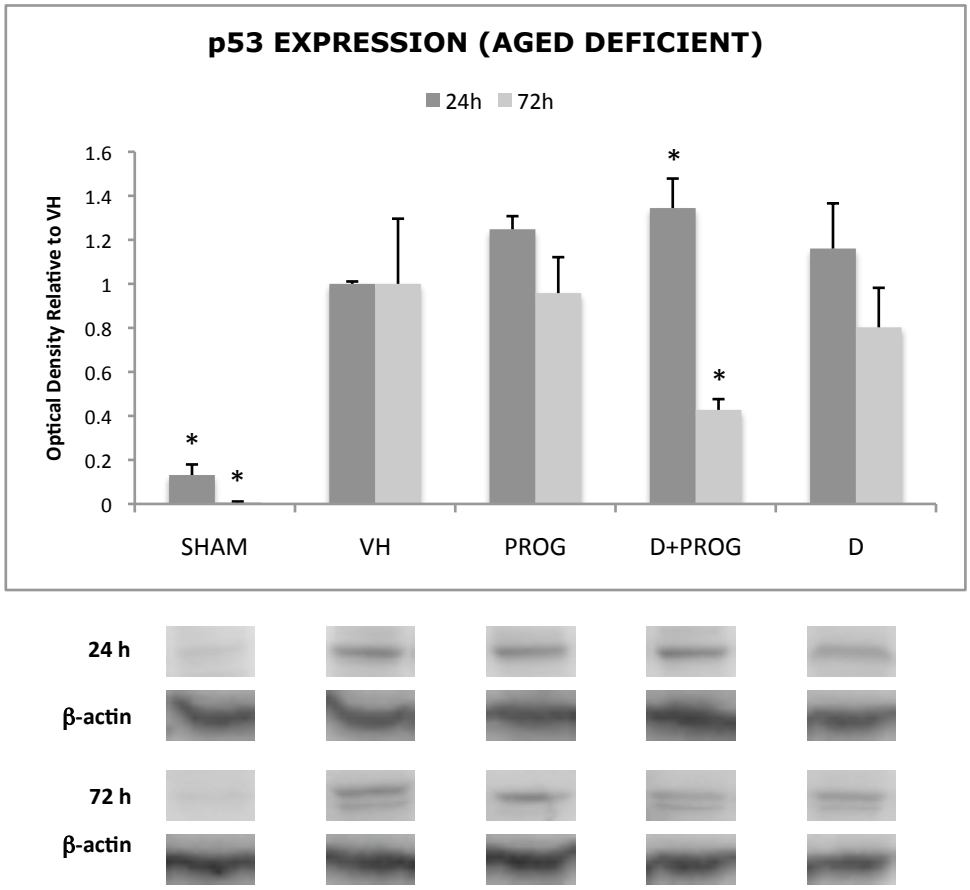
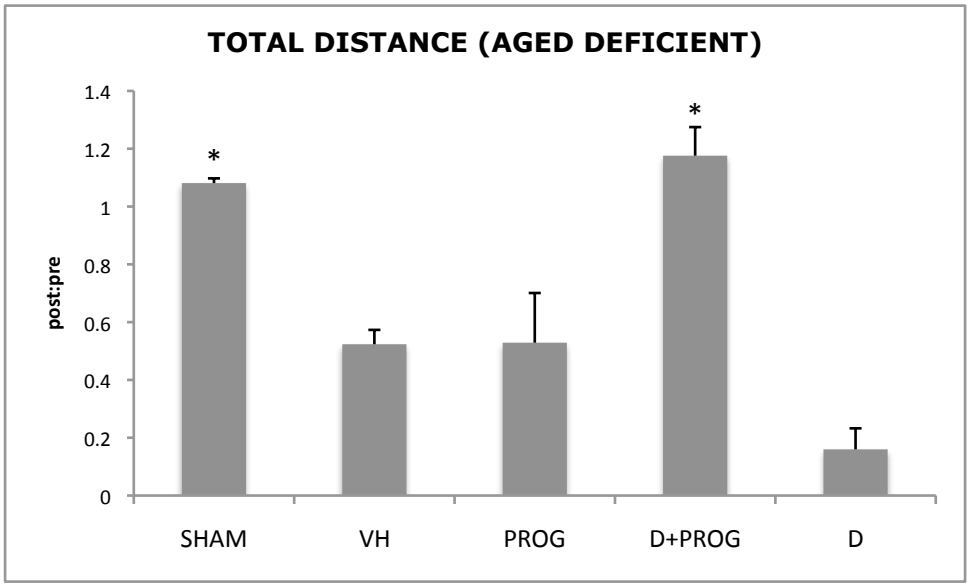
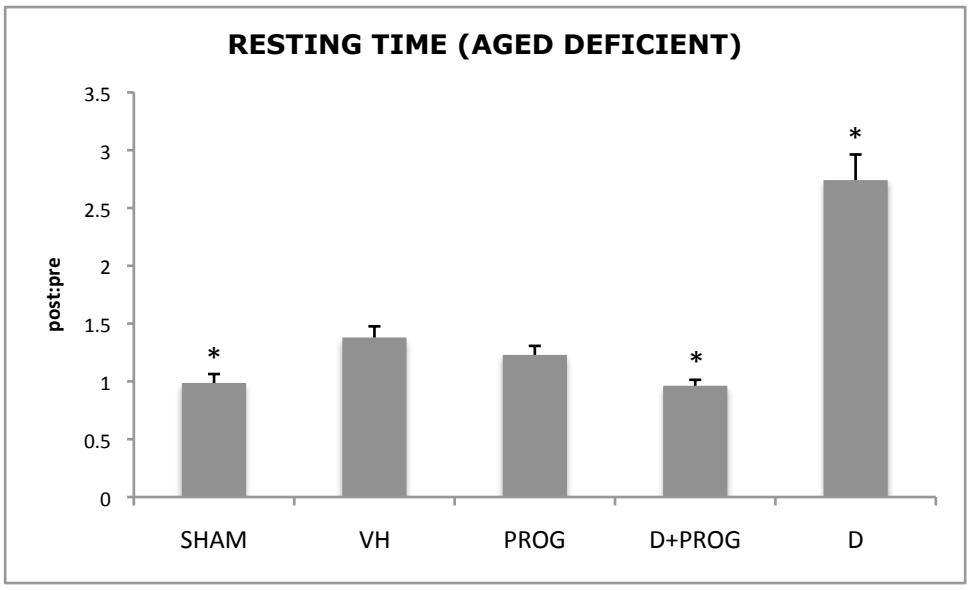


Figure 7.13. p53 levels in vitamin D deficient aged animals 24 (dark) and 72 (light) hours after injury. All values are normalized to respective time-point VH values. * $p < 0.05$ vs. VH, # $p < 0.05$ vs. PROG.

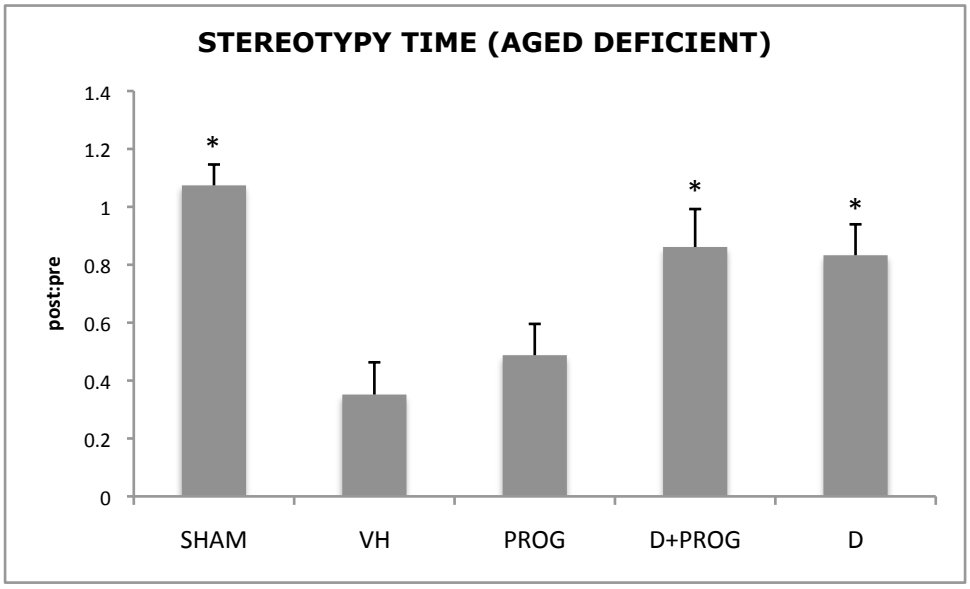
7.4.5. Combined Treatment with PROG and VDH Improves Behavioral Function Compared to Treatment with Vehicle, PROG, or VDH Alone

