NOTE TO USERS

The Pathophysiology of Equine Pituitary Pars Intermedia Dysfunction:

The role of dopaminergic neurodegeneration, oxidative stress and α-synuclein

A Thesis

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ABSTRACT

Equine pituitary pars intermedia dysfunction (PPID) is a common disease of aged horses. The pathogenesis of PPID is poorly understood, but a loss of dopaminergic inhibition of the pituitary pars intermedia is suggested. The goal of this thesis was to gain a better understanding of the pathologic mechanisms that result in PPID and to define factors that predispose horses to developing this condition. We hypothesized that PPID results from dopaminergic neurodegeneration of the periventricular dopaminergic neurons in response to accumulation of oxidatively damaged nerve terminal protein, α-synuclein; the same mechanism proposed in nigrostriatal dopaminergic neurodegeneration with Parkinson's disease. Immunohistochemical examination of pituitary and hypothalamic tissue demonstrated a loss of dopaminergic neurons in PPID affected horses compared to age-matched controls. In addition, 3-nitrotyrosine (an oxidative stress marker) and α-synuclein were increased and co-localized in the pars intermedia of horses with disease. These findings suggest a role for nitration of overexpressed α-synuclein in the pathogenesis of neurodegeneration in PPID. To assess whether horses developed PPID due to poor antioxidant defense mechanisms, biochemical assays were used to compare accumulation of oxidative damage to systemic and local (pituitary) antioxidant capacity. Accumulation of markers of oxidative stress was not associated with a deficiency in antioxidant capacity. However, we did observe an age-associated decrease in manganese superoxide dismutase (MnSOD) activity and a failure of MnSOD activity to increase in response to oxidative stress. These findings suggest MnSOD may contribute to disease susceptibility. In summary, evidence from our laboratory is consistent with earlier published data indicating PPID is due to a loss of dopaminergic inhibition and furthermore

suggests that PPID is a dopaminergic neurodegenerative disease. The role of oxidative stress and α-synclein expression in the pathophysiology of PPID is a new finding. These results suggest the pathogenesis of PPID may be mechanistically similar to that of Parkinson's disease.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
CLIP	corticotropin-like intermediate lobe peptide
CRH	corticotropin releasing hormone
DST	dexamethasone suppression test
β-end	beta endorphin
GnRH (R)	gonadotropin releasing hormone (receptor)
IHC	immunohistochemistry
α-MSH	alpha melanocyte stimulating hormone
MPTP	methyl-phenyl-tetrahydropyridine
(i) or (n)NOS	(inducible) or (neuronal) nitric oxide synthase
PCR	polymerase chain reaction
PD	Parkinson's disease
PI	pars intermedia
POMC	proopiomelanocortin
PPID	pituitary pars intermedia dysfunction
PVN	periventricular nucleus
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
TH	tyrosine hydroxylase
TRH (R)	thyrotropin releasing hormone (receptor)

CHAPTER 1:

INTRODUCTION:

Equine Pituitary Pars Intermedia Dysfunction and Parkinson's Disease, a shared mechanism of pathogenesis?

1.0 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID, equine Cushing's disease) is the most common endocrine disease of horses and ponies (Schott, 2002). Despite being first described more than 70 years ago (Pallaske, 1932), the etiology and pathophysiology of PPID remains poorly understood. The broad objectives of this thesis project were to develop a better understanding of the physiology of the equine pituitary pars intermedia, to investigate the pathologic mechanisms that result in PPID and to define factors that predispose horses to developing this condition.

1.1 The equine pituitary pars intermedia

The equine pituitary gland is comprised of distinct lobes (Figure 1), including the pars distalis (anterior lobe), pars nervosa (posterior lobe), pars tuberalis and a well developed pars intermedia (intermediate lobe). While the function of the pars distalis and pars nervosa are well known, the physiologic role of the pars intermedia and pars tuberalis are less well understood.

In the horse, the pars intermedia is comprised of a single endocrine cell type, the melanotrope (Schott, 2002). The melanotrope expresses the hormone precursor protein, proopiomelanocortin (POMC) (Saland, 2001). POMC is also expressed in the corticotropes of the pars distalis. However, due to differential post-translational processing by proteases called prohormone convertases, each cell type secretes a different complement of POMC-derived peptides (Figure 2) (Day et al., 1992). Due to the action of prohormone convertase I, POMC in corticotropes is primarily processed into adrenocorticotropin (ACTH). ACTH

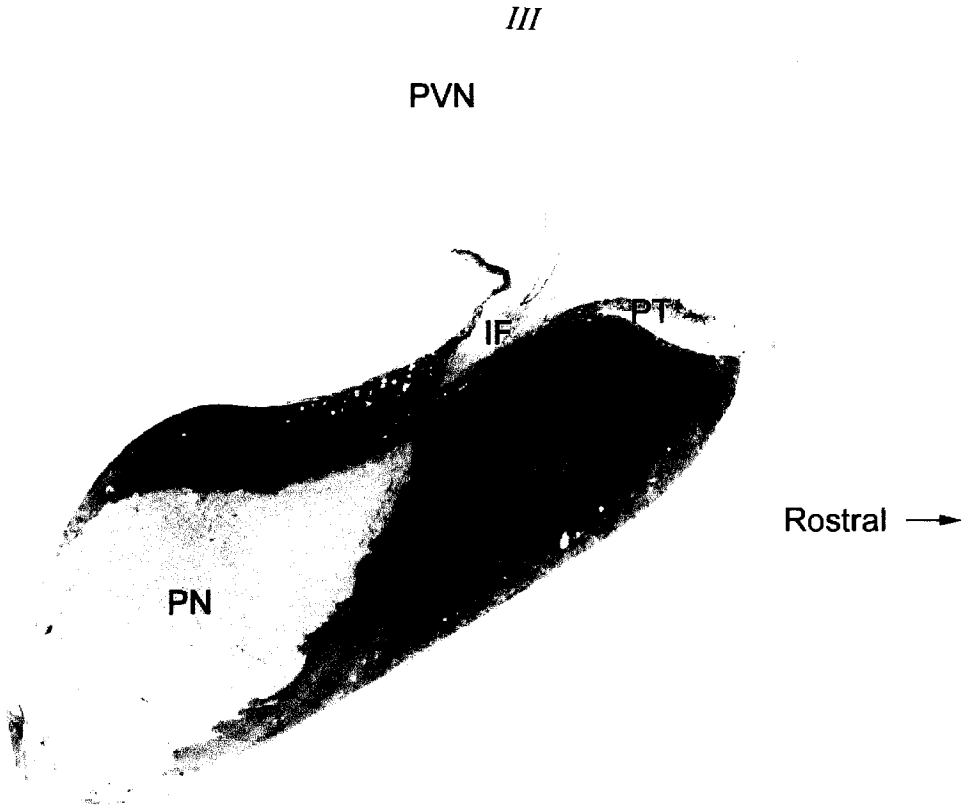

Figure 1: Saggital section of the equine pituitary gland and hypothalamus.

Pituitary section from a normal horse, stained with hematoxylin and eosin. The hypothalamus has been drawn by hand to illustrate the anatomical relationship between the pituitary and the brain. Abbreviations: PVN, periventricular nucleus; PD, pars distalis; PI, pars intermedia; PN, pars nervosa; PT, pars tuberalis; IF, infundibulum; III, third ventricle. (Drawing courtesy of David Sims)

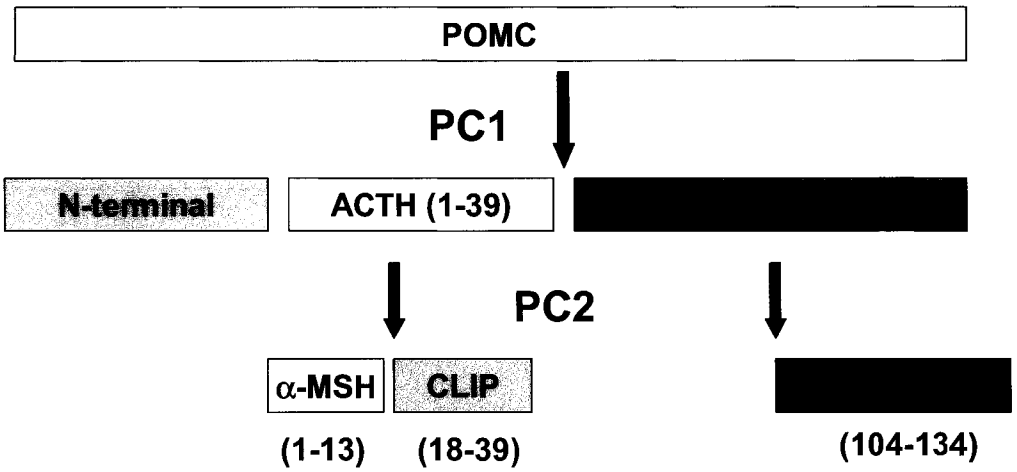

Figure 2: Cleavage products of proopiomelanocortin (POMC).

Products of proopiomelanocortin (POMC) cleavage are dependent on the presence of prohormone convertases I and II. In the corticotropes of the pars distalis only prohormone convertase I is present and the primary products of post translational processing of POMC are ACTH and β-lipotropin. Melanotropes of the pars intermedia have both prohormone convertase I and prohormone convertase II activity. The major products of POMC cleavage are therefore α–melanocyte stimulating hormone (α-MSH), corticotrophin-like intermediate lobe peptide (CLIP) and β-endorphin (β-END).

circulates to the adrenal cortex where it stimulates secretion of cortisol. Melanotropes contain active prohormone convertase I and II and therefore POMC in the pars intermedia (PI) is cleaved into the secretory peptides, α-melanocyte stimulating hormone (α–MSH), β-endorphins (β-end) and corticotropin-like intermediate lobe peptide (CLIP). A small amount of ACTH may also be produced. The physiologic role of the pars intermedia POMC derived-peptides in the horse has not been extensively studied.

Melanotropes are under inhibitory control by dopamine. Systemic administration of dopamine or dopamine agonists in horses results in decreased plasma concentrations of pars intermedia POMC-derived peptides (Orth et al.,1982). Dopamine in the pars intermedia is released directly from nerve terminals. These neurons originate in the periventricular nucleus of the hypothalamus, project through the infundibulum and terminate in the pars intermedia (Goudreau et al., 1995; Saland et al.,2001). Dopamine released from the nerve terminals interacts at dopamine (D2) receptors on the melanotropes to inhibit transcription of POMC and release of POMC-derived peptides (Cote et al., 1982; Kemppainen and Peterson, 1999). This dopaminergic pathway, known as the periventricular hypophyseal dopaminergic neuronal pathway, differs from the pathway that regulates prolactin release, the tuberinfundibular dopaminergic neuronal pathway (DeMaria et al., 1999). These latter neurons originate in the arcuate nucleus and terminate at the median eminence where they release dopamine into the hypophyseal portal system for delivery to the pars distalis lactotropes (DeMaria et al., 1999). Although many of the endocrine cells are regulated by both releasing and inhibitory factors, releasing factors for equine melanotropes have not been previously identified.

1.2 Equine pituitary pars intermedia dysfunction

Equine pituitary pars intermedia dysfunction (PPID), previously referred to as equine Cushing's disease, is a naturally occurring, progressive condition of aged horses and ponies. Although a prospective epidemiologic study of PPID is lacking, the literature (case series and reports) suggests an increased prevalence of disease in ponies and Morgan horses (reviewed in Schott, 2002). The disease is most common in aged (>15 years) horses, although animals as young as 7 years have been affected (Orth et al., 1982). Although early reports suggested mares were affected more frequently than males (Heinrichs et al., 1990), data accumulated more recently has not confirmed this finding. In 199 PPID horses described in 7 case series, genders were approximately equally distributed (106 mares, 93 males) (van der Kolk et al., 2004).

Prevalence of PPID in the general or aged equine population has not been established. Early estimates were based on casual observation of hirsute (abnormal curly hair coat) horses in general (0.075%, Evans, 1972) or referral (0.5%, van der Kolk et al., 1995) equine practices. However, hirsutism is considered to be a late change in horses with PPID. It has been estimated that horses may have disease as long as 4 years before developing clinical signs (Field and Wolf, 1988). Therefore determination of prevalence based only on the presence of a single clinical sign may greatly underestimate disease frequency. Several recent studies suggest a higher prevalence of PPID in horses, many without clinical signs. In a study of 40 adult horses with laminitis (inflammation of the sensitive lamina that attaches the hoof wall to the third phalanx) of unknown etiology, 50% had increased plasma ACTH concentration (Donaldson et al., 2004). ACTH does not typically increase with pain or disease, suggesting these horses had laminitis as a sequelea

of a primary endocrinopathy (Couetil et al., 1996; Xie et al., 2001). In a Japanese study, 3% of Thoroughbred mares (n= 165, 17 years or older) with no clinical evidence of disease had an adenoma or adenomatous hyperplasia detected during necropsy (Okada et al., 1997). In a recent study of 44 randomly selected animals, prevalence of PPID was 36% in all aged horses and 50% in horses older than 11 years of age, based on presence of a PI mass (Andrews et al., 2004). An epidemiological study of a large population of randomly selected horses and ponies of various breeds is needed to determine the incidence and prevalence of PPID.

Clinical signs of PPID contribute to generalized debilitation of affected animals. Signs include hirsutism, laminitis, weight loss, lethargy, infertility, polydipsia and polyuria. Hirsutism, often considered pathognomonic for PPID, has been cited in several case reports as the most common clinical sign of disease (reviewed in Schott, 2002). This is likely biased by the ease with which this clinical abnormality is recognized and the non-specific nature of the other clinical signs of disease. Approximately 50- 80% of affected horses develop laminitis, often necessitating euthanasia (Schott et al., 2001, Hillyer et al., 1992). PPID also causes altered energy metabolism and may result in muscle wasting, weight loss, or abnormal fat distribution. Horses with PPID often develop insulin resistance (Garcia and Beech, 1986; Reeves et al., 2001). Although true diabetes mellitus (β-cell depletion) is uncommon, hyperglycemia and hyperinsulinemia are frequent findings in horses with PPID (Hillyer et al., 1992; van der Kolk et al., 1995). Alteration in immune function of affected horses increases the risk of infections. Susceptibility to both parasitic and bacterial infections results in high incidence of endoparasitism, sinusitis, dermatitis,

and pneumonia in horses with PPID (Heinrichs et al., 1990; Hillyer et al.,1992; van der Kolk et al., 1993; Couetil et al., 1996).

Most hematological and biochemical parameters are not altered in PPID. The only common abnormality is hyperglycemia, present in 50-90% of affected horses (Hillyer et al., 1992; Love, 1993; van der Kolk et al., 1995). Mature neutrophilia, elevated liver enzymes, hypercholesterolemia and hypertriglyceridemia occur infrequently, as horses are relatively resistant to steroid hepatopathy and fatty liver (Cohen and Carter, 1992).

1.3 Diagnostic testing

Although the clinical signs of this disease are well recognized, ante mortem diagnosis of PPID is problematic, with all available diagnostic tests having limitations. In aged horses with advanced disease, the presence of clinical signs including hirsutism, laminitis, muscle atrophy, abnormal fat distribution, polydipsa and polyuria, and secondary infections may be sufficient for diagnosis (Schott, 2002). However, if treatment is to be pursued, testing is warranted as a tool to monitor response to therapy and to allow individualized titration of drug dose (Donaldson et al., 2002; Perkins et al., 2002). In horses with early disease, the clinical signs overlap with those of aging, making disease recognition difficult. Diagnostic confirmation of disease is therefore necessary.

The overnight dexamethasone suppression test (DST) is considered the "gold standard" method of ante mortem PPID diagnosis (Dybdal et al., 1994). In the unaffected horse, intramuscular administration of dexamethasone decreases the release of ACTH from the pars distalis, resulting in a serum cortisol concentration of less than 1 μg/ dl 19 hours later (Dybdal et al., 1994). Horses with PPID fail to suppress serum cortisol concentration,

presumably due to ACTH production from the pars intermedia (Figure 3). Originally this test was reported to have a sensitivity and specificity of 100% (Dybdal et al., 1994). However, a recent report suggests the reliability of the test has been over-estimated (Meisner et al., 2003). In addition, seasonal variation in response to dexamethasone has been documented in normal horses (Donaldson et al., 2005). Clinically normal ponies and horses had a normal DST in spring but when the same animals were tested in the fall, they failed to suppress. Although it has not been critically assessed, a loss of feedback inhibition by glucocorticoid may be a late event in the disease progression, and the high sensitivity originally reported may reflect a case selection bias towards horses with advanced disease. Other limitations of the dexamethasone suppression test include the need for multiple day sampling and the potential exacerbation of laminitis in horses with current or historical laminitis (Dybdal et al., 1994; Schott, 2002).