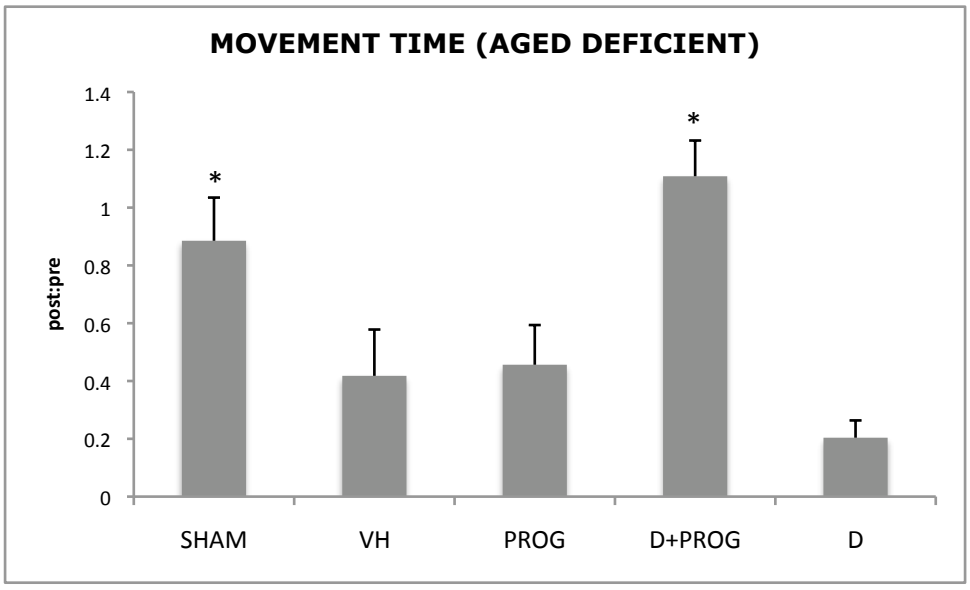
In addition to molecular measures of inflammatory cytokines, we examined the behavioral effects of the various treatments in aged deficient animals. Since this study was limited to the short-term effects on inflammation, only short-term Spontaneous Locomotor Activity was used. The results are shown in Figures 7.14 through 7.17 as the ratios of post-injury:pre-injury measurements. The basic parameters examined were total distance (Figure 7.14: $F_{4,20} = 9.179$, $p = 0.001$), resting time (Figure 7.15: $F_{4,20} = 42.393$, $p < 0.001$), stereotypy time (Figure 7.16: $F_{4,20} = 7.686$, $p = 0.002$), and movement time (Figure 7.17: $F_{4,20} = 5.097$, $p = 0.009$) 72 h after injury. We observed a significant improvement in locomotor activity with combination D+PROG treatment, but little or no benefit with either PROG or VDH alone.





Figures 7.14 and 7.15. Spontaneous locomotor behavior measurements of total distance traveled and resting time in vitamin D deficient aged animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values. * $p < 0.05$ vs. VH.





Figures 7.16 and 7.17. Spontaneous locomotor behavior measurements of stereotypy and movement times in vitamin D deficient aged animals 72 hours after injury expressed as the ratio of post-injury to pre-injury values. * $p < 0.05$ vs. VH.

7.4.6. Models and Correlations

Correlations within the vitamin D deficient animal data were very similar to those seen in Chapter 5, with significant positive correlations between molecular and behavioral data and between the different molecular measures themselves. Total distance was significantly negatively correlated with TNFα (r = -0.655, $p = 0.009$), IL-6 (r = -0.559, $p = 0.030$), NFκB p65 (r = -0.520, $p = 0.039$), and caspase-3 (r = -0.638, $p = 0.008$), but not IL-1β (r = -0.269, $p = 0.174$). Resting time was correlated significantly only with NFκB p65 (r = 0.660, p = 0.004) and caspase-3 (r = 0.638, p = 0.008). Stereotypy time was negatively correlated to TNFα (r = -0.685, p = 0.002), IL-1β (r = -0.664, p = 0.001), IL-6 (r = -0.599, p = 0.011), NFκB p65 (r = -0.601, $p = 0.006$), and caspase-3 (r = -0.479, $p = 0.038$). Movement time was negatively correlated with TNFα, (r = -0.557, $p = 0.016$), IL-6 (r = -0.580, $p = 0.015$), NFκB p65 (r = -0.521, $p = 0.022$), and caspase-3 (r = -0.614, $p = 0.005$), but not with IL-1β (r = -0.426, $p = 0.061$).

A number of significant correlations were also observed between our behavioral and molecular data when the entire data set was used (vitamin D sufficient and deficient aged animals with all 5 treatment groups combined). 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 negatively 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). In general, the behavioral impairment was negatively correlated with inflammatory cytokine levels.

To explore the interaction between inflammatory molecular events and behavior in more detail, we constructed a GLM using deficiency/injury/treatment as fixed factors (Type I SS due to logical priority within the factors) and normalized molecular measures as covariates for two behavioral outcomes, total distance traveled and resting time. The model for total distance was significant (corrected model: F = 5.069, p = 0.043, R^2 = 0.918), with most of the variability accounted for by TNFα (F = 30.841, p = 0.003, η_p^2 = 0.860), with a trend for deficiency (η_p^2 = 0.543). Resting time (corrected model: F = 16.930, p = 0.001, R^2 = 0.971) showed significant effects of TNFα (η_p^2 = 0.881), NFκB p65 (η_p^2 = 0.842), caspase-3 (η_p^2 = 0.757), deficiency (η_p^2 = 0.903), and treatment (η_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.

Finally, we also looked at a GLM with each molecular measure (72 h) as outcome using only the fixed factors (deficiency/injury/treatment). The significant ($p < 0.05$) results were: TNFα (corrected model: F = 32.401, $p < 0.001$, R^2 = 0.919; main effects: deficiency, partial η^2 = 0.554; injury, partial η^2 = 0.893; treatment, partial η^2 = 0.477; interaction effects: deficiency*treatment, partial η^2 = 0.410); IL-1β (corrected model: F =

7.844, $p < 0.001$, $R^2 = 0.696$; main effects: injury, partial $\eta^2 = 0.580$; treatment, partial $\eta^2 = 0.418$); IL-6 (corrected model: F = 37.690, $p < 0.001$, $R^2 = 0.930$; main effects: deficiency, partial $\eta^2 = 0.771$; injury, partial $\eta^2 = 0.865$; treatment, partial $\eta^2 = 0.492$; interaction effects: deficiency*injury, partial $\eta^2 = 0.401$; deficiency*treatment, partial $\eta^2 = 0.642$); NFκB p65 (corrected model: F = 11.065, $p < 0.001$, $R^2 = 0.787$; main effects: deficiency, partial $\eta^2 = 0.657$; injury, partial $\eta^2 = 0.533$); cleaved caspase-3 (corrected model: F = 8.158, $p < 0.001$, $R^2 = 0.713$; main effects: deficiency, partial $\eta^2 = 0.375$; injury, partial $\eta^2 = 0.470$; treatment, partial $\eta^2 = 0.432$; interaction effects: deficiency*injury, partial $\eta^2 = 0.188$).

These results suggest a complex relationship between D-deficiency, injury, treatment, and outcome, and one that varies significantly with regard to the different cytokines of the acute phase response, especially TNFα and IL-6. Our data are consistent with the fact that the molecular effects observed for our significant behavioral parameters (total distance and resting time) were also associated with TNFα, IL-6, and caspase-3 levels.

7.5. DISCUSSION

Insults to the CNS, including TBI, induce significant neuroinflammatory and oxidative stress responses, which further activate the cascade of secondary brain damage. Both PROG and VDH are pleiotropic hormones acting on several common, as well as on independent, CNS pathway mechanisms to reduce CNS damage and enhance CNS repair after TBI. Many studies now show that treatment with PROG significantly improves functional outcome after TBI in rats and humans (Gibson et al., 2008; Schumacher et al., 2007; Singh et al., 2008; Stein, 2008a). PROG has been shown to reduce inflammatory responses (He et al., 2004b; Pan et al., 2007) and oxidative stress. In addition PROG can activate protective pathways and increase the expression of genes and proteins associated with neuroprotection after brain damage.