Thyrotropin releasing hormone (TRH) stimulation is a second functional test used for diagnosis of PPID. An increase (30-66%) in serum cortisol concentration 15-90 minutes following TRH administration is considered diagnostic for PPID. Normal horses lack this response (Beech and Garcia, 1985; Schott, 2002). The performance of this test has not been critically evaluated in a large number of horses, particularly those with early disease. Further, this test is currently impractical due to the lack of approved TRH for use in the horse and the high price of human-approved products. The mechanism of this response has not been established. We hypothesized TRH is a releasing factor of the equine pars intermedia and the increase in cortisol observed in affected horses is the result of release from the hyperplastic PI. Chapter 4 presents data from experiments testing this hypothesis.

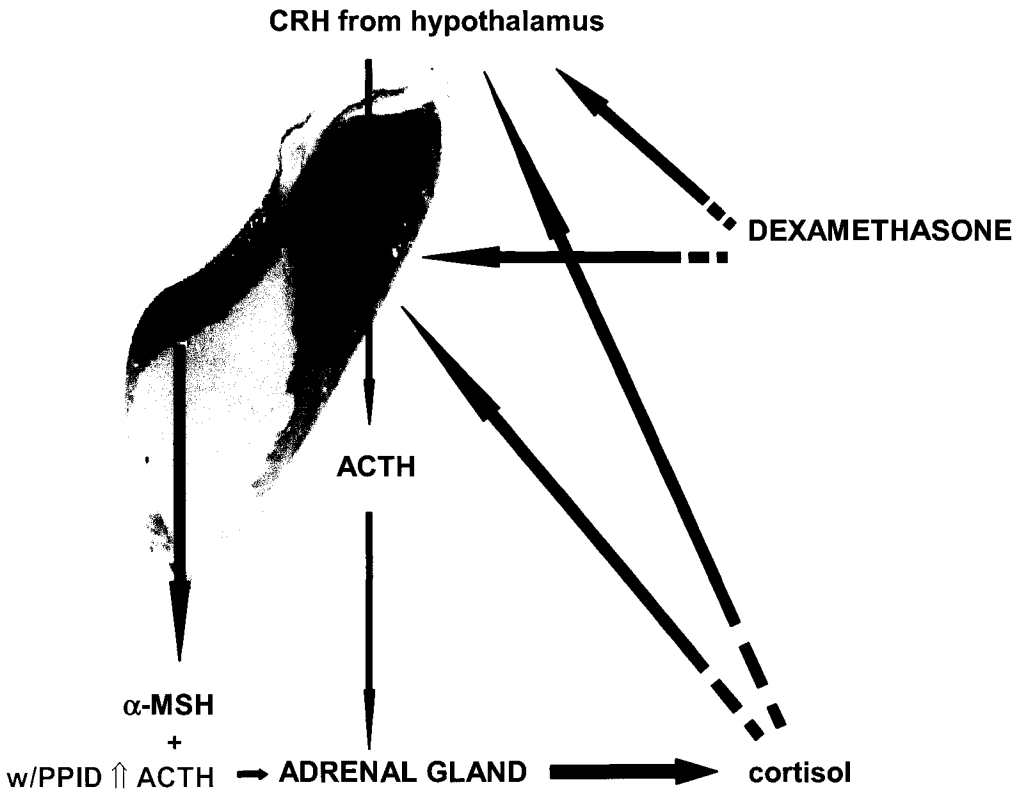

Figure 3: Cortisol and dexamethasone inhibition of ACTH release from the pituitary.

ACTH is a normal product of the pars distalis (PD) while α-MSH is a normal product of the pars intermedia (PI). In the healthy animal, ACTH released from the PD stimulates the adrenal gland to release cortisol. Endogenous cortisol and exogenous dexamethasone feedback on both the hypothalamus and the corticotropes of the PD to decrease corticotrophin releasing hormone (CRH) and ACTH release. This results in a suppression of serum cortisol concentration. In horses with PPID plasma ACTH is increased due to PI production. ACTH production in the PI is not regulated by cortisol feedback, therefore horses with PPID fail to suppress cortisol when administered exogenous dexamethasone. Red arrows are inhibitory.

Measurement of endogenous concentration of ACTH is also considered useful in the diagnosis of PPID. ACTH plasma concentration determined by radioimmunoassay was reported to have a sensitivity of 82-90% and a specificity of 90-100% in the diagnosis of PPID when overnight dexamethasone suppression was used as the reference test (Couetil et al., 1996; Donaldson et al., 2002, Perkins et al., 2002; Horowitz et al., 2003). Again, the utility of endogenous ACTH concentrations in diagnosis of early PPID is unclear and seasonal variation has been reported with false positive test results occurring frequently in the fall (Donaldson et al., 2005). Limitations of endogenous ACTH measurement include the need to process samples within 3 hours of collection and ship samples frozen, ideally on dry ice (Couetil et al., 1996).

Measurement of endogenous concentrations of other POMC peptides, such as α-MSH and β-endorphin, has been investigated as a diagnostic tool for PPID (Horowitz et al., 2003). Measurement of α-MSH and β-endorphin were found to have similar diagnostic power as measurement of ACTH (Horowitz et al., 2003). All three assays had a sensitivity and specificity of 85-90% when the overnight dexamethasone suppression test was used to diagnose PPID. We validated a commercial radioimmunoassay for α-MSH for use in the horse and assessed the influence of sample collection and handling in hormone measurement variation (Chapter 2).

Horses with PPID are typically insulin resistant and have an increased fasting serum insulin concentration (Garcia and Beech, 1986; Reeves et al., 2001). Measurement of serum insulin concentration has been investigated as a potential diagnostic test for PPID. In a study of 12 horses with histologically confirmed PPID, measurement of fasting insulin had a 92% sensitivity in diagnosing disease, using cut-off values (> 57 μU/ml) determined

in 7 apparently healthy horses (van der Kolk et al., 1995). Results from two other studies however revealed a high rate of both false positives and negatives. In a study of laminitic ponies with hyperinsulinemia, 7 of 8 had combined dexamethasone suppression / thyrotropin releasing hormone stimulation tests non-supportive of PPID (Reeves et al, 2001). In a larger study, 8 of 30 horses with clinical signs of PPID and supportive dexamethasone suppression test results had normal fasting serum insulin concentrations (Schott, 2002). Therefore, fasting serum insulin concentrations as a single endocrine test is of little value in the diagnosis of PPID.

Sensitivity of the combined dexamethasone suppression/ TRH stimulation test has recently been critically evaluated in 44 horses using histology to confirm diagnosis status (Andrew et al., 2004). Disease was defined as the presence of a discrete mass in the pars intermedia. Horses were included into the study based on availability (horses donated during the time span of the project). This differs from previous studies in which inclusion was based on presence or absence of clinical signs of disease. The more random method of selection used in this study is more appropriate for determining performance of a diagnostic test. Sensitivity of the combined test was reported as 88%, specificity as 79%.

Several testing strategies designed to assess cortisol regulation have been investigated for diagnostic usefulness. Measurement of endogenous serum cortisol concentration has been shown repeatedly to be of no value in diagnosis of PPID. Serum cortisol concentration is typically within normal reference ranges in horses with PPID and serum cortisol concentration may be affected by many physiologic and pathologic conditions, such as exercise, fasting, disease or stress (Heinrichs et al., 1990; Dybdal et al., 1994; van der Kolk et al., 2001). Circadian rhythm of cortisol has been shown to be

abolished by disease (Dybdal et al., 1994). It has been suggested that evaluation of cortisol fluctuation may be useful in identifying PPID. Demonstration of less than a 30% difference in morning (09:00) and afternoon (16:00) serum cortisol concentration has been promoted for diagnosis of disease, however this test has not been validated (Douglas, 1999). Urinary cortisol:creatinine ratios were compared in 12 normal, 13 PPID and 8 grass sickness affected horses (Chandler and Dixon, 2002). Using a cut-off value of 6.9×10^6, cortisol:creatinine ratios had a sensitivity of 92.3% and specificity of 75% in differentiating PPID from normal horses, indicating this may be a useful assay for identifying PPID. However, the test did not perform well in differentiating horses affected with PPID from those with grass sickness, suggesting its use may be limited by the presence of non-endocrine disease. ACTH stimulation of cortisol has also been shown to be of minimal clinical value (Dybdal et al., 1994). This is not unexpected as most horses with PPID do not have adrenal hyperplasia (Heinrichs et al., 1990; Boujon et al., 1993; Okada et al., 1997).

Recent efforts to validate ante mortem diagnostic tests for identification of horses with early PPID often use histology as a "gold standard" for diagnosis (Andrews et al., 2004; Dybdal et al., 1994). However, the histological changes associated with healthy aging, physiological stimulation or early PPID are not well defined. Also, accuracy of postmortem diagnosis in horses with early disease has not been previously evaluated. Therefore, we evaluated the degree of concordance among seven veterinary pathologists in interpretation of pituitary sections from ten aged horses with mild clinical signs of PPID to determine if histological assessment is valid as a gold standard for diagnosis (Chapter 3).

1.4 Pathology and histology

PPID is characterized by enlargement of the pituitary gland due to hyperplasia, hypertrophy and adenoma formation of the pars intermedia of the pituitary. Pituitary glands from horses with PPID are enlarged as much as 5 times the normal size (van der Kolk et al., 2004). This enlargement is due to expansion of the pars intermedia. The expansion, when severe, compresses adjacent lobes and the hypothalamus resulting in functional loss of adjacent tissue. The increase in size of the PI is associated with increased expression and secretion of pars intermedia POMC-derived peptides (Orth et al., 1982). Increased POMC peptide concentrations are detectable in the pars intermedia tissue as well as peripheral blood of horses with PPID (Wilson et al., 1982).

Microscopic findings in the pars intermedia of horses with disease include adenomatous hyperplasia and hypertrophy of melanotropes with well developed rough endoplasmic reticulum and Golgi apparatus reminiscent of an activated pars intermedia from a normal horse (Heinrichs 1990; Boujon 1993). The melanotropes are frequently spindle or polyhedral shaped and organized into nodules, nests or clusters by fine septa of connective tissue. Nuclei are pleomorphic, mitotic figures uncommon. Hemosiderin-laden macrophages and lipofuscin accumulation are common in the adjacent pars nervosa (Figure 4).

1.5 Pathophysiology

Two general pathologic models have been proposed to explain PPID. First, it has been suggested PPID results from spontaneous formation of a pars intermedia adenoma. In this model, the melanotrope, the endocrine cell in the equine pars intermedia, is the site

of origin of disease. Alternatively, PPID may be primarily a hypothalamic disease, resulting in loss of dopaminergic inhibition of the pars intermedia. In this model, adenoma formation is not due to a primary change in the melanotrope, but rather a loss of an external inhibitory factor, dopamine. While there have been only a few studies investigating the pathology of PPID, what has been reported suggests it is of a hypothalamic origin. Melanotropes are under inhibitory control by dopamine released directly in the pars intermedia from local nerve terminals (Kamppainen et al., 1989; Saland et al., 2001). These neurons originate in the periventricular nucleus of the hypothalamus, adjacent to the third ventricle (Luppi et al., 1986; Goudreau et al., 1995). Dopamine inhibits the secretion of POMC-derived peptides through interaction at D2 receptors on the melanotropes (Saiardi and Borrelli, 1998). Surgical disruption of the periventricular hypophyseal dopaminergic tracts in rats results in increased pars intermedia melanotrope expression of POMC peptides (Goudreau et al., 1995). In addition, D2 receptor knockout mice develop pars intermedia lesions similar to PPID (Saiardi et al., 1998). Studies in horses also suggest a central role for loss of dopamine inhibition in PPID. Loss of dopaminergic neurotransmission results in decreased concentration of the neurotransmitter dopamine and its metabolites, 3,4 dihydroxyphenylacetate and homovanillic acid in the nerve terminals. Dopamine and its metabolites can be measured either by high performance liquid chromatography or gas chromatography/ mass spectrophotometry (Meineke et al., 1989; Hows et al., 2004). Using HPLC, Millington et al. demonstrated a 9-fold decrease in dopamine and dopamine metabolites in the pars intermedia of horses with PPID compared to age matched controls (Millington et al., 1988). Orth et al. showed systemic administration of dopamine or a dopamine agonist to horses with PPID resulted

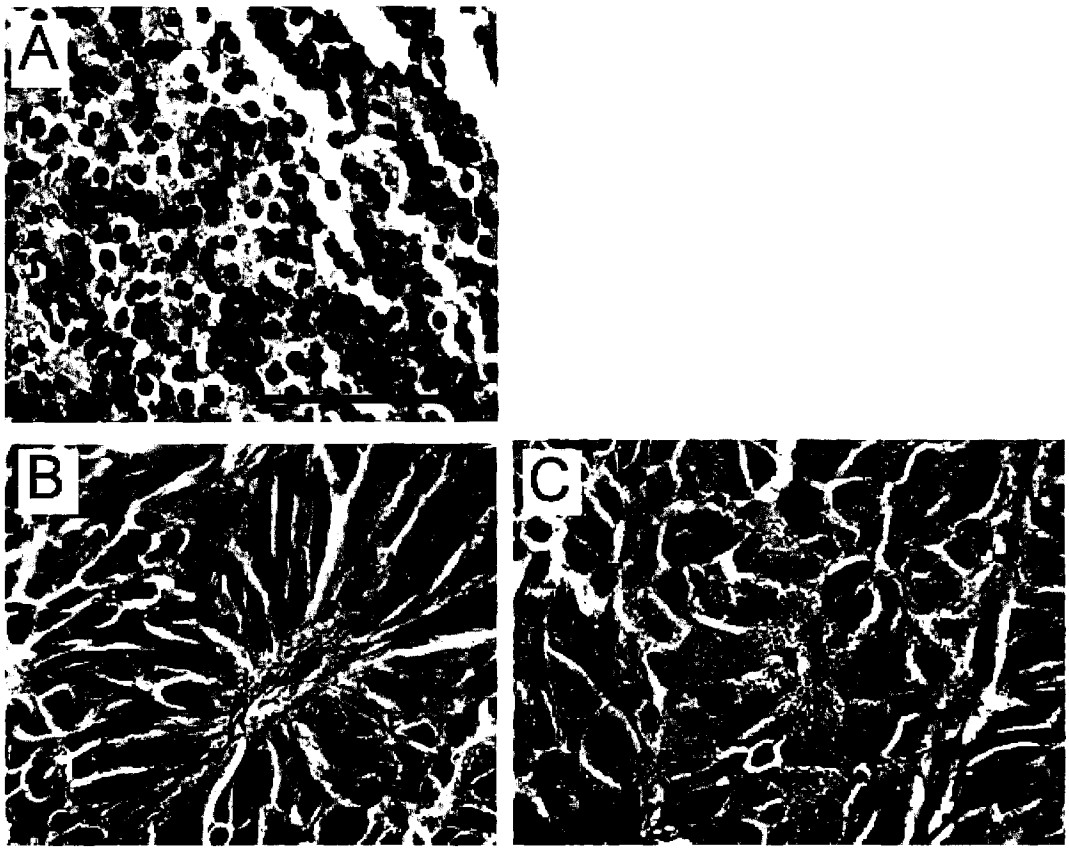

Figure 4: Histology of pars intermedia from normal and PPID horse.

(A) Pars intermedia from healthy, 15 year old horse. Cytoplasm of melanotropes is lightly to non- stained and nuclei are of similar size between cells. (B) Pars intermedia from affected horse, at same magnification as in A. Note the spindle shaped cells, organized in a nest or rosette around a venule. Cytoplasm of melanotropes stained intensely with eosin. C) Pars intermedia of affected horse at same magnification as in (A). Note the hypertrophic melanocytes, variation in size of nuclei, and acidophilic stained cytoplasm, all reminiscent of activated endocrine cells. BAR= 30μm. All sections hematoxylin and eosin stained.

in a decrease in plasma concentration of POMC peptides (Orth et al., 1982). A number of investigators have reported that horses treated with the dopamine agonist pergolide show improvement in both clinical signs and biochemical abnormalities associated with disease (Watson et al., 1998; Schott et al., 2001; Donaldson et al., 2002; Perkins et al., 2002). Based on these studies, we hypothesized equine PPID results from a loss of inhibition of the pars intermedia due to degeneration of the periventricular hypophyseal dopaminergic neurons.