Likewise, VDH has also 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 (Lin et al., 2003), EAE (Garcion et al., 2003b), LPS-induced oxidative stress (Garcion et al., 1999) and Parkinson's disease models (Shinpo et al., 2000; Wang et al., 2001). VDH exerts immunomodulatory effects and regulates the differentiation, growth, and function of a broad range of immune system cells (Adorini, 2002). A growing literature demonstrates that VDH restriction impairs a number of physiologic processes associated with healthy CNS functions such as mitosis, mitogenesis, neurite outgrowth, adult neurogenesis, and mitochondrial dysfunction (Almeras et al., 2007). Treatment with VDH induces the expression of NGF, GDNF, pro-apoptotic proteins (Kiraly et al., 2006), and upregulates OH-1 and reduces GFAP immunoreactivity in the injured brain (Oermann et al., 2004). Given the wide spectrum of action of both

hormones it is likely that a combination of the two, operating through unique and slightly different but compatible molecular mechanisms, might be synergistic in reducing the cytotoxic events associated with the injury cascade and increasing the neuroprotective events related to anti-apoptotic signaling and brain repair.

In this study we examined the efficacy of VDH as a treatment for TBI. We show "proof of concept" data that demonstrate its effectiveness in reducing inflammation after TBI in D-normal, young rats alone and in combination with PROG. We also show the benefits of combining VDH with PROG in treating TBI in vitamin D deficient, aged rats. This latter finding is potentially important to clinical practice because TBI is on the rise in the elderly (Adekoya et al., 2002) and most elderly are D-deficient (Holick and Chen, 2008). If we are to treat this age group with PROG after TBI, and there is evidence that PROG is a viable potential treatment (Chapter 4), we need to understand the circumstances that may affect both the extent of the injury and the effectiveness of acute post-injury treatment. We therefore asked three basic questions: 1) does VDH alone work to reduce acute inflammation, DNA damage, and cell death in young rats with TBI under "normal" nutritional conditions? 2) Is there evidence that VDH interacts with PROG, positively or negatively, in reducing acute inflammation, DNA damage, and cell death after TBI, also in young, vitamin D adequate rats? And, 3) since we have seen that D-deficiency attenuates the benefits of PROG treatment after TBI in old rats (Chapter 5), is it possible to counteract this effect with VDH co-administration? Based on the data, we can now answer these questions in the affirmative.

The data in young animals are relatively straightforward: at the dose we examined (1μg/kg), VDH appears to have similar effects to PROG when used alone, and shows

synergy in combination with PROG in reducing levels of TNFα, NFκB p65, and p53 protein. The combination is more effective than either compound alone. These effects are not unexpected. Given the discussion in Chapter 6, it is likely that VDH is activating mechanisms complementary to PROG in reducing DNA damage and cell death, including Ca^{2+} regulation, growth factor induction, increased antioxidant capacity, and cell cycle control. Based on the known immunomodulatory role of VDH, it was also expected that it would reduce levels of inflammatory cytokines in the tissue around the injury. While the results are not unequivocal and are based on a relatively small sample size, we believe it is reasonable to deduce that VDH alone can confer substantial neuroprotection after TBI and that it interacts with PROG in reducing inflammation.

The synergistic effect of VDH and PROG treatment was much more pronounced in aged, D-deficient than in young vitamin D sufficient animals, and our data show that in aged animals with D-deficiency a combination of PROG and VDH is needed to reduce the levels of TNFα, IL-1β, IL-6, NFκB p65, activated caspase-3, and p53 after TBI. One thing to note is the instability of the results 24 hours after injury, when inflammatory processes are in a very different state than at 72 hours. This is not surprising given the evidence that inflammation can be beneficial in the very early stages after injury (Chan, 2008). Here we see again the strong effect of combined treatment on apoptotic signaling (both p53 and activated caspase-3 levels), suggesting that multiple neuroprotective mechanisms may be in effect. It is also likely that the underlying disruption in T_H1/T_H2 balance common in the elderly and exacerbated by D-deficiency can account for the observed increased inflammatory response.

Since old individuals "tend" toward a pro-inflammatory state even before injury, the traumatic event can result in an overwhelming inflammatory response that can lead not only to increased long-term damage but also severe life-threatening multi-organ system dysfunction (Pape et al., 2007). Our data show evidence for elevated levels of IL-6 in D-deficient animals, suggesting a much more severe acute phase reaction. Since IL-6 is the key cytokine in the systemic acute phase reaction initiated by TNFα, this increased response could have potential implications for more general systemic inflammation and human survival. Also, since both IL-6 and TNFα figured prominently in regression models connecting short-term behavioral outcomes with the molecular data and deficiency/injury/treatment interactions, this can be interpreted to suggest that the elevated expression cluster of acute inflammatory factors, and especially TNFα and IL-6, can account for the short-term functional impairments observed after TBI; thus they are likely to be major components of the alterations in sickness behavior that often occur after brain injury.

What may be necessary to overcome this complex and manifold reaction to injury is a combination of agents that affect similar but different mechanisms. Our data show that VDH alone cannot overcome the effects of D-deficiency in the short term without some additional treatment (in this case PROG), since D-deficient animals treated with VDH still generally showed worse molecular and behavioral outcomes than D-normal animals treated with VH. While the results are not completely univocal in supporting a combination treatment (e.g. both PROG and VDH alone significantly reduce levels of IL-1β at the 72 hour time-point), only the combination treatment shows effectiveness in *all* cases examined.

Finally, we would like to mention the odd result of decreased $25OHD_3$ serum levels 72 hours after injury in animals given VDH compared to controls. While far from conclusive, as evidenced by the fact that the levels are not significantly different between 24 and 72 hours, the data can be taken to suggest that perhaps standard measurements of vitamin D status (or the status of other hormones) may not apply under injury conditions due to the short-term alteration in metabolism, signaling, and catabolic processes. As there is evidence that stress can both reduce the clearance (Crivello, 1988) and increase the signaling activity of VDH (Li et al., 2007b), it may be that acute systemic administration of VDH under altered systemic conditions (D-deficiency) and severe stress (TBI) can alter metabolic balance and make it difficult to interpret what serum $25OHD_3$ levels actually mean. This could have significant implications for determining D-deficiency in the human population.

In summary, we show that VDH has neuroprotective effects after TBI, that it interacts synergistically with PROG in reducing inflammation and cell death, and that it is an effective supplement to PROG in treating injured aged subjects with vitamin D deficiency. Since the demographic we are concerned with (aged, vitamin D deficient) is increasingly susceptible to TBI, these results have important translational implications for clinical practice and management of elderly patients with brain injury.

CHAPTER 8

CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

8.1. ABSTRACT

In this series of studies, we have extended the potential application of progesterone (PROG) as a treatment for TBI to older subjects by demonstrating its effectiveness in reducing acute inflammation and cerebral edema, and improving short-term behavioral outcome in aged rats. Vitamin D deficiency appears to increase baseline inflammation, exacerbate injury, and attenuate the beneficial effects of PROG when administered after TBI in aged rats. This effect can be reversed by co-administration with 1,25-hydroxyvitamin D_3 (VDH). One general point we can take from this is that the endogenous hormonal environment can affect treatment outcome, a potentially clinically important finding. A second point is that, compared to giving just a single agent, combination therapies that affect at least partially divergent mechanisms may be better suited to the treatment of heterogeneous disease processes such as human TBI. A third point is that although a combination of VDH and PROG has benefits beyond those of either compound alone, these compounds are themselves examples of "polypharmacologies" due to their pleiotropic effects on multiple systems. In this chapter we summarize our experimental results and place them within the larger context of human disease, explore some potential mechanisms for the observed effects, and suggest future directions for this research.

8.2. GENERAL SUMMARY AND CLINICAL IMPLICATIONS

8.2.1. Translational Context

Human TBI is a notoriously complex and heterogeneous disease process, a fact that may account for the current lack of treatment for this serious public health problem (Schumacher et al., 2007; Thurman et al., 1999). This complexity may also explain why most drugs designed as "monotherapies" that modulate single receptors or related groups of mechanisms have failed in clinical trials despite showing preclinical promise (Margulies and Hicks, 2009; Narayan et al., 2002). One of the key problems in trying to develop a specifically targeted treatment is the widely divergent complex of mechanisms that are known to be involved in the evolution of the injury, from the initial insult to the highly destructive secondary damage cascade to eventual reorganization and recovery (Povlishock and Katz, 2005). Each phase of the injury cascade may involve a different set of processes, which sometimes overlap and sometimes do not.

The most significant events impacting survival that occur during the acute phase of injury appear to be related to inflammatory processes, specifically the production of inflammatory cytokines, which are a well-recognized aspect of the physiological response to trauma (Harwood et al., 2005) and are the most consistent prognostic markers of outcomes in patients with systemic inflammatory response syndrome (SIRS), sepsis, or multi-organ dysfunction syndrome (MODS) and multi-organ failure (MOF) (Pape et al., 2007). As discussed in Chapter 2, it is primarily these systemic effects that appear to be the proximate cause of death after CNS injury (Keel and Trentz, 2005; Zygun, 2005; Zygun et al., 2005), a fact suggesting that TBI should be considered a systemic and not just a focal problem.

Inflammatory markers seem to be a good index of the extent of injury or traumatic insult (Bochicchio et al., 2001; Pape et al., 2007) as well as reliable independent predictors of injury outcome (Malone et al., 2001; Napolitano et al., 2000; Nast-Kolb et al., 1997; Nuytinck et al., 1988). There is also evidence showing that patients who eventually develop MOF have elevated cytokine levels at day one (Napolitano et al., 2000) followed by a second spike later (Pape et al., 1994) compared to patients who follow an uneventful clinical course. Specifically, although TNFα and IL-1β levels increase after severe injury, the level of IL-6 that is currently considered the most accurate for prognosis because it is the chief regulator of the hepatic acute phase response (Gabay and Kushner, 1999) and correlates with systemic inflammation and outcome (Pape et al., 2007). There is also literature showing that early increased levels of IL-6 can be a marker of high-risk of complication and organ failure (Gebhard et al., 2000).

Modulating the production of inflammatory cytokines during the acute phase after injury could be of benefit in reducing mortality. Given the evidence of gender differences in patients with multiple trauma, with premenopausal women showing significantly lower plasma cytokines as well as less MOF and sepsis compared to age-matched males (Frink et al., 2007), one of the reasons for increased survival in moderately and severely injured patients with TBI given PROG (Wright et al., 2007; Xiao et al., 2008) may be a reduction in systemic inflammation and consequent prevention of MODS and MOF. This is in addition to the specifically neuroprotective effects of PROG, which have been reviewed elsewhere (Schumacher et al., 2007; Stein, 2008b; Stein and Hurn, in press).

In the experiments described here, we focus on the acute phase events occurring between 24 and 72 hours after injury as a potentially key period in patient outcome after

TBI. Since rats have a much better survival rate after severe injury than humans, we looked at brain inflammation as indicative of the systemic inflammatory response. Overall, this research tries to answer three basic questions: 1) Does PROG work in aged subjects? 2) Does vitamin D deficiency (D-deficiency) affect the injury and the effectiveness of PROG after TBI? 3) Can the effects of D-deficiency be overcome by combining PROG with 1,25-dihydroxyvitamin D_3 (VDH), the active form of vitamin D, after TBI?

8.2.2. PROG is Effective in Aged Subjects with TBI

Our working hypothesis was that PROG is effective in treating TBI in elderly individuals. While this may appear trivial, it is far from obvious that a treatment that works in young adult animals would also work in the aged, and even if it did, that it would work equally well. For example, a number of systemic physiological changes associated with the aging process such as altered metabolism and endogenous hormonal milieu (Araujo et al., 2004; Lanfranco et al., 2003; Rammos et al., 2008; Vermeulen and Kaufman, 1995; Vermeulen et al., 2002), impaired immune function known as immunosenescence (Bulati et al., 2008; Gruver et al., 2007; Vasto et al., 2007), increased levels of systemic inflammation or "inflammaging" (Franceschi et al., 2007), and reduced physiological reserve or "frailty" (Fried et al., 2001; Walston et al., 2006) could alter drug effectiveness. This is especially notable since IL-6 levels are increased in the brains of healthy aged animals (Godbout and Johnson, 2004) and are associated with frailty in the aged (Topinkova, 2008). Numerous changes within the CNS itself, including reduced plasticity (Hof and Morrison, 2004) and more subtle chemical, structural, morphological, and network

alterations (Dickstein et al., 2007; Mattson and Magnus, 2006), can also significantly affect the ability of an older animal or person to survive and recover from severe injury.

The motivation for this experiment comes from data on the human population showing that, although the incidence of TBI has decreased in most age groups due to improved primary prevention (such as seat belts and safety helmets), it has increased by 21% in people over the age of 65 (Adekoya et al., 2002). Age is itself an independent predictor of mortality due to TBI, which in the geriatric population is twice that of younger victims (Mosenthal et al., 2002). These facts are confirmed by further data showing that the age distribution of hospitalizations due to TBI has the most pronounced peak in the late 70s (McArthur et al., 2004), and that the highest mortality rates from TBI, ranging from 60.9% to 86.8% depending on the study, occur in people 75-80 years old (Gomez et al., 2000). Given the significance of the problem, we considered it important to specifically address the effectiveness of PROG in senescent subjects in improving parameters associated with survival.

Based on these translational considerations, we measured levels of inflammatory proteins, cell death, edema, and short-term behavior during the acute phase of the injury (24-72 hours post-TBI) in aged rats (22-months old, the "human" equivalent of ~60) in order to determine the effectiveness of PROG in reducing mortality after TBI in the elderly clinical population. Injured animals treated with 8mg/kg and 16mg/kg PROG showed decreased expression of COX-2, IL-6, and NFκB at all time-points examined, indicating a reduction in the acute inflammatory process compared to the old rats given vehicle. The 16mg/kg PROG group also showed reduced neuronal apoptosis at all time-points as well as decreased edema and improved locomotor outcomes. Of special note

here are the effects on reduction of cerebral edema and IL-6 levels. A second finding is that, although the lowest PROG dosage used in previous studies in younger animals (8mg/kg) was also found to be effective in the aged, it was not as good as 16mg/kg, suggesting potentially altered metabolism in the older animals.

Another novel finding in this study was the (albeit not monotonic) association between increased levels of p-glycoprotein (Pgp) and reduced edema, suggesting that PROG may reduce cerebral edema through its anti-inflammatory effects on cytokine levels and through direct effects on blood-brain barrier (BBB) integrity. Pgp is regulated through the pregnane X receptor (PXR) (Bauer et al., 2004; Miller et al., 2008), and PROG has been shown to exert neuroprotective effects through the PXR (Langmade et al., 2006). This raises the intriguing possibility that some of the post-TBI benefits of PROG may be effected through this relatively novel signaling mechanism.

8.2.3. Vitamin D Deficiency Exacerbates Injury and Attenuates PROG Benefits after TBI in Aged Subjects

An estimated 1 billion people worldwide exhibit vitamin D deficiency (D-deficiency) or insufficiency (Holick, 2007). The demographic specifics vary, but studies show a prevalence that ranges from 32% in healthy adults (Tangpricha et al., 2002) to around 50% for adolescents and preadolescents in the northeastern United States (Gordon et al., 2004; Sullivan et al., 2005). Similar numbers are seen in Europe and even the Middle East, India, and Australia, where, despite abundant year-round sunlight, deficiency is widespread due to the common practice of shielding most of the skin from the sun (El-Hajj Fuleihan et al., 2001; Marwaha et al., 2005; McGrath et al., 2001; Sedrani, 1984). The most dramatic statistics for D-deficiency, however, come from studies in the elderly,

which show that 40 to 100% of community-dwelling American and European older men and women are D-deficient (Holick, 2007). Even higher averages are seen in the ill and institutionalized (Chatfield et al., 2007; Corino et al., 2007; Eyles et al., 2003; Eyles et al., 2005; Hewison et al., 2000; Holick, 1994, 2003b; Larrosa et al., 2001; McGrath et al., 2004; Townsend et al., 2005).

D-deficiency has been associated with a plethora of systemic disorders such as infectious, inflammatory, and autoimmune conditions (Cantorna et al., 2004; DeLuca and Zierold, 1998; Griffin et al., 2003; Hayes et al., 2003; Holick, 2003a, b; Mahon et al., 2003), cardiovascular disease (Martins et al., 2007; Melamed et al., 2008), hypertension and atherosclerosis (Onyszchuk *et al.,* 2008), neuromuscular function (Pfeifer *et al.,* 2002), cancer (Peterlik and Cross, 2005), neurodegenerative diseases (Garcion et al., 2002; Kalueff et al., 2004b), and functional outcomes in the elderly (Dawson-Hughes, 2008). In fact, D-deficiency appears to be correlated with most problems associated with advanced age, especially those with an inflammatory component (Cantorna et al., 2004; Hayes et al., 2003). This fits with the fact that VDH is known be a potent modulator of the immune system (Dusso et al., 2005; van Etten and Mathieu, 2005). There is also evidence that the level of serum vitamin D is one of the key markers of "frailty" or *systemic* insufficiency (Morley et al., 2006; Topinkova, 2008), and that it is associated with elevated levels of IL-6 (McCarty, 2005).