Immunohistochemistry is a second method routinely employed to demonstrate dopaminergic neurodegeneration in brain sections from other species. Antibodies directed against tyrosine hydroxylase (the rate limiting enzyme in dopamine synthesis), dopamine transporter or vesicular monoamine transporter can be used to quantify dopaminergic neuronal density (Pickel et al., 1975; Ciliax et al., 1995; Miller et al.,1999). Immunohistochemistry was used to quantify tyrosine hydroxylase immunoreactive nerve terminals and cell bodies in the periventricular neurons to assess dopaminergic neurodegeneration in PPID in this study (Chapter 5).

1.6 Parkinson's disease

Dopaminergic neurodegeneration is associated with several diseases in other species, most notably Parkinson's disease. Parkinson's disease is an age-associated neurodegenerative condition that affects 1 - 3% of the human population older than age 65, and 10% over 80 (Beal, 2001). The clinical signs are motor dysfunction including bradykinesia, muscle rigidity, postural instability and resting tremors. Parkinson's disease is a progressive condition, for which no curative treatment is available. While drugs can

provide symptomatic relief, their benefits are short lived, side-effects many and they do not prevent disease progression (Weiner, 2004). Characteristic pathological features of Parkinson's disease (PD) are degeneration of the dopaminergic nigrostriatal neurons and intraneuronal cytoplasmic inclusions known as Lewy bodies and Lewy neurites (Beal, 2001). Despite aggressive research efforts, the precise cellular and molecular events that result in the development of dopaminergic neuronal degeneration remain poorly understood. Factors suggested to be important include oxidative stress, mitochondrial dysfunction, ubiquitin- proteosome pathway dysfunction and α-synuclein overexpression, nitration and aggregation.

1.7 Dopaminergic neurodegeneration and oxidative stress

Oxidative stress is a condition in which toxic compounds formed from oxygen (reactive oxygen species, ROS) damage cells by disrupting cell membranes, proteins and DNA (reviewed in Kohen and Nyska, 2002). ROS are oxygen derived radicals, compounds that possess an unpaired electron and therefore are highly reactive. Important biological oxygen metabolites include superoxide ion radical ($O^{\bullet}_{2}^{-}$), nitric oxide radical ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2). Although these metabolites may damage cells directly, they are poorly reactive and not responsible for extensive cellular damage. However, indirectly they are more deleterious because they serve as sources for highly reactive intermediates. One of the most important reactions that occur in physiological conditions is the interaction of superoxide and nitric oxide radicals to form the highly reactive intermediate, peroxynitrite ($ONOO^-$). Another biologically critical reaction is the

Fenton reaction. This converts hydrogen peroxide to the extremely reactive hydroxyl radical ($OH^{\cdot}$) by transfer of an electron from reduced iron (Fe^{+2}) (Figure 5).

ROS are produced constitutively by the mitochondria as a consequence of normal oxidative metabolism. ROS production is increased following exposure to toxins and inflammation (Kohen and Nyska, 2002). Cells are protected from ROS by the co-existence of an antioxidant system comprised of both antioxidant enzymes and non-enzymatic antioxidants. Oxidative stress occurs when there is an imbalance between ROS exposure and antioxidant function, either due to increase production of free radicals or a decrease in antioxidant capacity (Figure 5).

Accumulation of oxidatively damaged cell components is considered to be an important contributing factor in the development of neurodegeneration. The brain is particularly susceptible to oxidative stress due to its high oxygen consumption, high concentration of iron (a catalyst in the Fenton reaction), high concentrations of oxidation substrates (such as cellular membranes and catecholamines) and low levels of antioxidant enzymes (Andersen, 2004). There is abundant evidence to support a role for oxidative stress in the pathogenesis of neurodegenerative diseases (Calabrese et al., 2000).

Numerous findings suggest a role for oxidative stress in the pathogenesis of Parkinson's disease. Postmortem examination of brains from PD patients reveals an accumulation of oxidatively damaged lipid breakdown products, such as 4-hydroxynonenal (Yoritaka et al., 1996), and of oxidatively damaged proteins, both nitrited (Good et al., 1998) and carbonylated (Floor and Wetzel, 1998). Antioxidants such as glutathione, glutathione peroxidase and superoxide dismutase may be decreased in brains from PD patients (Fahn and Cohen, 1992; Schulz et al., 2000; Kunikowska and Jenner, 2003).

Conversely, in some PD patients, manganese superoxide dismutase activities increase, possibly in response to ROS exposure (Saggu et al., 1989). Contributors to oxidative stress found in the brains of PD patients include an increase in iron concentration (Dexter et al., 1989) and a decrease in substantia nigra mitochondrial complex I activity (Schapira et al., 1990). Together, these events would act synergistically to induce oxidative damage.

High oxygen consumption in the brain generates high concentrations of superoxide radicals. A decrease in mitochondrial function, as occurs in Parkinson's disease (described below), further increases intracellular superoxide radical production. Superoxide, in the presence of nitric oxide, is rapidly converted to peroxynitrite, an extremely reactive intermediate. In addition, superoxide dismutase activity metabolizes the superoxide radical to hydrogen peroxide. Hydrogen peroxide is typically metabolized to water in the brain by the glutathione antioxidant system. In a state of glutathione and glutathione peroxidase deficiency, as occurs in the substantia nigra in Parkinson's disease, hydrogen peroxide will accumulate (Schulz et al., 2000). Hydrogen peroxide, in the presence of high concentrations of iron, is converted through the Fenton reaction to hydroxyl radicals, an extremely reactive and damaging intermediate. Thus, in patients with Parkinson's disease, the substantia nigra has all the right perturbations to put it at high risk for damage secondary to oxidative stress. Further evidence for the role of oxidative stress in dopaminergic neurodegeneration has been obtained in animal models of PD. Several chemicals when administered either systemically or directly into the substantia nigra or striatum of rodents produce pathological changes similar to those seen in PD. These chemicals include 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP, described

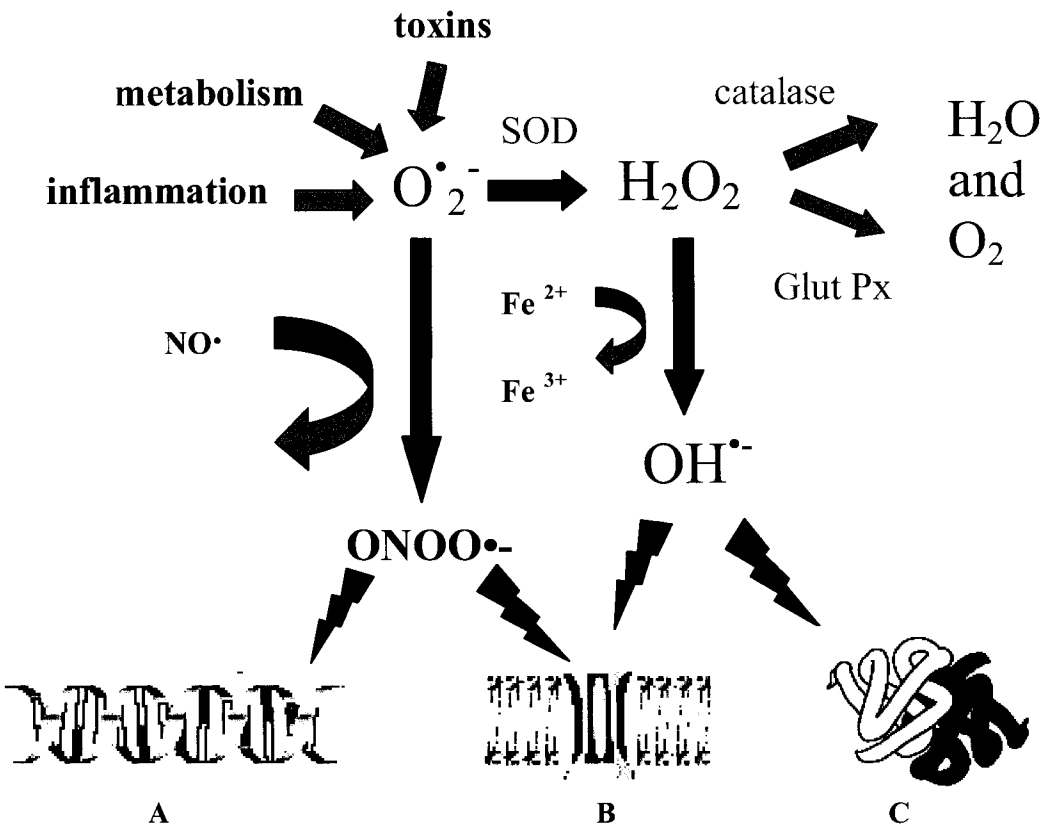

Figure 5: Oxidative stress pathway of cellular damage.

Oxidative stress is the result of imbalance between antioxidant capacity and exposure to reactive oxygen species (ROS). ROS is produced by mitochondria during normal aerobic metabolism. Metabolism of dopamine also results in generation of ROS. Pathologic conditions may increase ROS, such as systemic metabolic disease, mitochondrial dysfunction, exposure to toxins and inflammation. When the capacity of the antioxidant system is exceeded, reactive intermediates such as peroxynitrite ($ONOO^-$) and hydroxyl radicals (OH^-) can damage DNA (A), cell membrane lipids (B), and proteins (C). Abbreviations: superoxide dismutase (SOD), glutathione peroxidase (Glut px)

below), 6- hydroxydopamine, dopamine, and free 3-nitrotyrosine (Beal, 2001; Mihm et al., 2001). Administration of these neurotoxins results in dopaminergic neurodegeneration of the substantia nigra with decreased dopamine concentration at the striatum. Treated animals develop motor abnormalities similar to PD. Animal models have been used extensively to study the factors that protect or promote neurodegeneration following toxin exposure. Animals genetically altered to overexpress antioxidant enzymes, or animals pretreated with antioxidants are protected from neurodegeneration following exposure to the chemical neurotoxins (Przedborski et al., 1992; Fallon et al., 1997). Conversely, animals with mutations in the genes for manganese superoxide dismutase or glutathione peroxidase exhibit increased susceptibility for dopaminergic neurodegeneration following exposure to the oxidative stress inducing neurotoxin, MPTP (Klivenyi et al., 2000; Andreassen et al., 2001). The protective effect of antioxidants and deleterious effect of antioxidants loss in animal models of PD supports a role for oxidative stress in disease.

Interaction of the superoxide radical with nitric oxide, as described above (Figure 5), yields the strong radical peroxynitrite. Peroxynitrite can damage cells by at least two different mechanisms. Peroxynitrite can be further converted into the strong hydroxyl radical or it can transfer a nitrosyl group to proteins, catechols or other cellular components, damaging them directly. 3-nitrotyrosine, the product of nitration of tyrosine containing proteins, accumulates in brain tissues from Parkinson's patients (Good et al., 1998). Activity of nitric oxide synthase (NOS), the enzyme responsible for nitric oxide production, has been shown to contribute to neuronal death in animal models for Parkinson's disease (Schultz et al., 1995; Przedborski et al., 1996; Liberatore et al., 1999; Zhang et al., 2000). Inhibitors of nNOS (neuronal NOS) protect mice from MPTP induced dopamine loss and

dopaminergic neurodegeneration (Schultz et al., 1995; Przedborski et al., 1996). nNOS and iNOS (inducible) knockout mice are protected from MPTP (Przedborski et al., 1996; Liberatore et al., 1999). Drugs, such as minocycline, which decrease gliosis and subsequent iNOS upregulation also protect against MPTP induced striatial dopamine loss and dopaminergic neurodegeneration (Du et al., 2001). These findings suggest a role for nitric oxide in PD.

One reason the dopaminergic system is particularly susceptible to oxidative stress is dopamine itself. Dopamine can react through several mechanisms to produce oxygen radicals. Dopamine can react non-enzymatically with oxygen to form quinones, semi-quinones, hydrogen peroxide and oxygen radicals, all of which are directly or indirectly toxic to the cell (Luo and Roth, 2000). Oxygen radicals can be further modified to peroxynitrite in the presence of nitric oxide, which is increased in both clinical cases and animal models of PD (Good et al., 1998; Liberatore et al., 1999). In addition, the enzymatic degradation of dopamine by monoamine oxidase B produces hydrogen peroxide as a by-product. Hydrogen peroxide can form hydroxyl radicals in the presence of reduced iron. Neuromelanin, which is abundant in the substantia nigra, enhances hydroxyl radical production by reducing iron and thereby facilitating the Fenton reaction (Youdim et al., 1989).

Because dopamine is cytotoxic, neurons are equipped with several mechanisms to maintain cytosolic dopamine within a safe concentration range. Dopamine can be stored in vesicles, metabolized by monoamine oxidase to dihydroxyphenyl acetic acid (DOPAC), or transported out of the cell via reverse transport using the dopamine transporter (Sulzer, 2001). When cytosolic dopamine concentrations exceed the elimination pathways'

capacity, dopamine accumulates. Excessive dopamine can be stored as the pigment neuromelanin. Neuromelanin first appears in the substantia nigra of humans within three years of birth and increases with age (Sulzer et al., 2000). Neuromelanin is comprised of dopamine, its metabolites, dopamine quinones and semi-quinones, iron and proteins all contained in a two- layer membrane enclosed, intracellular vesicle (Sulzer et al., 2000). In the presence of oxygen radicals or iron, intraneuronal dopamine forms dopamine quinones. Quinones are cytotoxic intermediates that produce reactive oxygen species through redox cycling to semiquinones. Quinones can also be incorporated in neuromelanin. The sequestering of dopamine, its metabolites and dopamine quinones serves to protect the cell from the cytotoxicity of all of these compounds. Therefore, it is likely that an imbalance in any part of the protective system results in dopamine accumulation and cytotoxicity.

We hypothesized horses develop dopaminergic neurodegeneration of the periventricular neurons secondary to oxidative stress due to a systemic or local (pars intermedia) decrease in antioxidant capacity. Experiments were designed to test this hypothesis (Chapters 4 and 5).

1.8 Mitochondrial Dysfunction

The first evidence that mitochondrial dysfunction contributes to development of PD resulted from the investigation of a cluster of several young heroin users who developed clinical signs of disease in 1982. It was discovered they had inadvertently been exposed to a contaminant in synthetic heroin, 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP) (Langston et al., 1983). MPTP crosses the blood brain barrier, where it is converted to 1-methyl- 4-phenylpyridinium (MPP+) in glial cells by monoamine oxidase B (MOA-B,

Figure 6). MPP+ is selectively taken into dopaminergic neurons by the dopamine transporter. MPP+ inhibits the activity of the enzyme NADH-CoQ reductase, a component of complex I of mitochondrial oxidative metabolism. By inhibiting mitochondria function, energy (in the form of ATP production) decreases and oxygen radical production increases (Beal, 2001). Cell death follows.

The discovery that MPTP had the ability to induce Parkinson's-like signs in humans led to its use in animal models of PD. MPTP administered to rodents and non-human primates results in clinical signs that mimic PD (Beal, 2001). Histological examination shows dopaminergic neuronal loss in the substantia nigra following acute or chronic MPTP administration. The synthetic chemical, MPTP, and its metabolite, MPP+, are not found in the environment, and therefore are unlikely causes of Parkinson's disease. However, other chemicals with similar properties are environmental contaminants such as the pesticide rotenone and the herbicide paraquat. These chemicals, alone or in

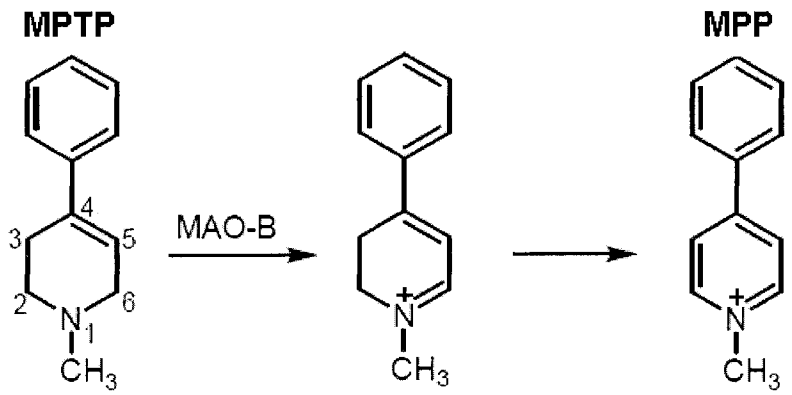

Figure 6: The structure of MPTP and MPP

combination with other agricultural chemicals, have been found to induce Parkinson's–like symptoms in animals (Betarbet et al., 2000; Thiruchelvam et al., 2002). This finding led to the hypothesis that exposure to pesticides may increase risk of Parkinson's disease. This is supported by epidemiological data linking PD to rural living, farming, drinking well water, and occupational exposure to agricultural chemicals (Rajput et al., 1987; Semchuk et al., 1992; Gorell et al., 1998).

The role of mitochondrial dysfunction in development of disease has been explored using specialized cell lines known as cybrids. Cybrids are created by transplanting mitochondrial DNA from an affected donor into neuronal cell lines depleted of their native mitochondria. Cybrids have a uniform genetic (nuclear) and environmental background and differ only in mitochondria DNA. Therefore, differences between cybrid lines in growth characteristics or response to experimental treatments may be attributed to differences in mitochondrial DNA. Cybrids created using mitochondrial DNA from patients with sporadic PD have a decrease in complex 1 activity, increased ROS production and increased antioxidant activity compared to cybrids created with mitochondrial DNA from non-affected individuals (Gu et al., 1998). These cybrids also spontaneously develop inclusion bodies mimicking Lewy bodies (described below) in both composition and structure (Trimmer et al., 2004). These experiments provide evidence that mitochondrial defects alone can invoke oxidative stress and inclusion body development similar to that occuring in Parkinson's disease. The role of mitochondria in development of disease is supported by identification of defects in complex I activity in both the brain (Schapira et al., 1990) and platelets (Parker et al., 1989) from PD patients. In addition,

mitochondrial DNA polymorphisms have been identified that are significantly associated with risk of disease (Smigrodzki et al., 2004).

1.9 Familial Parkinson's Disease

Most cases of PD are "idiopathic" or "sporadic" and are considered to be the result of the cumulative effect of genetic and environment factors, as discussed. However, recently several rare autosomal mutations have been identified that are linked to inheritance of Parkinson's disease in predisposed families. These mutations have provided important clues to the pathogenesis of PD. Two mutations in the α–synuclein gene (A30P, A53T) have been identified (Polymeropoulos et al., 1997). Additionally, a family with inherited Parkinson's disease was discovered to have triplication (three additional copies) of the gene for α–synuclein and a 2-fold increase in α–synuclein protein expression (Singleton et al., 2003; Miller et al., 2004). Parkinson's disease is also associated with mutations in the parkin and ubiquitin C terminal hydrolase genes (Kitada et al., 1998; Leroy et al., 1998). These proteins are both involved in the ubiquitin/ proteasome pathway that clears damaged proteins from cells. The discovery of these mutations highlights the importance of α–synuclein, and the ubiquitin-proteasome pathway in the pathogenesis of PD.

1.10 Ubiquitin-proteasome pathway

Intracellular proteins must be removed from a cell after they are no longer needed or when they are no longer functional. This process must be very specific to prevent removal of vital proteins and prevent fatal accumulation of unnecessary or damaged

proteins. Different intracellular proteins have different functions and thereby require different half-lives. The ubiquitin-proteasome pathway is responsible for degradation of intracellular proteins. This pathway involves two discrete steps 1) tagging the substrate to be removed with a covalently linked ubiquitin molecule and 2) degradation of the tagged protein by the 26S proteasome complex (Glickman and Ciechanover, 2002). This process releases and recycles the ubiquitin molecule for future use. There are three enzyme groups involved in the covalent linkage of ubiquitin to a protein marked for removal. The first is ubiquitin-activating enzyme, E1, which activates ubiquitin in an ATP dependent process. Next, an ubiquitin-carrier protein, E2, catalyzes the attachment of ubiquitin to the target protein. Finally, the E3s are the ubiquitin- protein ligases, which serve as a scaffold to bring together the E2 protein and the specific substrate to facilitate transfer of the activated ubiquitin (Glickman and Ciechanover, 2002).

The identification of two mutations in the ubiquitin-proteasome pathway, which result in familial Parkinson's disease, highlights the importance of this pathway in PD. Mutations in parkin, an ubiquitin-protein ligase (E3), were identified in an autosomal, recessive juvenile form of Parkinson's disease (Kitada et al., 1998). Interestingly, people with this mutation do not form Lewy bodies. The reason for this anomaly is not known. Parkin mutations are the most frequent cause of familial PD. A second, rare mutation has been found in UCH L1, an enzyme involved in ubiquitin recycling (Leroy et al., 1998). People with this mutation develop clinical evidence of PD. The presence (or absence) of Lewy bodies with this mutation has not been reported. To further investigate the role of parkin and proteasomal protein degradation in Parkinson's disease, parkin knockout mice were developed. Surprisingly, these mice are viable and show no reduction in

dopaminergic neurons in the substania nigra (Goldberg et al., 2003). It is likely additional contributing risk factors must be present in addition to proteasomal impairment for development of PD.

Impaired proteolytic degradation also has a role in the pathogenesis of sporadic Parkinson's disease. Proteasomal activity and expression of proteasomal subunits have been reported to be decreased in substania nigra postmortem tissue from PD patients (McNaught et al., 2003). The function of the ubiquitin-proteasome system was studied in rats treated chronically with the complex I inhibitor, rotenone. In this model, rats develop increased oxidative stress, α-synuclein inclusions and nigrostriatal neurodegeneration (Betarbet et al., 2000). Proteasomal activity was decreased and ubiquitin-conjugated proteins were increased in treated rats, suggesting impairment of proteasomal protein degradation (Yao et al., 2004). In neuroblastoma SH-SY5Y cells treated with rotenone, proteasomal function was reduced as a result of cellular oxidative damage to proteins including those of the proteasome itself (Shamoto-Nagai et al., 2003).

1.11 α-Synuclein and Lewy bodies

Lewy bodies are large non-membrane bound eosinophilic inclusions composed of ubiquitinated, aggregated protein fibrils (halo) surrounding a vesicular (lipid) core. α-synuclein is the major component of the halo. Other components that may be present in Lewy bodies include mitochondria, cytoskeletal proteins and synphilin (Chung et al., 2001; McNaught et al., 2002; Trimmer et al., 2004). Synphilin is a newly identified protein, whose function is unknown. Synphilin has an ATP binding site, several protein binding sites and has been demonstrated to strongly bind synuclein (Kruger, 2004). Antibodies to

ubiquitin and the oxidative stress marker 3-nitrotyrosine also react with Lewy bodies (Gai et al., 2000; Trimmer et al., 2004). Both synuclein and synphilin are substrates for ubiquitation (Chung et al., 2001). Synuclein and ubiquitin are frequent targets for nitration.

The importance of α-synuclein in the pathology of Parkinson's disease is supported by the recent finding of autosomal dominant mutations in α-synuclein that are associated with familial PD (Polymeropoulos et al., 1997). Following identification of these mutations, transgenic mice and *Drosophila* that overexpress either wild type or mutated α-synuclein have been developed (Masliah et al., 2000; Feany and Bender, 2000). Both transgenics, those expressing wild type and those expressing mutated α-synuclein, develop PD-like dopaminergic neuronal degeneration and neuronal inclusions. Chronic administration of MPTP or rotenone, models for dopaminergic neuronal loss, also result in the formation of α-synuclein containing inclusions (Betarbet et al., 2000; Fornai et al., 2005). Together, these observations implicate α-synuclein in the pathogenesis of PD.

1.12 Structure and function of α-synuclein

Alpha-synuclein is a member of the synuclein family of proteins that includes α, β, and γ synuclein. In humans, these proteins are the product of three genes on three separate chromosomes, 4, 5 and 10 respectively (Lucking and Brice, 2000; Goedert et al., 2001). Alpha and β synuclein are expressed primarily in nervous tissue. They are found abundantly in the neuronal cytosol, and are enriched at the presynaptic nerve terminals. Gamma-synuclein protein on the other hand, is primarily found in the peripheral nervous tissue and non-nervous tissue. Interestingly, an increase in expression of γ-synuclein is a

marker for progression in breast tumors, and was originally called "breast cancer-specific gene 1" (Lucking and Brice, 2000; Goedert et al., 2001).

Synucleins are small, natively unfolded proteins, ranging from 120-140 amino acids in length (Lucking and Brice, 2000; Goedert et al., 2001). They are heat-stable and relatively soluble. They lack any defined intracellular target sequences and also lack cysteine and tryptophan residues throughout the protein (Lucking and Brice, 2000; Goedert et al., 2001). The first 93 amino acids of α-synuclein are completely conserved between the mouse and rat, and the human differs only by 2 amino acids. This evolutionary constraint suggests an important and specific role for the n-terminus of the protein. The first 93 amino acids of the n-terminus contain a loosely repeated motif characteristic of an alpha helical secondary structure. Alpha helical structures typically confer an ability to bind phospholipids (Lucking and Brice, 2000; Goedert et al., 2001). Alpha-synuclein has been shown to bind rat vesicles via the first four n-terminal helixes *in vivo* (McLean et al., 2000). McLean et al. also demonstrated the α-synuclein protein was tightly associated with cellular membranes in intact neurons in culture (McLean et al., 2000). In addition to binding phospholipids, the n-terminus has been shown to bind synphilin (Chung et al., 2001). The c-terminus of synucleins contains an acidic amino acid tail and is the site of both nitration and cross-linking in the presence of reactive oxygen species (Giasson et al., 2000).

The function of α-synuclein remains unknown. Alpha-synuclein knockout mice are viable, fertile animals with a normal complement of dopaminergic neurons and normal neurotransmitter release and reuptake in response to simple electrical stimulation (Abeliovich et al., 2000). However, in response to repeated stimulation, knockout animals

exhibit increased dopamine release and decreased vesicle formation, suggesting a role for α-synuclein in modulation of dopamine neurotransmission during high intensity stimulation. In light of its binding properties, α-synuclein may function in neurotransmission through modulation of vesicular trafficking.

Alpha-synuclein has also been reported to have a role in the regulation of dopamine biosynthesis (Perez et al., 2002). Overexpression of α-synuclein in MN9D cells (a mouse mesencephalic dopaminergic cell line) reduced tyrosine hydroxylase activity (40%), tyrosine hydroxylase phosphorylation (20-30%) and dopamine synthesis (4-fold). No change was observed in tyrosine hydroxylase protein concentration. Tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis, must be phosphorylated to be active (Perez et al., 2002). A reduction in tyrosine hydroxylase activity results in decreased dopamine biosynthesis. Further studies using a rat mesencephalic dopaminergic cell line (MES23.5) and a human neuronal line (BE2-M17) found tyrosine hydroxylase protein expression and activity were decreased when α-synuclein was overexpressed (Bapista et al., 2003; Yu et al., 2004). Similar results were also found in transgenic mice that overexpress α-synuclein (Kirik et al., 2002).

There is some evidence that suggests α-synuclein may be protective to a cell, preventing apoptosis secondary to oxidative stress. Hashimoto et al. studied the effect of α-synuclein in c-jun-N-terminal kinase (JNK) activation following oxidative stress (Hashimoto et al., 2002). JNK activation and cell death was measured in neuronal cell lines following hydrogen peroxide treatment. Cells transfected with α-synuclein were resistant to hydrogen peroxide and JNK was not activated. Cells transfected with β-synculein were not protected. In an effort to gain insight into α-synuclein function, Nagano

et al. looked for binding of α-synuclein with proteins involved in apoptosis. They demonstrated that α-synuclein does not bind to apoptosis regulators Bcl-2 or BAD (Nagano et al., 2001). The role of α-synuclein in cytoprotection is likely a concentration dependent effect, as numerous other studies (below) have observed neurotoxicity secondary to overexpression of this protein.

1.13 The role of α-synuclein in the pathogenesis of Parkinson's disease

Several discoveries have lead to a greater understanding of the importance of α-synuclein in the pathogenesis of PD. Alpha-synuclein is the predominant component in Lewy bodies and Lewy neurites, a pathologic hallmark of PD. Lewy bodies are inclusions found in the dopaminergic neurons of the substania nigra in disease. Lewy neurites are found in the striatum. Both are aggregates of insoluble fibrils of α-synuclein. The accumulation of these inclusion bodies was presumed to have a causative role in the development of PD.

Native α-synuclein is an unfolded, soluble monomer. The formation of aggregated fibrils proceeds through an intermediate structure, the protofibril. Transition from the monomer to the protofibril involves the conversion from a natively unfolded protein to dimers and oligomers that interact to form a β-sheet. This protofibril is then further oligomerized to form non-soluble fibrils or Lewy bodies (Sulzer, 2001).

Animal models have provided insight into the role of α–synuclein in dopaminergic neurodegeneration. Transgenic mice that overexpress human α-synuclein and rodents chronically exposed to MPTP both develop inclusions that contain α-synuclein. The inclusions that develop however are organized in a non-fibrillar, protofibril configuration

(Masliah et al., 2000). Yet, despite the lack of fibril formation, animals in both models experience dopaminergic neurodegeneration and clinical motor deficits. This led to the hypothesis that it is the α-synuclein protofibrils that are toxic to neurons, and the accumulation of insoluble α-synuclein fibrils is a consequence and not a cause of cellular toxicity. Events that accelerate formation or impair removal of the intermediate protofibril would therefore be associated with risk of Parkinson's disease. Several lines of evidence support this hypothesis.

Three factors have been identified that promote the accumulation of protofibrils; oxidative stress, cytosolic dopamine and α-synuclein concentration. In order for protofibrils to accumulate, α-synuclein must exceed a critical concentration. Cytosolic α-synuclein concentration can increase due to increased expression or decreased protein removal. Protein expression may be increased due to genetic variation or due to stimulation by exogenous factors. Two types of genetic variants have been described that cause increased α-synuclein gene expression. Polymorphisms in a repetitive element upstream of the promoter region of α-synuclein, known as NACP-REP1, have been associated with increased synuclein expression and increased incidence of PD (Farrer et al., 2001). Triplication of the α-synuclein gene has been found in a family with a predisposition to early-onset PD (Singleton et al., 2003). Protein concentration was increased 2-fold in individuals with this mutation (Miller et al., 2004). Expression of α-synuclein may also increase following various inflammatory or stress events. Exposure of neurons to lipopolysaccharide (Tanji et al., 2002), interleukin 1β (Tanji et al., 2002), dopamine (Gomez-Santos 2003), MPTP (Vila et al., 2000), rotenone (Betarbet et al.,

2000), or permethrin (Gillette et al., 2003) are associated with induction of α-synuclein expression.

The second mechanism that may result in accumulation of α-synuclein is impairment of degradation. While it is clear cytosolic accumulation of α-synuclein decreases proteasomal function, a primary role of proteasome dysfunction in α-synuclein accumulation is more controversial (Snyder et al., 2003; Fornai et al., 2005). Certainly, mutations in the ubiquitin-proteasome pathway, such as parkin and UCH- L1, are associated familial Parkinson's disease (Polymeropoulos et al., 1997; Kitada et al., 1998). However, the effect of inhibition of proteasome function appears dependent on the experimental model. Proteasome inhibition in PC12 cells and primary mesencephalic neurons resulted in accumulation of α-synuclein inclusions (Rideout et al., 2001; McNaught et al., 2002b) yet the total concentration of α-synuclein was not altered (Rideout et al., 2001; Biasini et al., 2004).

Once a critical concentration of α-synuclein is exceeded, formation of protofibrils is dependent on the availability of dopamine. The importance of dopamine concentration is suggested by the increased susceptibility of the substantia nigra as compared to the ventral tegmentum to neurodegeneration. Dopamine concentration in the cytosol of the substantia nigra is greater than in the dopamine containing nuclei of the ventral tegmentum (Takahashi et al., 1997). The ventral tegmentum has greater expression of VMAT2, which promotes vesicle formation and lower expression of dopamine transporter protein, which enhances uptake of dopamine into the neuron. Additional evidence for the importance of dopamine cytosol concentration is demonstrated by work of Staal and Sonsalla (2002), showing inhibition of VMAT2 enhances the neurotoxicity of MPTP in mice.