Would this deficiency affect the outcome of a brain injury and would it interfere the benefits of PROG treatment for TBI in the elderly? We hypothesized that D-deficiency would exacerbate inflammation and reduce or altogether eliminate the benefits of PROG treatment after TBI in aged animals. Because early inflammation is a reliable

prognostic indicator of mortality in human patients with significant trauma, we measured acute inflammatory proteins, cell death, DNA damage, and short-term behavior as indicators of inflammation and secondary damage in vitamin D deficient aged animals after TBI.

We observed increased levels of inflammation in the brains of vitamin D deficient (D-deficient) uninjured animals compared to vitamin D sufficient (D-normal) counterparts. This confirms previous studies (Cantorna et al., 2004; Hayes et al., 2003; Lips, 2006) and suggests that D-deficiency establishes a higher baseline level of inflammation, in effect priming the system for an increased response after injury. This elevated response to injury was directly observed in vehicle-treated D-deficient compared to D-normal animals, showing that D-deficiency can increase inflammation and cell death in the perilesional area, thus exacerbating the injury (as measured by cell death and DNA damage) and in the case of human patients, potentially increasing mortality. A similar outcome was seen with PROG treatment in D-deficiency, which substantially reduced PROG's beneficial effects in aged subjects after TBI compared to D-normal counterparts. This finding has potentially serious implications for both clinical and preclinical research, since evidence that an endogenous deficiency in a common nutrient (such as but not limited to vitamin D) may not only exacerbate injury but also reduce the effectiveness of treatment could potentially create a serious confound for any clinical study investigating drug efficacy.

In general, we demonstrated increased inflammation in all cases with D-deficiency, whether our subjects were uninjured, injured but untreated, or injured and treated with PROG. This elevated acute phase response was not innocuous and correlated

with increased cell death and DNA damage, indicating a more severe secondary injury process. D-deficiency also affected sickness behavior, which was strongly correlated (general linear models, GLMs) with the expression of TNFα and IL-6, showing that short-term locomotor behavior can be a good indicator of the amount of brain inflammation in the acute phase after injury. Our GLMs also showed that both TNFα and IL-6 were significantly affected by deficiency and both showed deficiency-treatment interaction effects, suggesting that these cytokines may constitute important hubs in the network of mechanisms underlying the attenuation of PROG benefits in D-deficiency. Also notable is the fact that, while most of the variability in TNFα was accounted for by injury, the level of IL-6 was primarily affected by D-deficiency. This fits with other data connecting IL-6 levels with D-deficiency, frailty, and inflammation (McCarty, 2005; Miller et al., 2007; Morley et al., 2006; Van den Berghe et al., 2003) and suggests that IL-6 may be the key cytokine involved in the effects of D-deficiency. The elevation in IL-6 was most evident in our data when comparing D-deficient and D-normal animals after TBI and PROG treatment. While most of the other cytokines were elevated in D-deficient animals 2- or 3-fold, IL-6 was increased nearly 5-fold by 72 hours after injury. Given the data from the human population showing that the level of IL-6 is the most accurate prognostic indicator of survival in the acute phase after TBI, our findings could have significant implications in the clinical setting. Elderly people with D-deficiency would need to be identified and potentially treated differently after TBI because they could be more frail and thus more likely to die from their injuries. This is speculative, but the implications are intriguing and potentially important for clinical practice.

8.2.4. Combination of VDH with PROG Reverses the Attenuation of PROG Efficacy and Reduces Inflammation after TBI in Aged Subjects

Conceptually, the consideration of a different compound in relation to PROG (or any "primary" drug), and especially one that is endogenous and as pleiotropic in its effects as vitamin D (discussed in Chapter 6), introduces a logical dichotomy. On the one hand is the issue of *deficiency*, or altered baseline *prior* to an insult. In this case, systemic influences such as changes in endocrine metabolism or hormone level and potential inflammatory priming effects that can affect both outcome and the effectiveness of PROG become important. On the other hand is the issue of *combination*, in which the same compound is administered *after* injury and concurrently with PROG. We hypothesized that administration of VDH in combination with PROG would reverse the effects of D-deficiency and be beneficial in aged subjects after TBI, and that the combination would be more effective than either drug alone.

Combination therapy is a well-established approach in the pharmacological treatment of a number of diseases such as HIV/AIDS or tuberculosis, although most drug development still focuses on individual drugs targeting at most a few specific mechanisms. Two issues are involved: drug combination and target promiscuity, or pleiotropy. The rationale for combining drugs ranges from targeting multiple divergent mechanisms to overcoming single-drug limitations such as receptor kinetics, pharmacology, and signaling pathways (Faden, 2001; Gingrich and Traynelis, 2000). This idea has also recently gained ground in the treatment of TBI (Margulies and Hicks, 2009), and some of the issues involved are discussed in Chapter 6. As a simple example, one could use both PROG and VDH to target cell death after TBI. PROG would reduce

cell death by preventing release of cytochrome c from the mitochondria, upregulating the anti-apoptotic Bcl-2 protein, and downregulating the pro-apoptotic Bax protein (Brinton et al., 2008; Djebaili et al., 2005; Schumacher et al., 2007). VDH, on the other hand, would prevent the reactivation of cell cycle machinery (Chatterjee, 2001; Cozzolino et al., 2001; Jensen et al., 2001), a common step towards apoptosis in terminally differentiated neurons (Cernak et al., 2005; Di Giovanni et al., 2005), and would upregulate nerve growth factor (NGF), providing a strong external pro-survival signal (Brown et al., 2003; Wion et al., 1991).

The other issue is pleiotropy, or the ability of a drug to affect multiple systems. In an important study of current drug-development and approval practice, Yildirim and colleagues (Yildirim et al., 2007) performed a network analysis of all U.S. Food and Drug Administration (FDA) -approved drugs in the DrugBank database to determine the global relationships between drug targets and known disease-gene connections. The authors found that the 1,178 FDA-approved drugs targeted a total of only 394 human proteins (of the potentially ~80,000 proteins that constitute the "human druggable proteome" (Plewczynski and Rychlewski, 2009)), mostly consisting of membrane proteins, with an average of 1.8 target proteins per drug. The implications of the data are revealing: 1) most drugs target fewer than 2 proteins; 2) most new and approved drugs are targeted to "already validated target proteins, causing an abundance of 'follow-on' drugs"; and 3) most drugs target "inessential" proteins not involved in multiple sets of interactions (Yildirim et al., 2007). The authors conclude: "Our analysis…suggests a need to update the single drug-single target paradigm, just as single protein-single function relations are somewhat limited to accurately describe the reality of cellular

processes. Future attempts at rational drug design will eventually take into account the 'systems' effects of a drug on the greater network" (Yildirim et al., 2007).

Combining PROG and VDH, two pleiotropic drugs that work primarily through intracellular receptors/transcription factors, might therefore be considered, at least according to the current trends, a bit radical. We feel that our combinatorial approach is reasonable given the complexity and heterogeneity of human TBI and the fact that, of the 130 preclinical drugs that have shown promise in treating brain injury, all have failed when taken to clinical trial (Margulies and Hicks, 2009).

We demonstrated "proof of concept" with a combination of PROG with VDH in young adult rats with TBI and normal nutritional status. Using standard doses shown to be effective in previous studies (16mg/kg PROG, 1μg/kg VDH given on the same schedule), we found that the combination treatment was more effective than either compound alone in reducing the levels of TNFα, NFκB p65, cell death, and DNA damage after TBI. Combination treatment was as effective as either compound alone in reducing IL-1β. Combining VDH and PROG has since also been shown to be effective *in vitro* in increasing the survival of primary cortical neurons that had undergone a glutamate challenge, with the combination showing more neuroprotection than either compound alone at best dose (Atif et al., 2009).

We applied this treatment concept to D-deficient aged rats with TBI and showed that the only treatment that reduced proteins measured (TNFα, IL-1β, IL-6, NFκB p65, activated caspase-3, p53) in all cases by 72 hours after injury was the combination of PROG and VDH (5μg/kg in a single dose) compared to vehicle or either compound given alone. The combination treatment was also the only one that dramatically improved

behavioral parameters, which our statistical models showed to be strongly correlated with systemic inflammation and levels of TNFα and IL-6. We used a single large dose of VDH rather than a repeated smaller one for two reasons: 1) human data have shown that a single "megadose" of VDH can correct D-deficiency for up to a year (Diamond et al., 2005); 2) a single injection of VDH would be much easier to administer as an adjunct to intravenous PROG in the clinic. Should these results be translatable to humans, the clinical implications are obvious: a combination treatment of PROG and VDH given to elderly patients with TBI should improve survival over PROG given alone to the same population.