Work from Lansbury's group provides a potential mechanism by which the protofibrils may be toxic to the cell (Volles et al., 2001). Protofibrils were shown to bind vesicles *in vitro*, with a strong enough affinity to suggest the binding was by insertion into the lipid bilayer. To test the effect of this strong binding on the vesicles, vesicles were loaded with a calcium-sensitive fluorescent dye (that fluoresces when bound to calcium), and calcium was added to the reaction mixture. Vesicles were treated with the calcium ionophore ionomycin (which allows calcium to diffuse through the vesicle membrane) to determine the theoretical maximum fluorescence. Vesicle fluorescence was then measured in the presence of α-synuclein protofibrils, monomers or fibrils. The rate and intensity of vesicle fluorescence was measured. There was no enhancement of fluorescence by addition of monomer or fibrillar α-synuclein. However, the addition of α-synuclein protofibrils resulted in a rapid and enhanced vesicular fluorescence. This was both dose dependent and size dependent, as the smaller vesicles were more likely to fluoresce. In a dopaminergic neuron, protofibrils could increase cell membrane permeability, causing increase in cytoplasmic calcium that would signal cell death. Alternatively, the dopamine vesicle membrane might become leaky, releasing its toxic contents into the cytoplasm. Dopamine is a small molecule, therefore likely to escape. Finally, protofibrils might permeablize the mitochondrial membrane, signaling apoptosis.

Toxicity of the protofibrils was confirmed by demonstrating accelerated neuronal death when cell lines were treated with inhibitors of protofibril to fibril conversion (Conway et al., 2001). Inhibitors were identified by screening 169 chemicals of a commercially available compound library. Fifteen of these compounds inhibited α-synuclein fibrillization. Fourteen of the 15 effective compounds were catecholamines,

including dopamine and L-dopa. The inhibitory effect was reversed by treatment with antioxidants and reducing agents, suggesting the dopamine and L-dopa had to be oxidized to be inhibitory. Fibril formation *in vitro* was fastest in unmodified α-synuclein as compared to dopamine-quinone-α-synuclein adducts, and conversely, the lifetime of protofibrillar intermediates was longest with the presence of dopamine-quinone-α-synuclein adducts.

Oxidative stress also promotes protofibril accumulation by modification of α-synuclein. Exposure to nitrating agents selectively modifies three tyrosine residues located at the carboxyl terminus. Exposure to nitrating and oxidizing agents results in covalent cross linking of these residues, resulting in dimers and multimeric forms of the protein. Antibodies that recognize either nitrated or nitrated and oxidized α-synuclein selectively label Lewy bodies and Lewy neurites in PD (Giasson et al., 2000). Selective nitration of the c-terminal tyrosines of α-synuclein also occurs in MPTP treated mice (Liberatore et al., 1999). Other nerve terminal proteins are spared. Several groups have reported 3-nitrotyrosine modified α-synuclein inhibits conversion of protofibrils to fibrils (Souza et al., 2000; Conway et al, 2001; Hodara et al., 2004). It is unclear whether modification of synuclein is an early, initiating event in PD or the result of disease-generated ROS exposure.

Based on these results from his laboratory and others, Lansbury proposed a new model for the pathogenesis of PD (Conway et al., 2001). High concentrations of dopamine in the cytoplasm in the presence of oxidative stress results in the formation of dopamine-quinones that, in the presence of high concentrations of α-synuclein, trigger the formation of protofibrils. Protofibrils are cytotoxic to the cell, resulting in neuronal cell death.

1.14 Cell death and dopaminergic neurodegeneration in Parkinson's disease

It is often difficult to assess the mechanism of cell death in natural, chronic neurodegenerative disease because of the temporal separation between insult, cell death and death of the organism. At the time of organism death, the majority of the affected neurons are typically already lost, no longer in the process of dying. It is therefore often necessary to rely on animal and *in vitro* models to predict the molecular events surrounding neuronal cell loss. When studying neurodegeneration in this fashion, it is very important to recognize these models often employ an acute, severe neurotoxic insult. This may not be the nature of the insult that leads to spontaneous disease. Even models that employ chronic exposure to neurotoxins may not reflect the events that occur in spontaneous disease. Extrapolation of findings from studies performed in animal and culture models to naturally occurring PD should therefore be made with caution.

Evidence of apoptotic cell death in post mortem evaluation of brain tissue from Parkinson's patients includes both morphological and biochemical characteristic changes of cell shrinkage, chromatin condensation, DNA fragmentation as well as activation of caspase 3 (Mochizuki et al., 1994; Blum et al., 2001). Apoptotic cell death has also been shown in a variety of studies to be involved in neuronal death following MPTP exposure. DNA fragmentation and caspase 3 activation as well as caspase 9 activation and cytochrome c release have been reported in the substantia nigra of mice following MPTP administration (Hartmann et al., 2000; Viswanath et al., 2001). Mice in which pro-apoptotic protein BAX is ablated are resistant to MPTP neuronal death (Vila et al., 2000) as are mice in which anti-apoptotic protein Bcl 2 is overexpressed (Nagano et al., 2001). In addition, transgenic mice that express a general caspase inhibitor (baculovirus protein

p35) are resistant to MPTP-induced dopaminergic neuronal loss (Viswanath et al., 2001).

A common method used for determining cell death of dopaminergic neurons has been the demonstration of a loss of immunoreactive markers specific to these neurons, including tyrosine hydroxylase, vesicular monoamine transporter and dopamine transporter (Jakowec et al., 2004). However, expression of these proteins may be inhibited in neurons that have undergone a loss of function but not cell death. This phenomenon was seen in aging studies in which decreased tyrosine hydroxylase reactive neurons but not cell loss was associated with aging (McCormack et al., 2004). Therefore, we defined "dopaminergic neurodegeneration" as a loss of functional neurons for the studies described in this thesis.

1.15 PPID and Parkinson's disease

Our understanding of the pathogenesis of Parkinson's disease has expanded greatly in the past 5 years, largely due to the identification of several mutations that cause familial PD. It is becoming clear that the more common, non-familial PD is the result of interaction of genetics, environmental exposures and cellular circumstances. It is only when a collection of predisposing events converges in the correct combination that clinical PD occurs (Figure 7). Although the clinical endpoint is the same, the precise combination of initiating events varies among individuals. A severe, acute neurotoxic insult, such as the single exposure to MPTP of the heroin users in 1983 is sufficient to induce lifelong PD. Mild, chronic exposure to oxidants, such as environmental herbicides and pesticides, is also likely sufficient. Predisposition due to a genetic defect leading to a disruption of dopaminergic neuronal homeostasis or antioxidant capacity also contributes to risk of PD.

And certainly, autosomal mutations in the α-synuclein gene or genes in the ubiquitin-proteosome pathway cause PD. In every case, although the underlying cause differs, the clinical outcome does not. Patients suffer nigrostriatal dopaminergic neurodegeneration and progressive motor dysfunction.

We propose PPID, similar to PD, is the result of an interaction of genetics, environmental exposures and cellular conditions with affected animals developing periventricular dopaminergic neurodegeneration as a result of the accumulation of aggregated nitrated α-synuclein (Figure 8). The instigating events resulting in oxidative stress and α-synuclein overexpression are multifactorial and differ among affected horses, but the clinical outcome is the same. In this model, the underlying pathologic mechanisms of PPID are similar to those of Parkinson's disease but the clinical outcome differs. In Parkinson's disease, the loss of dopaminergic inhibition of the striatum results in clinical signs of motor dysfunction. In PPID, the loss of dopaminergic inhibition of the pars intermedia results in clinical signs of endocrine dysfunction.

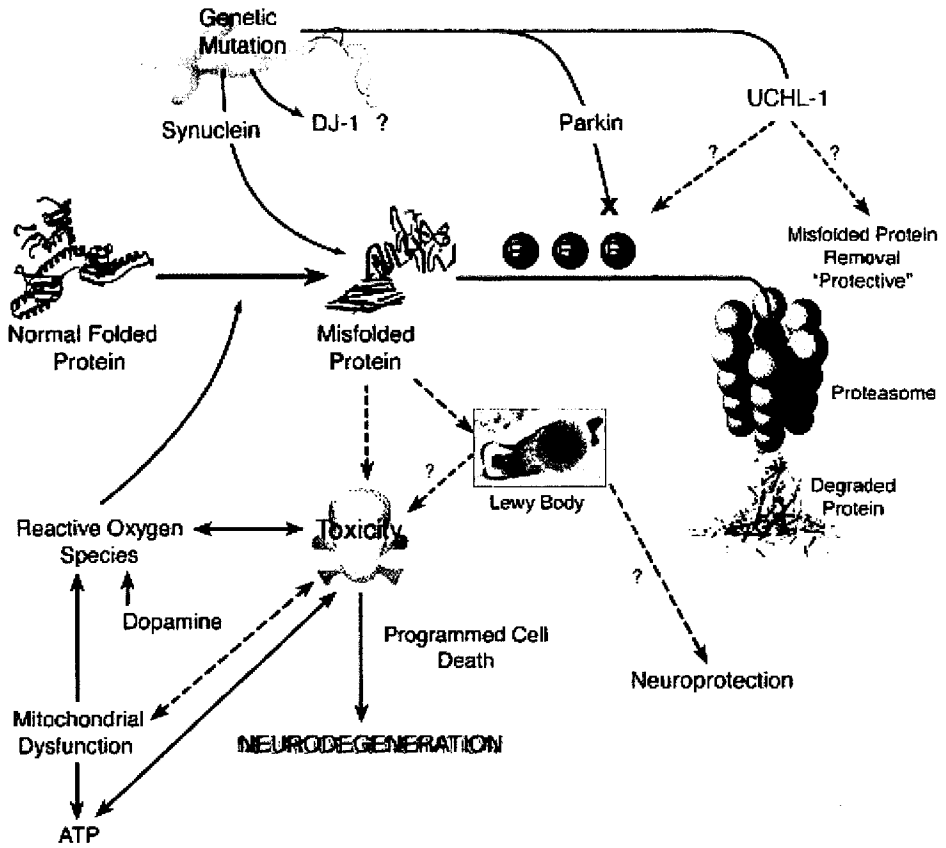

Figure 7: Model for the pathogenesis of Parkinson's disease

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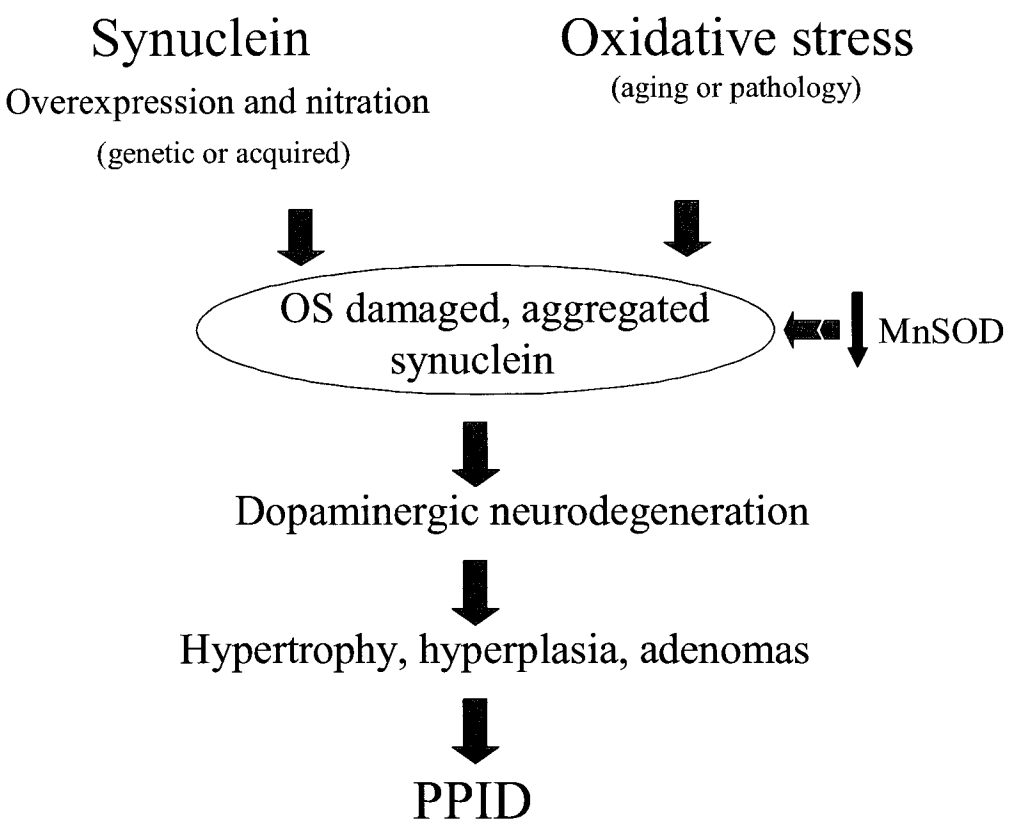

Figure 8: Model for PPID, based on Parkinson's disease model.

Horses with increased expression (either due to genetic or environmental factors) and nitration of alpha synuclein, in the presence of oxidative stress (either due to aging or pathology secondary to endogenous ROS production or environmental oxidants) accumulate aggregates of damaged alpha synuclein. Aggregated synuclein is neurotoxic, resulting in dopaminergic neurodegeneration of the periventricular neurons. Loss of dopamine at the pars intermedia results in hypertrophy, hyperplasia and adenoma formation, with overexpression of PI-derived POMC peptides and subsequently clinical signs of PPID.

1.16 HYPOTHESIS

We hypothesized that PPID results from periventricular dopaminergic neurodegeneration secondary to oxidative stress and α-synuclein overexpression, nitration and aggregation. In order to investigate this hypothesis it was first necessary to validate various test methods employed for diagnosis of PPID (Chapters 2-4). The specific aims of these initial studies were

1. To investigate the effect of sample handling, storage and collection time and season on plasma α-melanocyte stimulating hormone concentration in healthy horses (Chapter 2).
2. To investigate the mechanism responsible for the thyrotropin releasing hormone (TRH) response test and evaluate its usefulness in diagnosis of PPID (Chapter 3).
3. To validate postmortem histology as the "gold standard" of PPID diagnosis (Chapter 4).

Following assessment of diagnostic test methods we sought to test our hypothesis that PPID results from periventricular dopaminergic neurodegeneration secondary to oxidative stress and α-synuclein overexpression and nitration. Specifically, we aimed

1. To demonstrate a loss of periventricular dopaminergic neurons in horses with PPID (Chapter 5).
2. To demonstrate an accumulation of oxidative stress markers in the pars intermedia of horses with PPID (Chapter 5).

3. To demonstrate an increase in α-synuclein expression, nitration and aggregation in horses with pars intermedia oxidative stress or dysfunction (Chapter 5).
4. To demonstrate a decrease in antioxidants (glutathione, glutathione peroxidase, superoxide dismutase) or increase in systemic oxidative damage (3-nitro-tyrosine) in horses with pars intermedia oxidative stress or dysfunction (Chapter 6).
5. To determine if genetic polymorphisms occur in the equine manganese superoxide dismutase gene and if they are associated with decreased enzyme activity, increased oxidative stress and/or increased risk of PPID (Chapter 7).

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CHAPTER 2:

Effects of season and sample handling on measurement of plasma alpha-melanocyte-stimulating hormone concentrations in horses and ponies.

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2.1 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID) is a spontaneously occurring progressive disease affecting primarily aged ponies and horses. Despite recognition of this condition for more than 70 years, the cause and pathogenesis of PPID remains poorly defined (Pallaske, 1932). Although the clinical signs of this disease are well recognized, ante mortem diagnosis of PPID is problematic, with all available diagnostic tests having limitations. In aged horses with advanced disease, the presence of clinical signs including hirsutism, laminitis, muscle atrophy, abnormal fat distribution, polydipsa and polyuria, hyperhidrosis, and secondary infections may be sufficient for diagnosis (Schott, 2002). However if treatment is to be pursued, testing is still warranted as a tool to monitor response to therapy and to allow individualized titration of drug dose (Schott et al., 2001; Donaldson et al., 2002). In horses with early disease, the clinical signs often overlap with phenotypic changes observed with normal aging. Testing is therefore necessary to confirm the diagnosis.