Although it seems reasonable that supplementation with vitamin D in a deficient state will be equivalent to ongoing vitamin D sufficiency--i.e., acute correction of deficiency should be equivalent to no deficiency--there is no *a priori* reason for this assumption. At 24 hours after injury we observed no differences in inflammatory cytokines between VDH-treated deficient and D-normal animals, with the exception of IL-1β, which was lower in VDH-treated animals than in D-normal animals. By 72 hours after injury, however, while there was still no difference with TNFα and IL-1β was still lower than in D-normal animals, IL-6, NFκB p65, and cell death were all higher in VDH-treated deficient compared to D-normal animals. This suggests that, at least with reference to IL-6 levels, acutely correcting D-deficiency is not as good as staying D-normal. In other words, prevention is better than treatment. While D-deficiency is a public health issue that has received quite a bit of press lately, we can now add another benefit to maintaining a normal vitamin D status: it is not just good for the arteries and

protective against cancer, it can also be a primary intervention against TBI, in some ways the equivalent of wearing a seatbelt.

In this series of studies we showed that: 1) PROG is effective in reducing acute inflammation, a key indicator of survival in human patients, in aged rats with TBI; 2) D-deficiency increases acute phase inflammation and attenuates the benefits of PROG treatment in aged rats with TBI, suggesting that it might increase human mortality after brain injury; 3) a combination of PROG and VDH partially reverses the effects of D-deficiency and reduces post-TBI acute inflammation in old rats, suggesting that, should these results translate to the clinic, a simple vitamin D injection could help save human lives.

8.3. POTENTIAL UNDERLYING MECHANISMS

Although both PROG and VDH exert their effects through a wide variety of mechanisms (discussed in Chapters 3 and 6, respectively), here we would like to focus on the mechanisms that may underlie our observations, namely the exacerbation of inflammation and reduction of PROG benefit after brain injury by D-deficiency. Our general hypothesis, supported by the literature, is that D-deficiency (along with aging) induces a hyper-inflammatory underlying state, which, when triggered by a severe systemic injury (such as a TBI), results in overwhelming inflammation by feed-forward amplification that cannot be overcome by the same methods as in a normal healthy context. To help understand how this process occurs, we will discuss some systemic, cellular, and molecular mechanisms of vitamin D regulation of the immune system.

8.3.1. Systemic Cytokine Levels: IL-6 and TNFa

There is a direct relationship between D-deficiency and elevated levels of serum IL-6, and it may be mediated by parathyroid hormone (PTH), which is elevated in response to low levels of vitamin D (McCarty, 2005). Hyperparathyroidism is known to be associated with elevated levels of IL-6 (Grey et al., 1996) and other acute phase reactants such as C-reactive protein (CRP) (Richards et al., 2007). This association can be explained by the fact that PTH directly upregulates IL-6 production by osteoblasts (Greenfield et al., 1995), adipose tissue (Mohamed-Ali et al., 2001; Vicennati et al., 2002), and liver (Mitnick et al., 2001). Data in human patients show reduced levels of serum IL-6 and CRP with vitamin D administration (Van den Berghe et al., 2003). IL-6 is, as noted earlier, the critical hub in inflammatory amplification. It is the main regulator of the hepatic acute phase reaction (Gabay and Kushner, 1999) and its production is positively

regulated in a feed-forward manner by IL-6 itself, soluble IL-6 receptor (sIL-6R), TNFα, and IL-1β in both the brain and the peripheral immune system (Van Wagoner et al., 1999). Increased levels of IL-6 in the brain have been associated with serum levels and outcome in the brains of TBI patients (Kossmann et al., 1995; Minambres et al., 2003). It has been suggested that central nervous system (CNS) IL-6 levels may be crucial in initiating the systemic acute phase response to severe brain injury (Kossmann et al., 1995). In the brain, IL-6 is produced by neurons and astrocytes where it functions as a trophic factor under normal conditions (Van Wagoner et al., 1999). IL-6 production by astrocytes is increased by hypoxia (Maeda et al., 1994), and IL-6 levels are known to be elevated in patients suffering from stroke (Acalovschi et al., 2003). IL-6 also leads to increased levels of glial fibrillary acidic protein (GFAP), a key marker of astrocytosis and brain inflammation (Chen et al., 2003b).

Low serum levels of vitamin D are also directly associated with increased levels of serum TNFα in healthy (pre- and post-menopausal) women (Peterson and Heffernan, 2008). The same is the case in diseased populations. For example, six-month supplementation with VDH led to a significant reduction in serum TNFα levels in post-menopausal women (Inanir et al., 2004) and hemodialysis patients (Borazan et al., 2003). Another study also demonstrated increased serum concentrations of TNFα in congestive heart failure (CHF) patients over a nine-month period, while TNFα levels remained constant in CHF patients supplemented daily with vitamin D (Schleithoff et al., 2006). These data support previous research showing that vitamin D suppresses TNFα production (Cantorna et al., 1998; Cohen et al., 2001; Evans et al., 2006). Although the specific mechanism for this suppression is difficult to establish, since TNFα is produced

by a variety of cell types including monocytes, macrophages, T cells, adipocytes, fibroblasts, and smooth muscle cells (Popa et al., 2007), a study by Zhu et al. showed that VDH is capable of directly downregulating several genes involved in TNFα production such as IκBε, lipopolysaccharide-induced TNFα factor (LITAF), TNFα-induced protein 2, TNF receptor superfamily 2A, and TNFα itself in the colonic tissue of mice with inflammatory bowel disease (Zhu et al., 2005). Elevated levels of TNFα have also been observed in the serum of patients with other disorders associated with D-deficiency such as multiple sclerosis (MS), rheumatoid arthritis (RA), heart disease, osteoporosis, and inflammatory bowel disease (IBD) (Giovannoni et al., 2001; Prince, 2005). Serum TNFα levels have been directly associated with CNS TNFα after ischemic stroke (Zaremba et al., 2001), suggesting direct communication between serum cytokine levels and the degree of inflammatory activity in the brain.

This evidence suggests an association between D-deficiency and elevated systemic levels of both IL-6 and TNFα, and it is our hypothesis that this "proinflammatory" baseline is an important factor in the increased acute phase response we observed. Since the acute phase is amplified through positive feedback, "priming" of the inflammatory response due to D-deficiency could lead to a runaway reaction that overwhelms the ability of the organism to cope with the cascade of injury events. In human patients, this could lead to an increased likelihood of the systemic inflammatory response syndrome (SIRS) and MODS. This direct modulation of cytokine expression may also help to explain why VDH is a useful adjunct to PROG in the context of TBI treatment.

8.3.2. The Innate Immune System

D-deficiency and VDH have important effects on the innate immune system through monocytes and their derivative cells, macrophages and dendritic cells (DCs), all of which are important in the initiation and maintenance of inflammation (van Etten and Mathieu, 2005). These cells, along with B cells, are considered "professional" antigen-presenting cells (APCs) because they express major histocompatibility complex class II (MHC II) molecules on their surface and control naïve T cell differentiation and the stimulate helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) (van Etten and Mathieu, 2005). VDR is expressed constitutively by macrophages and DCs and inducibly by activated T-cells, providing a direct mechanism of action through gene regulation (van Etten and Mathieu, 2005).

A number of studies have shown that VDH controls the expression of MHC II molecules on monocytes in both human patients and experimental models. In human patients, VDH was found to control the expression of HLA-DRB1 genes through a direct vitamin D responsive element (VDRE) in the promoter of the gene. This fact is significant since HLA-DRB1 is the genetic locus most commonly found in Northern Europe and increases the risk of MS 3-fold (Ramagopalan et al., 2009). Although the details are still unknown, this may have implications for inflammation, since MS is an inflammatory process (Cantorna and Mahon, 2004). VDH has also been noted to reduce the expression of MHC II molecules in microglia in the rat hippocampus, correlating with lower inflammation (Moore et al., 2007), and in the CNS of animals with experimental immune encephalomyelitis (EAE), an animal model of MS, in which induced D-deficiency exacerbated disease symptoms (Garcion et al., 2003a). Inhibition of MHC II and co-stimulatory molecule expression also reduces the ability of monocytes to

stimulate T cells, an important factor in reducing inflammation (van Etten and Mathieu, 2005). The expression of MHC II molecules could also be significant for human TBI, since MHC II expression on peripheral blood monocytes has been most consistently correlated with septic morbidity and mortality after trauma among the early markers measured (Ayala et al., 1996). It is in fact more consistent in predicting mortality than serum IL-6, but cannot be measured reliably and is therefore not practically useful (Pape et al., 2007).