Overnight dexamethasone suppression testing is considered the "gold standard" method of ante mortem PPID diagnosis. Originally this test was reported to have a sensitivity and specificity of 100% (Dybdal et al., 1994). However, it was suggested in a recent report (Miesner et al., 2003) that the reliability of the test has been over-estimated. When the dexamethasone suppression test was repeated 3 times at 30-day intervals in 7 horses with clinical signs of PPID, only 1 of 7 horses tested positive for disease on all three days (Miesner et al., 2003). Following an initial positive result, 5 of 7 horses suppressed normally on each subsequent test date, indicating either false positive results at the initial

test period or false negative results during subsequent testing. Although it has not been critically assessed, a loss of feedback inhibition by glucocorticoid may be a late event in the disease progression, and the high sensitivity originally reported may reflect a case selection bias towards horses with advanced disease. Other limitations of the dexamethasone suppression test include the need for multiple days for blood sample collection and the potential exacerbation of laminitis in horses with active or historical laminitis (Eyre et al., 1979; Johnson et al., 2002).

A 50% increase in serum cortisol concentration 15-90 minutes following thyrotropin releasing hormone administration has also been proposed as a diagnostic test (Beech and Garcia, 1985). The performance of this test has not been critically evaluated in a large number of horses, particularly those with early disease. Further, this test is currently impractical due to the lack of approved thryotropin releasing hormone for use in the horse and the high price of human-approved products.

The endogenous concentration of ACTH is also high in horses with PPID (Orth et al., 1982; Couetil et al., 1996; Horowitz et al., 2003). Plasma ACTH concentration measured by radioimmunoassay was reported to have a sensitivity of 82 to 90% and a specificity of 90-100% in the diagnosis of PPID when overnight dexamethasone suppression was used as the reference test (Couetil et al., 1996; Horowitz et al., 2003). Again, the usefulness of endogenous ACTH concentrations in diagnosis of early PPID is unclear. Limitations of measurement of endogenous ACTH include the need to process samples within 3 hours of blood sample collection and to ship samples frozen, ideally on dry ice (Couetil et al., 1996).

Recently, the endogenous concentration of alpha-melanocyte stimulating hormone (α-MSH) was investigated as a diagnostic test for PPID (Horowitz et al., 2003). α-MSH is a primary product of proopiomelanocortin (POMC) produced in the pars intermedia. Orth et al. (1982) showed the increase of α-MSH in the blood of horses with PPID to be of a greater magnitude than the increase in ACTH. Therefore, a detectable increase in α-MSH might precede a measurable increase in ACTH. A sensitivity and specificity of 88 and 85%, respectively, were reported when using the dexamethasone suppression test results to define affected horses (Horowitz et al., 2003).

In addition to its potential usefulness as a diagnostic test, accurate measurement of α–MSH has application as a research tool. Because α-MSH is primarily a product of the pars intermedia, whereas ACTH is primarily a product of the pars distalis, concurrent measurement of these POMC peptides can be used to differentiate a pars intermedia response to an environmental or pharmacological challenge from a pars distalis response (Wilson et al., 1982). A better understanding of the physiological role of the pars intermedia in healthy horses may provide insight into the pathogenesis of the dysfunction that occurs in horses with PPID. For measurements of plasma α-MSH concentration to be a more useful diagnostic or research tool, a more complete understanding of factors that may influence its measurement is required. Therefore, we evaluated the effect of sample handling, season, and time of day on plasma α-MSH concentration in healthy horses and ponies.

2.2 METHODS AND MATERIALS

Study Population: All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care. Group 1 consisted of 11 clinically healthy, adult Standardbreds (2 geldings and 9 non-pregnant mares) housed together indoors at the Atlantic Veterinary College, Prince Edward Island, Canada. Horses were kept in individual stalls and fed grass hay twice daily and water *ad libitum*. Group 2 consisted of 13 semi-feral ponies (6 stallions and 7 breeding mares) maintained on 50 acres of natural grass pasture in Chester County, PA, USA. Mineral and salt blocks were provided and supplemental hay was provided in the winter. Water was available from natural ponds and streams. Natural brush and light forest was available for shelter. All ponies were healthy and had overnight dexamethasone suppression test results that were within reference range (Dybdal et al., 1994).

Measurement of α-MSH concentration: Plasma α-MSH concentration in ponies and horses was measured by use of a commercially available radioimmunoassay designed for use with human plasma (Euria-α–MSH RIA, American Laboratory Products Company, Windham, NH). α-melanocyte stimulation hormone is a 13 amino acid peptide, with an identical sequence found in horses and humans (Wilson et al., 1982). The absence of interfering substances in equine plasma and α-MSH homology between humans and horses makes this assay suitable for use with horse plasma. The absence of interfering substances in equine plasma was confirmed by demonstrating a similar predicted and observed α-MSH concentration of when a known quantity of α-MSH was added to pooled equine

plasma. Further, serial dilution of provided standards and equine plasma spiked with α-MSH resulted in the expected α- MSH concentrations. Sensitivity of the assay, as reported by the manufacturer and confirmed in our laboratory, was 3 pmol/L. Intra- and interassay variation reported by the manufacturer was 12% and 13% at 16 pmol/L, respectively, and 3% and 4% at 78 pmol/L, respectively. In our laboratory, both intra- and interassay variation was 5% for both high and low concentrations. Data provided by the manufacturer indicated low cross reactivity with related peptides (<0.002%). Specific control sera provided with each kit were included as positive controls for each assay. All samples were assayed in duplicate.

Sample Handling: Sample handling effects were assessed in samples collected from horses in Group 1. All conditions were assessed in groups of 6 randomly selected horses. Blood was collected by jugular venipuncture into evacuated glass tubes containing EDTA as an anticoagulant. Plasma was separated by centrifugation and transferred to polypropylene containers. The effect of freezing was assessed by comparing plasma that was separated within 1 hour of blood sample collection and assayed immediately with plasma that was frozen and stored at -80°C for 1 week. Samples were also stored for 9 months at -80°C to determine if α-MSH was stable under long-term storage. To assess the effects of delayed processing, plasma was either immediately separated from blood samples or blood samples were stored in EDTA-containing evacuated tubes at 4°C for 8 or 48 hours before plasma was separated. Plasma was then separated and frozen as before. The effects of suboptimal handling of plasma were determined by immediately storing

plasma after separation at 25°C for 24 hours, 4°C for 48 hours or 7 days, and at –20°C for 30 days prior to freezing at -80°C for batch analysis.

The effect of hemolysis was investigated by spiking pooled horse plasma with known concentrations of equine hemoglobin. Fresh lysate was prepared by mixing equine packed red blood cells with ice-cold distilled water at a 1:9 dilution. The lysate was centrifuged at 800X g at 4°C for 20 minutes. Lysate was added to unaltered pooled equine plasma, pooled equine plasma spiked with α-MSH (37.5 pmol/L), and pooled equine plasma from horses with plasma α-MSH concentration of > 100 pmol/L to reach final hemoglobin concentrations of 0.5, 1.0, 2.0, and 4.0 g/L.

Time of sample collection: The effect of blood sample collection time on plasma α-MSH concentration was assessed in 6 group 1 horses. Blood was collected by jugular venipuncture at 0800, 1200 and 1600 hours. Plasma was separated within 1 hour and stored at -80°C until analyzed.

Effect of Season on Plasma α-MSH: The effect of season on plasma α-MSH concentration was assessed using all group 1 horses and group 2 ponies. Blood was collected by jugular venipuncture, and plasma was separated and stored at -80° C until analyzed. All samples from group 1 were collected between 0730 and 0900 hours, samples from group 2 were collected between 0800 and 1400 hours. Samples for group 1 horses were collected in January, March, April, June, September, and November. Samples for group 2 ponies were collected in May and September.

Statistical analysis: All results are presented as mean (± standard deviation) values. All comparisons were made among samples collected from repeated blood sample collections from the same horses. For multi-point comparisons, a repeated measures ANOVA was used, with a Bonferroni correction for multiple comparisons for post hoc analysis. For 2-point comparisons, a paired t test was used. Values of $P < 0.05$ were considered significant. Commercially available software was used to perform analyses (Minitab Inc, Version 13.32, State College, PA; GraphPad Software, Version Prism 3.03, San Diego, CA).

2.3 RESULTS

Sample handling: The effect of storage of samples at –80° C on measured plasma α-MSH concentration was assessed. No significant difference ($P = 0.17$) in plasma α-MSH was found in measurements made immediately and measurements made after storage for 1 week (13.1 ± 2.2 pmol/L vs 11.9 ± 2.8 pmol/L, respectively). Further, storage for 9 months at –80° C did not result in a significant ($P = 0.22$) decrease in measured plasma α-MSH concentration compared with measurements made immediately (13.1± 2.2 pmol/L vs 14.1 ± 2.6 pmol/L, respectively). Therefore, in all following stability studies, plasma samples were frozen and stored at -80° C for batch analysis.

The effect of delayed separation of plasma on measured α-MSH concentration was assessed for various periods (Figure 1). Although the storage of blood samples at 4°C for 8 hours before plasma was separated did not significantly change measured concentrations, compared with that of plasma immediately separated (12.0 ± 2.7 pmol/L vs 11.1 ± 3.1 pmol/L, respectively), the storage of blood samples at 4°C for 48 hours before plasma was separated did result in a significant decrease in plasma α-MSH concentration (8.3 ± 2.1 pmol/L vs 11.1 ± 3.1 pmol/L, respectively).

Storage of plasma in polypropylene tubes at 25°C for 24 hours, at 4°C for 48 hours, and at – 20°C for 1 month prior to freezing at -80° C did not significantly affect the α-MSH concentration (Figure 1). However, storage of plasma at 4°C for 1 week did result in a significant decrease in measured plasma α-MSH concentration, compared with plasma stored immediately at -80° C (8.6 ± 1.4 pmol/L vs 11.1 ± 2.7 pmol/L).

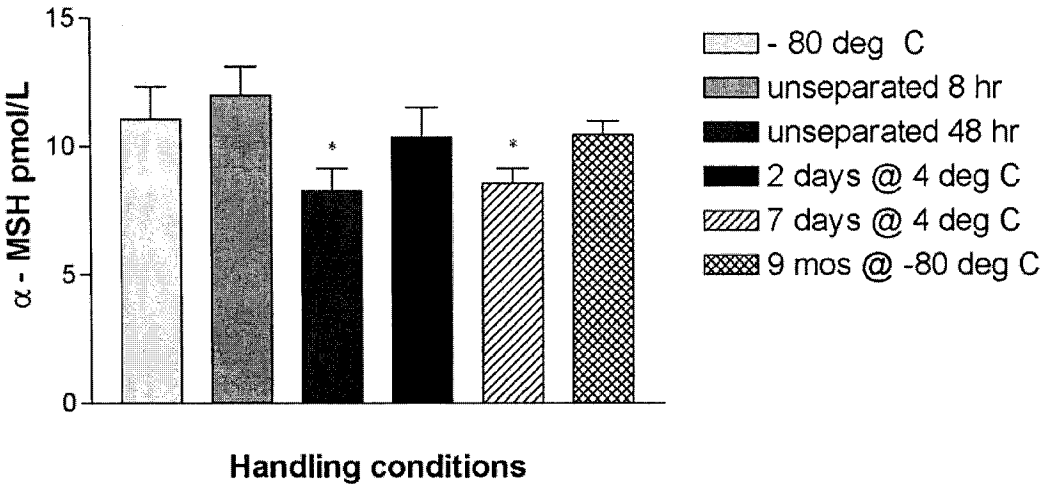

Figure 1: Effect of sample handling and storage conditions on α-MSH concentration.

When compared to samples processed immediately and stored at – 80 ° C, samples left unseparated for 48 hours at 4 ° C showed a decrease in α- MSH concentration (repeated measures ANOVA, * $P<0.05$). Plasma left 7 days at 4 ° C also showed a decrease in α-MSH concentration (repeated measures ANOVA, * $P<0.05$). There was no difference in α-MSH concentration with any other handling or storage conditions including storage at 25 ° C for 24 hours and at –20 ° C for 30 days (data not shown.)

Hemolysis: Addition of hemolysate to plasma samples did not significantly affect measurement of α-MSH concentration in unaltered equine plasma or equine plasma spiked with α-MSH (Figure 2), although a decrease of an approximately 10% was observed with the hemolysate concentration of 4 g/L. Findings for pooled plasma sample from horses with naturally high plasma α-MSH concentration were similar to findings for equine plasma spiked with α-MSH.

Time of day and time of year: No significant (P =0.9) difference in plasma α-MSH concentration was found among samples collected at 0800, 1200 or 1600 hours (mean 8.3 ± 2.6, 8.5 ± 3.2, 8.3 ±3.0 pmol/L, respectively). However, in horses and ponies, significant seasonal variation in plasma α-MSH concentration was found, with the highest hormone concentrations found in September (Figures 3 and 4). Comparing September to the spring months, ponies had an 11-fold greater plasma α-MSH concentration, whereas in the same comparison horses had a 2-fold greater concentration. The range of plasma α-MSH concentration was also greatest in the fall, with ponies having values that ranged from 40 to 185 pmol/L in September, compared to 3 to 19 pmol/L in May, and horses having values that ranged from 2.7 to 58 pmol/L in September, compared to 9.5 to 15.9 pmol/L in April. No significant difference in plasma α-MSH concentrations was found between male (n=6) and female (7) ponies in May (7.0 ± 2.3 vs 10.75 ± 4.6, respectively; P = 0.09) or September (95.9 ± 42.5 vs 99.3 ± 60.7, respectively; P =0.9).

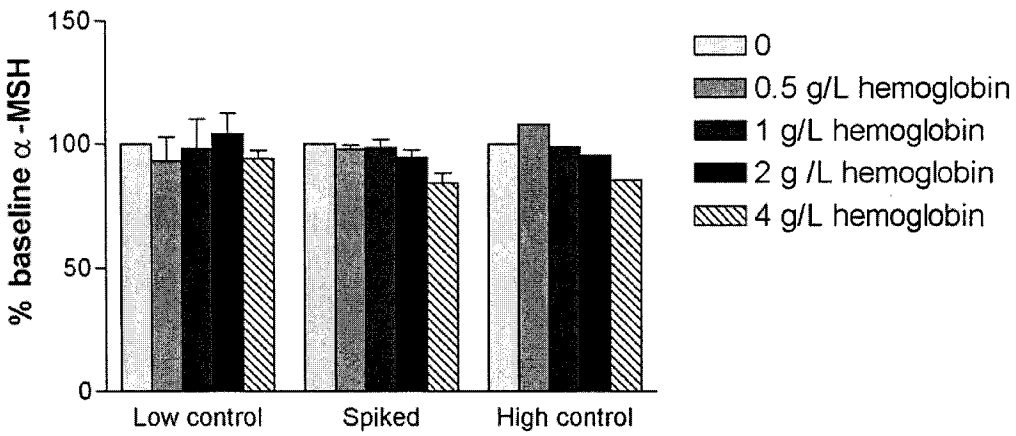

Figure 2: Effect of hemolysate on measurement of α-MSH.

Equine hemolysate was added to pooled equine plasma (low control, n=3, mean α-MSH= 16 pmol/L), pooled equine plasma spiked with α-MSH (spiked, n=3, mean α-MSH=40 pmol/L) and pooled equine plasma with endogenous α-MSH of 211 pmol/L (high control, n=1) to a final concentration of 0, 0.5, 1.0, 2.0 and 4.0 g/L. Bars represent the percent change in α-MSH compared to baseline at each concentration of hemoglobin for each group. There was no significant difference at any hemoglobin concentration.