VDH also decreases the expression of toll-like receptors (TLR) 2 and 4 in human monocytes, which show a VDH dose-dependent suppression of TLR2 and TLR4 mRNA and protein (Do et al., 2008). TLR activation directly induces the expression of VDR and the 1α-hydroxylase enzyme in macrophages (Stoffels et al., 2006), which in turn induce the production of cathelicidin (a protein toxic to *M. tuberculosis*), providing a mechanism for the bactericidal effect of vitamin D in tuberculosis (Liu et al., 2006), but also providing a feedback loop for local control of TLR expression and inflammation (Stoffels et al., 2007). Since TLRs are known to be upregulated by TBI and are associated with TNFα production and inflammation (Chen et al., 2008), D-deficiency is likely to "prime" monocyte hyperresponsiveness to inflammatory stimuli through disinhibition of TLR expression (Du et al., 2009; Sadeghi et al., 2006). TLR modulation is also one of the likely mechanisms of PROG action after TBI (Chen et al., 2008), so it is possible that this is an area of intersection between the actions of PROG and VDH.

Another mechanism for attenuation of immune responses by VDH is through upregulation of vitamin D_3 upregulated protein 1 (VDUP-1), which is a negative regulator of thioredoxin (Trx), an important mediator of intracellular antioxidant capacity

(Billiet et al., 2008). VDUP-1 also has important roles in cell cycle and differentiation (Kim et al., 2007). Upregulation of VDUP-1 has been shown to induce caspase-3 and apoptosis in macrophages, possibly through inhibition of the interaction between Trx and apoptosis signal-regulated kinase 1 (ASK1), a regulatory protein that induces programmed cell death through the activation of Jun N-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK) pathways (Song and Lee, 2003; Tobiume et al., 2001). Induction of apoptosis by VDUP-1 appears to be tissue-specific, but may also be one of the mechanisms by which VDH induces tumor cell death (Song et al., 2003).

Probably the most significant effect that VDH exerts in the immune system is on DCs, which are key APCs in the activation and differentiation of helper T cells and form the link between the innate and adaptive immune system. VDH is known to directly inhibit DC maturation and differentiation through a VDR-dependent mechanism (Penna and Adorini, 2000; van Halteren et al., 2002). VDH reduces the expression of MHC II and other co-stimulatory molecules such as CD40, CD80, and CD86 by DCs (van Etten and Mathieu, 2005), significantly reducing their ability to initiate T cell-mediated immunity. VDH also directly inhibits production of IL-12 by DCs and other APCs (D'Ambrosio et al., 1998). Since IL-12 stimulates T_H1 and inhibits T_H2 helper T cell differentiation, this is a key event in determining the pro- or anti-inflammatory direction of immune response activation (Matsuzaki et al., 2006). VDH further increases the production of IL-10, an immunosuppressive cytokine that opposes the effects of IL-12 and promotes differentiation of the Treg phenotype (Penna and Adorini, 2000).

8.3.3. The Adaptive Immune System

VDH and PROG are both known to induce a skew towards the anti-inflammatory T_H2 phenotype (Matsuzaki et al., 2005). VDH exerts effects not only through its actions on DCs, but also directly through its effects on T cells themselves, where it inhibits proliferation, secretion of T_H1 cytokines, and progression through the cell cycle (van Etten and Mathieu, 2005). VDH also directly targets transcription of T_H1-associated cytokines such as IFN-γ and IL-2. IFN-γ is the major positive feedback signal for APCs, and by blocking its production VDH inhibits further antigen presentation and additional T lymphocyte recruitment (Cippitelli and Santoni, 1998), an important feature of the anti-inflammatory effects of VDH (Topilski et al., 2004). IL-2 is a growth factor for T cells; by blocking it, VDH prevents further proliferation and activation of T_H1 cells and indirectly induces T cell differentiation in the T_H2 direction (Takeuchi et al., 1998). VDH also inhibits the production of IL-12 by T_H1 cells, directly reducing neural antigen-specific T_H1 response (Cantorna and Mahon, 2005; Imazeki et al., 2006). VDH is also capable of not only inducing but also stimulating regulatory T cells (Treg), which inhibit T_H1 responsiveness and are an important mechanism in preventing autoimmunity (Penna and Adorini, 2000).

In addition to its indirect effects on T_H2 immunity through T_H1 inhibition, VDH also directly induces the production of T_H2 cytokines such as IL-4, IL-5, and IL-10 by T cells, thereby pushing T cell differentiation towards T_H2 (Boonstra et al., 2001). VDH directly inhibits the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) production by T cells, thereby also blocking inflammatory amplification by inhibiting the feedback activation of macrophages by T cells (Imazeki et al., 2006; Tobler et al., 1987; Towers et al., 1999). VDH can also modulate activation-induced apoptosis of

T lymphocytes through its effects on the expression of Fas ligand (FasL) (Cippitelli et al., 2002). Overall, the effects of VDH on the immune system are to reduce the activity of DCs, T_H1, T_H17, and B lymphocytes and the expression of IL-2, while stimulating T_H2 and Treg T cells and increasing the levels of IL-4 and IL-10. All of these mechanisms are involved in skewing the immune system towards a T_H2 response and reducing inflammation (Cantorna and Mahon, 2005).

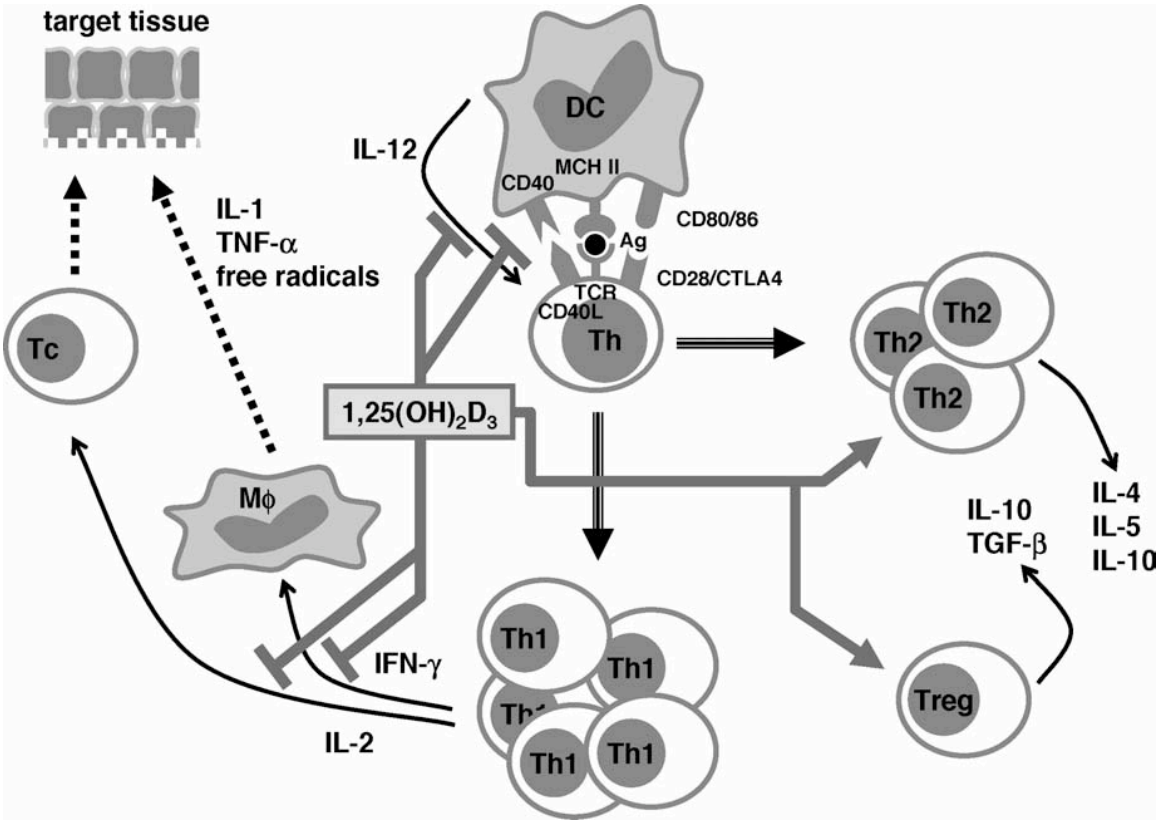


Figure 8.1. A summary of VDH effects on T_H1/T_H2 cytokines and immune responses. From van Etten and Mathieu, 2005.

Notably, PROG also appears to skew immune responses towards the T_H2 phenotype. This seems to be an important mechanism in the protection of embryos from attack by the mother's immune system during pregnancy (Dealtry et al., 2000; Faas et al., 2000; Raghupathy, 1997), but has also been cited as a mechanism in the improvement of autoimmune disease symptoms with increased PROG levels (Wilder, 1998). The molecular mechanisms are unclear, but direct induction by PROG of progesterone-induced blocking factor (PIBF) and leukemia inhibitory factor (LIF), both of which directly stimulate the production of T_H2 cytokines, have been suggested (Piccinni et al., 1998; Szekeres-Bartho et al., 2001). Another mechanism is related to the fact that T_H2 but not T_H1 cells express the key PROG-metabolizing enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) (Matsuzaki et al., 2005). The ability to metabolize PROG may be an element of immune cell survival, and T_H2 cells were able to survive cytotoxic action of PROG at the same concentration that induced apoptotic cell death in T_H1 cells (Matsuzaki et al., 2005). Additionally, PROG directly promotes T_H2 differentiation from naïve T cells (Matsuzaki et al., 2005), and even induces IL-4 production in already committed T_H1 cells (Miyaura and Iwata, 2002). PROG can also enhance the development of IL-10-producing T_H2 cells (Piccinni et al., 1995). What is notable in this case is that, like VDH, PROG biases the immune response towards T_H2 behavior, but does so through a different set of cellular mechanisms. This provides an example of the benefits of combination therapy and shows how two different compounds might synergistically affect a system-level process (T_H1 vs. T_H2 induction) through modulating divergent local mechanisms.

8.3.4. Model Summary

Although PROG acts through a number of mechanisms to reduce cell death and improve functional recovery after TBI (Schumacher et al., 2007; Stein, 2008b), here we propose that a key process in its ability to reduce inflammation after injury is its ability to inhibit pro-inflammatory T_H1 and stimulate anti-inflammatory T_H2 immune responses. Although the T_H1/T_H2 paradigm is not unproblematic (Kidd, 2003), it is a useful construct in understanding the role of the adaptive immune system in inflammation. Our explanatory model is based on the fact that T_H1/T_H2 differentiation marks a decisive event very much like a phase transition in the development of extended inflammation after severe injury.

Under standard age and dietary conditions, the pleiotropic effects of PROG are sufficient to produce a generally anti-inflammatory phenotype with a T_H2 skew, which then affects observables such as levels of TNFα, IL-1β, IL-6, and edema and decreases mortality by reducing the systemic effects of inflammation after brain injury (Brinton et al., 2008; Schumacher et al., 2007). As we suggested earlier, this reduction in systemic inflammation may be one of the key mechanisms by which PROG significantly increased survival after TBI in human patients (Wright et al., 2007; Xiao et al., 2008). With age, the general response of the immune system moves towards a pro-inflammatory condition (Franceschi et al., 2007; Godbout and Johnson, 2006), which is associated with frailty and the increased susceptibility of aged organisms to external insult (Fried et al., 2001; Paganelli et al., 2006; Walston et al., 2006). This increased inflammation and decreased immune flexibility with age could explain why we generally needed a larger dose of PROG in older animals in order to obtain similar effects on acute phase reactants. In other words, the anti-inflammatory effects need to be maximized in order to overcome the underlying predisposition towards inflammatory hyper-reactivity.

Under conditions of D-deficiency, however, the pro-inflammatory T_H1 bias is further elevated to the point where PROG alone is unable to counteract the amplification of the inflammatory cascade after injury. This could especially be the case since the inflammatory system shows complex non-linear behavior whereby a small initial difference can lead to dramatically different outcomes (Godin and Buchman, 1996; Neugebauer et al., 2001; Seely and Christou, 2000). A higher dose of hormone in this case may not work due to the bimodal activity typical of steroids (including VDH), which often cause very different tissue responses at low vs. high concentrations (Abraham et al., 2006; Buckstrom et al., 2008; Samuel and Sitrin, 2008). This divergent effect may be related to the decision between cellular proliferation vs. differentiation programs, which appear to be the two most fundamental modes of cellular behavior (Helikar et al., 2008; Samuel and Sitrin, 2008; Xia et al., 2006). One way to prevent overwhelming inflammation after injury, then, is to combine pleiotropic drugs such as PROG and VDH. Although the various gene regulatory and signaling mechanisms will be different, they will presumably synergize at the systemic level to inhibit T_H1 differentiation, reduce the acute phase, and potentially improve outcome and survival.

A very general approach to conceptualizing aging views it as a loss of systemic integration and adaptability that leads to a reduced ability to resist environmental perturbation, eventually leading to death (Lipsitz, 2004). This decline of complex (fractal) functioning in aging and disease has been observed in various systems, from cardiac rhythms and gait to cerebral autoregulation and large-scale brain network integration (Andrews-Hanna et al., 2007; Costa et al., 2002; Goldberger et al., 2002; Hausdorff et al., 2004; Lipsitz and Goldberger, 1992; Novak et al., 2004; Schulte-

Frohlinde et al., 2002). Due to its association with frailty and various other age-related diseases, D-deficiency appears to have a similar destabilizing effect (Topinkova, 2008; Villoslada et al., 2009).

PROG and especially VDH can then be viewed as system stabilizers, as is evident from their beneficial effects on processes that range from DNA stability and cell cycle control to systemic immune function and hormone production (Adorini and Penna, 2008; Chatterjee, 2001; Rammos et al., 2008). Since severe TBI is essentially a massive perturbation that leads to activation and probably extreme over-activation of defensive responses (as in the case of inflammation, which although beneficial in the short term can also be highly damaging when overactive or prolonged) the introduction of stabilizing factors may allow for a reduction of this early damage and an improved ability to recover (Villoslada et al., 2009). Although this model may be oversimplified, a top-down approach could be useful in conceptualizing notions of injury and treatment that can be combined with a bottom-up understanding of mechanisms to generate new hypotheses and potentially lead to new treatments (Brown, 2006; Villoslada et al., 2009).

8.4. CLOSING REMARKS AND FUTURE DIRECTIONS

Several general points emerge from this research. One is that TBI is not limited to the CNS, but is rather a systemic, "organismic" problem. Another is that multiple approaches and combinations of drugs may be necessary for the development of appropriate treatments for complex disorders such as TBI. Finally, while an understanding of specific disease mechanisms is clearly of fundamental importance, emergent properties like system behavior (or the phenomenological "injury" itself), which arise through non-linear complex interactions of individual mechanisms, should be given more attention in characterizing the systemic "disease" associated with a TBI. In predicting and "explaining" outcomes as well as in the treatment of TBI, translational research needs to take both mechanistic (bottom-up) and systemic (top-down) constructs into account simultaneously because the goals are different from those of pure bench research—translational research seeks more than a clarification or elucidation of knowledge. The goal is to provide an effective intervention at the systems level or, in other words, don't just treat the injury site—make the patient better. To quote Ludwig von Bertalanffy, one of the founders of modern systems theory: "Virchow's programme of 'cellular pathology,' claiming to resolve disease into functional disturbances of cells, is to be supplemented by the consideration of the organism-as-a-whole, as it appears clearly in such fields as theory of human constitutions, endocrinology, physical medicine and psychotherapy…Thus it seems necessary to expand our conceptual schemes if we wish to deal with these complex realms, and to make it possible for them to be included in the exact sciences" (Bertalanffy, 1950).

Our results indicate a number of directions for future research, but here we would like to outline only those that seem to be most important.

1. The long-term effects of D-deficiency in aged rats with TBI and PROG/VDH treatment need to be explored. In the studies presented here we focused on acute phase inflammation, which may be related to survival in the short term, but it is clearly of prime importance to elucidate the effects of D-deficiency on the development of injury and functional recovery in the long term.
2. A complete characterization of combination treatment effects needs to be performed, perhaps including different populations of animals (young/old, male/female, etc.) as well as more detailed dose-response curves for both drugs, or even different, more effective combinations than those used here.
3. An application of the issues discussed here (age, gender, D-deficiency, PROG/VDH combination) to the treatment of stroke. This is a potentially important issue since the human population most susceptible to stroke is also aged and likely to be D-deficient. Furthermore, since the injury process in stroke has many characteristics that are similar to those seen in TBI, combination therapy that proves to be effective in TBI could be beneficial in treating stroke as well.

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