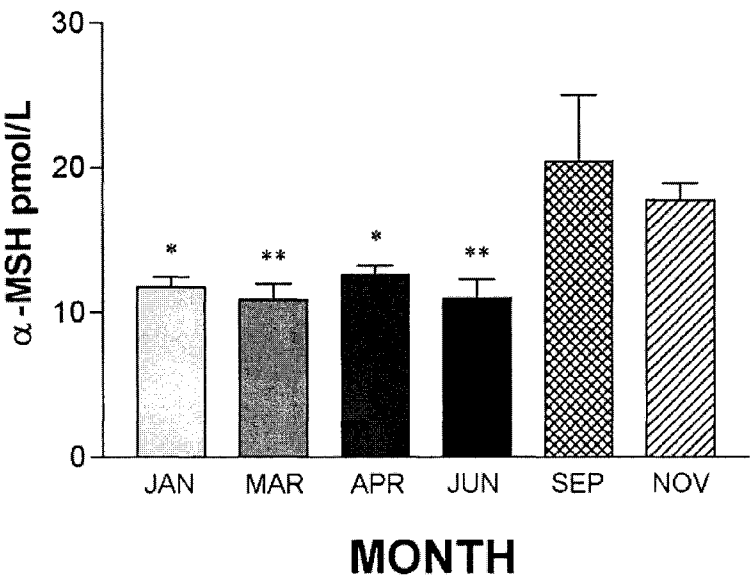

Figure 3: Seasonal variation of plasma α-MSH concentration in healthy horses.

Plasma α-MSH concentration in healthy standardbred horses is significantly lower in winter, spring and summer compared to September (Jan and Apr, * $P<0.05$; Mar and Jun, ** $P<0.01$). The range was also greatest in September (2.7 to 58 pmol/L) compared to April (9.5 to 15.9 pmol/L).

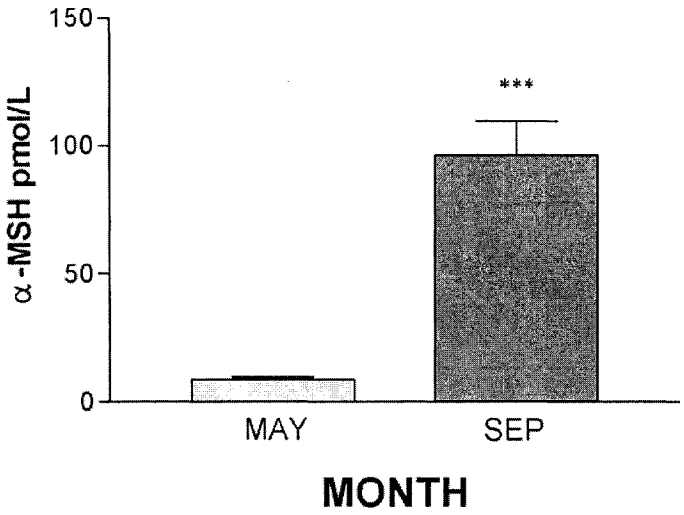

Figure 4: Seasonal variation of plasma α-MSH concentration in healthy ponies.

Plasma α-MSH concentration in healthy ponies was significantly greater in September compared to May (*** $P<0.001$). The range in ponies was greatest in September (40 to 185 pmol/L) compared to May (3 to 19 pmol/L).

2.4 DISCUSSION

As a result of the limitations of the currently available diagnostic tests for PPID, an improved testing method for this disease is needed. Pituitary pars intermedia dysfunction is a disease diagnosed primarily by practitioners in the field. Therefore, to be useful, a diagnostic test should be applicable to blood samples collected in the field. Ideally, blood samples should be able to be collected at a single farm visit, collected any time of the day and transported to the clinic without requiring special handling. In addition, the test should pose little to no risk to the patient. Our findings indicate measurement of plasma α-MSH concentration meets these requirements.

To assess the effect of delayed separation of plasma, whole blood in EDTA containing evacuated tubes was held for 8 or 48 hours at 4°C prior to centrifugation and the separation of plasma. Although no difference was found in samples held for 8 hours, 48 hours of storage resulted in a significant decrease in plasma α-MSH concentration (Figure 1). Therefore, we suggest that plasma be separated within 8 hours of blood collection to avoid sample deterioration.

To assess the impact of storage conditions of plasma on hormone concentration, separated plasma was stored at 25°C for 24 hours, at 4°C for 48 and 7 days, and at – 20 ° C for 1 month (Figure 1). When plasma was held 1 week at 4°C, a decrease in α -MSH concentration occurred. All other conditions did not affect the hormone concentration. Therefore, plasma samples should be stored frozen until assayed, and overnight shipment of frozen plasma samples is preferable. Storage of plasma samples at – 80°C did not adversely affect hormone concentration. We therefore conclude that storing plasma samples at – 80°C for later batch analysis is acceptable.

Hemolysis is known to interfere with a number of diagnostics assays (Alleman, 1990). The effect of hemolysis on measurement of plasma α-MSH concentration was assessed by adding a known quantity of equine hemolysate to pooled plasma samples containing low and high concentration of hormone. No significant effect of hemolysate was found up to a hemoglobin concentration of 4 g/L. Although a decrease in measured plasma α-MSH concentration was found when hemolysate was added at a final hemoglobin concentration of 4 g/L, this difference was not significant. Nonetheless, it would be prudent to avoid severely hemolyzed samples for plasma α-MSH concentration measurements.

To determine the effect of sample collection time on α-MSH concentrations, we compared plasma hormone concentration in samples collected at 0800, 1200 and 1600. No difference in hormone concentration was found between samples collected at any of these times throughout the day. This is consistent with the lack of circadian rhythm of α-MSH in horses reported by Horowitz et al. (2003). Therefore, it does not appear that time of day is important in collecting blood samples.

In contrast to time of day, a distinct seasonal effect was found in horses and ponies. In horses and ponies, the measured plasma α-MSH concentration was considerably higher in September, compared to samples collected in the winter, spring, and early summer (Figures 3 and 4). An effect of season on α-MSH concentration has been described for humans, hamsters, sheep, and weasels (Logan and Weatherhead, 1979; Altmeyer et al., 1986; Lincoln and Baker, 1995). For Siberian hamsters and short tailed weasels, α-MSH concentration is reported to increase during the summer, at a time when coat changes color (Norman et al., 1972; Logan and Weatherhead, 1979). In humans living in Germany,

plasma α-MSH concentration peaks in August, with lowest concentrations reported in January (Altmeyer, et al., 1986). In Soay sheep, a feral breed of sheep in Scotland, plasma concentration of α-MSH is greatest in late summer and fall (Lincoln and Baker, 1995). The functional importance of the seasonal cycle is unknown, but several physiological events occur in parallel with the α-MSH cycle. In sheep, body weight, voluntary food intake, and condition all peak simultaneously with α-MSH, with seasonal maximums occurring in September. Soay sheep with surgically created hypothalamic-pituitary disconnection have an increase in circulating concentration of α-MSH and chronic increase in body weight (Lincoln et al., 2001). These findings considered together suggest that α-MSH or other POMC derived peptides may play a role in metabolic preparation for winter in Soay sheep. It is possible that horses and ponies have a seasonal increase in POMC-derived peptides to metabolically prepare them for a decrease in accessible food observed in the wild in winter. If so, dysregulation of this pathway might be associated with abnormalities in body weight and fat storage. Weight loss and abnormal fat distribution are 2 clinical signs associated with equine PPID (Boujon et al., 1993; van der Kolk et al., 1993; Donaldson et al., 2002).

Development of a winter coat also begins as length of day decreases in the fall. The development of hirsutism in horses with PPID leads one to speculate that the naturally occurring seasonal increase in POMC-derived peptides contributes to development of winter coat growth. This has not been assessed in equids.

In our study, ponies had a more robust increase in plasma α-MSH concentrations in the fall when compared with horses (11-fold vs. 2-fold). Several possible explanations exist for this finding. The ponies in our study were sexually intact and in natural breeding

conditions under the influence of reproductive hormones, whereas the horses were nonbreeding females or geldings. When blood samples were collected in September, the female ponies were either in late pregnancy (> 8 months; n=4) or had foaled within the last 2 weeks (3). In May, they were either nonpregnant (n=2) or in early pregnancy (<4 months: n=5). Despite these variables, no difference in plasma α-MSH concentration was found between male and female ponies. Therefore, it seems unlikely that the observed difference between horses and ponies was solely the result of reproductive influences. Another possibility is the difference may reflect a metabolic and endocrinologic difference between horses and ponies. Ponies are more predisposed to obesity than Standardbreds (Jeffcoat et al., 1986; Freestone et al., 1992). In addition, they are predisposed to glucose intolerance, insulin insensitivity and PPID (Jeffcoat et al., 1986; Freestone et al., 1992; Schott, 2002; Rijnen and van der Kolk, 2003). A greater seasonal change in pars intermedia expression may be beneficial in preparing animals for winter, allowing survival when conditions are harsh and food is sparse. It is possible that ponies are more evolved to survive harsh winter elements, compared to horses. The difference in management conditions between the 2 groups may also have contributed to the observed difference in seasonal plasma α-MSH concentration. The Standardbreds in our study were housed indoors and feed intake was strictly controlled. The ponies were outside, under natural light with free access to pasture. Finally, it is possible the seasonal hormonal peaks occur at different times of year in different geographical locations, and the actual peaks may have been missed due to sample collection interval.

The findings of our study are important for several reasons. First, as a result of the seasonal regulation of the pars intermedia, it is important to consider the season when

testing equids for PPID. In blood samples collected in September from the pony group, 6/13 had concentrations of α-MSH considered diagnostic for PPID (> 91 pmol/L, Figure 4) (Horowitz et al., 2003). In all animals, concentrations from samples collected in spring and summer were within reference range. A diagnosis for PPID determined in the late winter, spring or early summer is likely more reliable than a diagnosis made in the fall, when the pars intermedia is highly active. This would likely be true regardless of method of testing used, as all testing is aimed at detecting dysregulation or overactivity of the pars intermedia. Further, the establishment of reference range values for diagnostic endocrine testing in equids must be done with consideration of the season. At a minimum, seasonally specific reference range values may increase sensitivity and specificity of endogenous plasma α-MSH concentration for the diagnosis of PPID. However, the range of α-MSH concentrations in September was much greater than in the spring, making differentiation of reference and diagnostic ranges more problematic. Finally, results of our work are consistent with a physiological role of the equine pars intermedia of horses and ponies in metabolic preparation for winter. If seasonal variation in plasma POMC-derived peptides serves to regulate lipogenesis in horses and ponies similar to sheep, then it is possible that its dysregulation may play a role in the development of chronic obesity. The role of the POMC-derived peptides in obesity is an area of intense research in humans and warrants more critical evaluation in the horse.

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2.6 SIGNIFICANCE OF FINDINGS

Plasma α-melanocyte stimulating hormone (α-MSH) concentration in horses and ponies was observed to be significantly greater during the fall compared with other seasons. Therefore, if plasma α-MSH concentration is to be useful in diagnosis of PPID, seasonally specific reference ranges are needed. To facilitate the usefulness of plasma α-MSH concentration in diagnosis of PPID for studies in this thesis, we determined seasonally specific reference ranges using data from Chapter 2. These results were confirmed using data collected from horses euthanized at the Atlantic Veterinary College.

Establishment of seasonal-specific reference ranges for plasma α-melanocyte stimulating hormone concentration in the horse

In the study of Horowitz et al. (2003), a plasma α-MSH concentration > 91 pmol/L was considered to be diagnostic of PPID. This reference value was established with 63 horses, (38 unaffected and 25 affected horses) using the overnight dexamethasone suppression test to determine disease status and receiver-operating characteristic (ROC) analysis to select cut-off values that optimized sensitivity (88%) and specificity (84.2%). However, seasonal variation in endocrine function was not considered when determining this reference range. In fact, all the control horses were tested in the fall, while the affected horses were tested throughout the year (personal communication, J Watson).

Plasma α-MSH concentration from the healthy horses and ponies in our study (see Methods, Chapter 2.2) was much lower (10.9 ± 3.6 pmol/L, n=25) in the spring, summer or winter than in the control horses reported by Horowitz (59.6 ± 91.6 pmol/L, n=38) whereas

concentrations in the fall were similar in the two studies (49.2 ± 49.3 pmol/L, n= 25 versus 59.6 ± 91.6 pmol/L, n=38) (Horowitz et al., 2003; McFarlane et al., 2004). This is consistent with the controls in the Horowitz study being collected in the fall, and further emphasizes the need to consider season when interpreting plasma α-MSH concentration in the horse. Therefore, we determined seasonally specific reference ranges in healthy horses and ponies using data collected from client owned animals and from horses euthanized at the Atlantic Veterinary College.

To determine whether separate reference ranges were needed for horses, ponies or individual seasons, plasma α-MSH concentrations measured seasonally in 11 healthy horses and 13 healthy ponies (See Methods, Chapter 2) were evaluated. There was no significant difference in the mean plasma α-MSH concentration from horses or ponies during the non-fall months (Jan: 11.8 ± 2.4, Mar: 10.9 ± 3.6, Apr: 12.6 ± 2.2, Jun: 11 ± 4.4, horses; May: 8.9 ± 4, ponies) or in horses in the fall (Sep: 20.5 ± 15.2, Nov; 17.8 ± 4). However ponies had significantly greater plasma α-MSH concentrations in September (mean 96.5 ± 49.2, n=13) compared to horses. Therefore, ponies and horses were considered together in determining non-fall reference range values and separately for fall reference range values.

Blood samples were collected in the non-fall months (December 1- August 31) from 58 horses and ponies that remained clinically normal for > 2 years following sampling. Using a value of mean concentration plus 2 standard deviations (10.0 ± 4.0 pmol/L) as the upper limit of normal, non-fall plasma α-MSH concentrations > 18 pmol/L would be considered greater than the normal reference range. Blood samples were collected in the fall (September 1- November 30) from 24 horses and 14 ponies that

remained clinically normal for > 2 years following sampling. Using a value of mean concentration plus 2 standard deviations as the upper limit of normal, fall concentrations > 83 pmol/L in horses (mean: 31 ± 26) and > 194.5 pmol/L in ponies (mean: 96.5 ± 49.2) were considered higher than normal.

A disadvantage of this method of selection is the use of "apparently healthy" animals, rather than animals confirmed disease free by postmortem examination. Therefore, plasma α-MSH seasonal reference range values were also determined using horses donated for euthanasia, selected based on the absence of clinical signs and confirmed free of pituitary pars intermedia disease (adenomatous hyperplasia or adenoma) at postmortem examination. Similar to the first study, plasma α-MSH was lower in the spring, summer and winter (mean= 9.2 ± 5.6; n= 36) compared to α–MSH in the fall (mean= 17 ± 22; n= 18 horses). Using a value of mean concentration plus two standard deviations as the upper limit of normal, non-fall plasma concentrations > 20.4 pmol/L and fall concentrations > 61 pmol/L were considered greater than normal reference range. There was no significant difference between the mean plasma α-MSH concentrations in either study. Therefore, the two study populations were combined and the upper limit of normal reference range for plasma α-MSH concentration was determined (Table 1) for use in future studies. Plasma α-MSH concentration greater than the upper limit of the normal reference range would be considered consistent with a diagnosis of PPID.

Table 1. Seasonally-specific upper limit reference values for plasma α-melanocyte stimulating hormone (α-MSH) in horses and ponies

Horse/ pony	Season	Mean α-MSH pmol/L	SD	Number of animals	Cut-off
Horse or pony	Winter, spring, summer	9.7	± 4.6	94	> 19
Horse	Fall	25	± 22.3	42	> 70
Pony	Fall	96.5	± 49.2	14	> 195

CHAPTER 3:

Alpha-melanocyte stimulating hormone release in response to thyrotropin releasing hormone in healthy horses, horses with pituitary pars intermedia dysfunction and equine pars intermedia explants.

In press as:

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3.1 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID, equine Cushing's disease) is the one of the most common endocrine disease of aged horses and ponies (Schott, 2002). Horses with PPID are at high risk for life-threatening complications, including laminitis (inflammation of the sensitive lamina that attaches the hoof wall to the third phalanx), secondary infections and metabolic derangements such as insulin resistance and chronic hyperglycemia (Schott, 2002). Although the clinical signs of this disease are well recognized, ante mortem diagnosis of PPID is problematic. Early disease recognition is obscured by the overlap between phenotypic changes accompanying age and disease. Testing is therefore necessary to confirm the diagnosis.

PPID is characterized by hypertrophy, hyperplasia and adenoma formation of the pars intermedia of the pituitary (Schott, 2002). The horse has a well-developed pituitary pars intermedia (PI) comprised of a single endocrine cell type, the melanotrope, which produces the hormone precursor protein, proopiomelanocortin (POMC). In the PI, POMC is cleaved into the secretory peptides, α- melanocyte stimulating hormone (α–MSH), β-endorphin and corticotropin-like intermediate lobe peptide (CLIP) due to the activity of prohormone convertases I and II (Millington et al., 1988). A small amount of ACTH may also be produced. Horses with PPID have increased expression and secretion of PI POMC-derived peptides detectable in the peripheral blood as well as pars intermedia tissue (Wilson et al., 1982).

One ante mortem test for diagnosis of PPID is the thyrotropin releasing hormone stimulation test. An increase in plasma or serum cortisol concentration 15-90 minutes

following thyrotropin releasing hormone (TRH) administration has been associated with disease (Beech and Garcia, 1985). Plasma cortisol concentration did not change in normal horses in response to TRH (Beech and Garcia, 1985). While the TRH response has been used in the diagnosis of PPID, the mechanism of this response remains unknown.

A similar response has been documented in human patients with Cushing's disease or Nelson's syndrome (Krieger and Luria, 1977; Pieters et al., 1979; Ishibashi and Yamaji, 1981). Some, but not all, patients with these conditions have been reported to have an increase in circulating concentration of both ACTH and cortisol following TRH. Human pituitary adenoma tissue in culture also releases ACTH in response TRH treatment, whereas tissue from healthy individuals does not (Ishibashi and Yamaji, 1981). This suggests aberrant receptor expression or function in adenomatous cells, although this was not directly investigated.

Horses with Cushing's disease (PPID), unlike humans, have almost exclusively adenomas of pars intermedia rather than pars distalis origin. Therefore, the mechanism responsible for cortisol secretion following TRH administration in horses likely differs from that of humans. In frogs and pigs, TRH has been shown to be a physiological releasing factor of the pars intermedia leading to α-MSH release both *in vivo* and in cultured melanotropes (Tonon et al., 1983; Trouslard et al., 1989). However, rats have well developed intermediate lobes yet do not release pars intermedia peptides when treated with TRH (Kraicer, 1977). Therefore, it is necessary to investigate pituitary regulatory mechanisms in each species individually.

We hypothesized that thyrotropin releasing hormone directly stimulates the equine pars intermedia melanotrope, resulting in release of POMC-derived peptides. Specifically,

we sought to determine 1) if plasma α-MSH and ACTH concentrations increase following TRH administration in normal horses and horses with PPID; 2) if pars intermedia explants from normal horses release α-MSH and ACTH following TRH treatment; and 3) if TRH receptor is expressed in pars intermedia melanotropes.

3.2 METHODS AND MATERIALS

Animals:

All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care, following approval by the UPEI Animal Care Committee. The study group for the *in vivo* TRH response test was comprised of sixteen clinically healthy horses, (median age: 11 years; age range: 3-24 y; 6 mares, 10 geldings) and 7 affected horses (median age: 24 years; age range: 16- 30 y; 4 mares, 3 geldings.) All healthy horses were confirmed free of pituitary disease by postmortem gross and histologic examination. All affected horses were selected based on overt clinical signs of PPID and confirmed by either or both the dexamethasone suppression test or the presence of pars intermedia adenomatous hyperplasia or an adenoma on postmortem examination. All affected horses had an increase in plasma cortisol concentration following TRH administration. Tissue for PI explant studies was obtained from three clinically healthy horses, euthanized for unrelated reasons (ages: 12, 17 and 20 years; 2 mares, 1 gelding). PI obtained from an additional four normal horses (ages: 18, 18, 19 and 24; 3 mares, 1 gelding) and two horses with histologic evidence of adenomatous hyperplasia (ages: 14 and 24; 1 mare, 1 gelding) were used to assess the expression of TRH receptor mRNA.

Assay methodology:

Alpha-melanocyte stimulating hormone (α-MSH) was measured in equine plasma and in culture media using a commercial radioimmunoassay (Euria-α-MSH RIA, American Laboratory Products Co, Windham, NH) previously validated for use with equine plasma

(McFarlane et al., 2004). Intra- and interassay variation was 5% at both high and low concentrations. Samples with α-MSH concentrations outside the range of the standard curve were diluted as necessary. Plasma adrenocorticotropin and cortisol were measured by radioimmunoassay at a commercial veterinary diagnostic laboratory (Michigan State University, Diagnostic Center for Population and Animal Health, DCPAH) using assays validated for use in the horse. Sensitivity of the ACTH assay, as reported by the manufacturer, was 0.3 pmol/L (Nichols Institute Diagnostics, San Clemente CA), and intra- and interassay variation was < 8% (personal communication, KR Refsal, Michigan State University, DCPAH). Data provided by the manufacturer indicated no cross reactivity with α-MSH or β-endorphin. Protein content of samples was determined using a commercial assay, using bovine serum albumin as a standard (Biorad DC Protein Assay, Hercules, CA).

In vivo TRH response test:

Thyrotropin releasing hormone (1 mg; Sigma Chemical Co, Oakville, Ontario, Canada) was dissolved in sterile 0.9% saline (1 ml) and administered intravenously. Control horses received only 0.9% saline. Venous blood was collected into vacuum tubes containing EDTA as an anticoagulant prior to and 30 minutes after treatment. Samples were stored on ice until processed. Plasma was separated within 1 hour of collection and stored at -80°C until assayed for α-MSH, ACTH and cortisol.

TRH response in pituitary explants:

Pituitaries were collected within 15 minutes following euthanasia of horses with

pentobarbital (125mg/kg). The pituitary was bisected sagittally along midline. Half of the pituitary was fixed in 10% buffered formalin for histology. The other half was washed 3 times in ice cold PBS. The pars distalis and pars intermedia were dissected, finely minced, then divided into two aliquots each. Tissue was transferred to 9.5 cm^2 culture wells containing 2.5 mls of RPMI media supplemented with 10% horse serum, 5% fetal calf serum, 2mM glutamine, 10 U/ml of penicillin and 10 U/ml streptomycin. One aliquot of PD and PI was supplemented with 500 nM of TRH (44 µl of 10ug/ml stock solution in phosphate buffered saline, PBS; pH 7.4). The other aliquot received an equal volume of PBS. Plates were incubated at 37°C, in 5% CO_2 for 24 hours. At 24 hours, culture media and tissue samples were transferred to separate 2 ml polypropylene tubes and spun at 1700*g* for 10 minutes at 4°C. The supernatant was transferred to a new tube for hormone determination and the tissue pellet was homogenized in 250 µl of PBS for protein determination. α-MSH and ACTH were expressed in pmol/gm protein and results from TRH treated explants were expressed as a percent of the corresponding PBS control.

RT-PCR:

The pituitary was removed from four normal and two affected horses immediately following euthanasia. Pars distalis and pars intermedia tissue were isolated using a dissecting microscope. Tissue samples were finely minced. Tissue was placed in 500 µl TRIzol™ (Molecular Research Center, Cincinnati, OH) then snap frozen in liquid nitrogen for total RNA isolation. Total RNA was extracted, and treated with DNase (Ambion, Austin, Texas) to remove any genomic DNA contamination. RNA was reverse transcribed into cDNA using random primers (Thermoscript RT PCR kit, Invitrogen,Carlsbad, CA).

Polymerase chain reaction was performed for TRH and gonadotropin releasing hormone (GnRH) receptors using the following primer sets:

TRHR forward: TCTGTCACCCCATCAAAGCCCAG;

TRHR reverse: GGGTTGATGGCACTGTTGAGATA;

GnRHR forward: GTATGGCAAACAGTGCCTCTCCTG;

GnRHR reverse: CTGGATCTGACACCCTGTTTAAC.

TRHR primers were selected from regions of the TRH receptor 1 gene found to be highly conserved in other species (http://www.ncbi.nlm.nih.gov/BLAST.) GnRHR primers were selected based on published sequence for the equine GnRH receptor (Porter et al, 1997). RT controls included a sample in which the mRNA was replaced with water. PCR controls included the RT water control, a sample in which the RT reaction was omitted, and a sample where the cDNA was replaced with water.

Standard PCR reactions were performed in a thermal cycler (MJ DNA engine, Watertown, MA) using 30 cycles of annealing at 56°C for 20 seconds, extension at 76°C for 1 min, and denaturation at 94°C for 20 seconds. To confirm the amplification of TRH receptor, the resulting PCR products were gel purified and the amplified cDNA was sequenced in the forward and reverse direction (Guelph Molecular SuperCentre, Ontario, CA, ABI Prism 3100). The sequences were compared to known published sequences in other species (nucleotide- nucleotide BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

Real-time PCR was performed in a Rotor-Gene 6 (Corbett Research, Mortlake, Australia) using the primers listed above and a quantitative PCR kit (Platinum® SYBR® Green qPCR SuperMix-UDG; Invitrogen, Carlsbad, CA) with the following reaction conditions: 50°C for 2 min; 95°C for 2 min; then 45 cycles of 95°C for 15 sec, 55 °C for

30 sec, 72 °C for 30 sec. Dynamic tube correction was applied and number of cycles to threshold was calculated for both mRNA and the expression of TRH receptor mRNA corrected to GnRH receptor mRNA.

Statistics:

All statistical calculations were performed using commercial statistical software (Minitab, Version 13.32, Minitab Inc, State College, PA; Prism Version 3.03, GraphPad Software, San Diego, CA). Normality of the data were confirmed using the Kolmogorov-Smirnov test. Paired data (*in vivo* stimulation and explant data) were compared using a paired t-test. Data from normal horses were compared to PPID-affected horses using an unpaired t-test. For all statistical comparisons, a $P \leq 0.05$ was considered significant. All results are expressed as mean ± standard error of the mean.

3.3 RESULTS

Healthy horses showed a marked increase in plasma α-MSH 30 minutes after administration of TRH (10 ± 1.9 versus 42.8 ± 7.4 pmol/L, $P<0.001$; baseline and post-TRH, respectively) (FIG 1A). Plasma α-MSH concentration in PPID horses also increased significantly 30 minutes after TRH administration (111.8 ± 31.9 versus 619 ± 176 pmol/L, $P<0.05$; baseline and post-TRH, respectively) (FIG 1B). Both the baseline and post-TRH plasma α-MSH concentrations were significantly greater in PPID compared to normal horses ($P<0.05$).

Plasma ACTH concentration increased in healthy horses 30 minutes after administration of TRH (3.6 ± 0.5 versus 6.2 ± 0.5 pmol/L, $P<0.001$; baseline and post-TRH, respectively) (FIG 1A). Plasma ACTH concentration in PPID horses also increased significantly 30 minutes after TRH administration (16 ± 3.7 versus 42.1 ± 9.2 pmol/L, $P<0.01$; baseline and post-TRH, respectively) (FIG 1B). As with α-MSH, baseline and stimulated ACTH concentrations were significantly higher in horses with PPID ($P<0.05$).

Plasma cortisol concentration following TRH stimulation increased in normal horses (153 ± 14.8 versus 205 ± 9.3 nmol/L, $P<0.001$; baseline and post-TRH, respectively) and horses with PPID (128 ± 20 versus 227 ± 22 nmol/L, $P<0.001$; baseline and post-TRH, respectively). Baseline and post-TRH plasma cortisol concentrations were not significantly different between normal and PPID horses. Plasma cortisol concentration increased more than 50% in 7 of the 16 normal horses and 5 of the 7 PPID horses (FIG 2). To confirm that the plasma increase in α-MSH was the result of a direct stimulation of the pars intermedia, pars intermedia explants were exposed to TRH. Explants were treated for

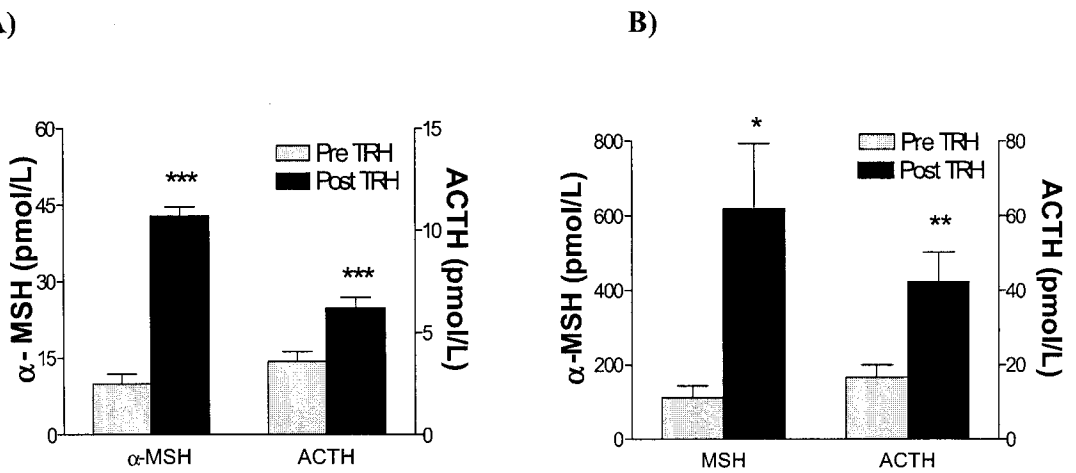

Figure 1: Thyrotropin releasing hormone (TRH) response *in vivo*.

(A) Plasma α- MSH or ACTH concentration increased significantly in healthy horses (n=16) 30 minutes after intravenous administration of 1mg TRH compared to resting concentration . Lack of pars intermedia disease was confirmed at necropsy. (B) Plasma α- MSH or ACTH concentration in horses with clinically overt PPID (n=7) increased significantly 30 minutes after intravenous administration of 1mg TRH. Pars intermedia disease was confirmed at necropsy (n=5) or by an abnormal dexamethasone suppression test (n=2). *P<0.05, **P<0.01, ***P<0.001

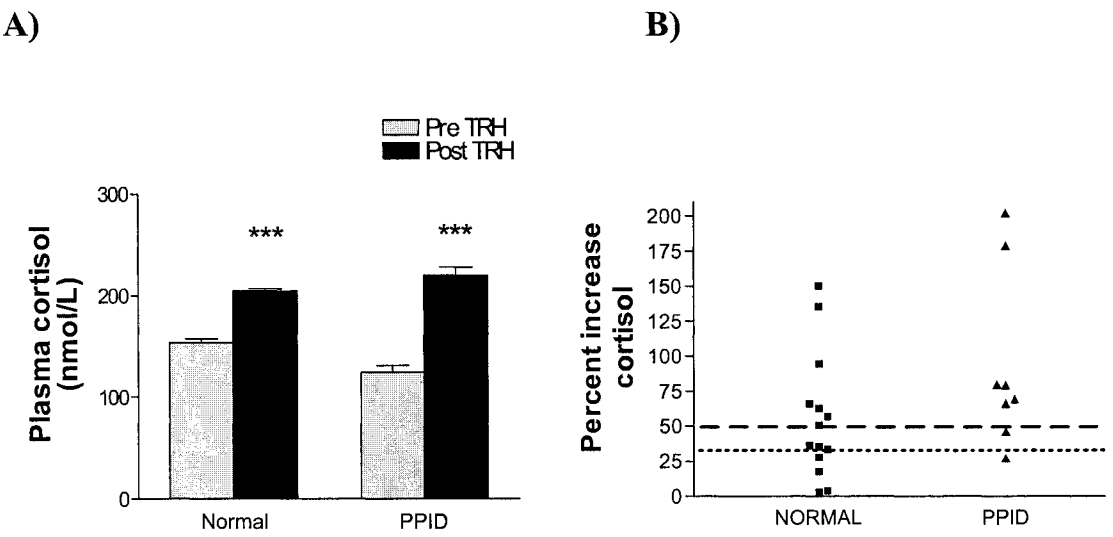

Figure 2: Plasma cortisol concentration following thyrotropin releasing hormone (TRH).

(A) Absolute plasma cortisol concentration after TRH increased significantly in normal (n=16) and PPID affected (n=7) horses compared to resting concentration ($P<0.001$). (B) Relative plasma cortisol response following TRH stimulation in healthy (n=16) and PPID (n=7) horses. Cortisol concentration increased following TRH in all affected horses as expected, however the concentration increased in many of the normal horses as well. Dotted line= 30% increase; Dashed line=50% increase

24 hours to ensure maximal response with the limited tissue supply, and because TRH half life was not an issue using an *in vitro* system. Pars intermedia tissue treated 24 hours with TRH showed a 2.9 fold increase in α–MSH concentration in the supernatant ($P<0.05$), whereas no α–MSH was produced by the pars distalis explants (FIG 3). Following TRH treatment, all pars distalis explants showed an increase in supernatant ACTH concentration (median 2.7 fold increase, range 2.4-231), while no ACTH was produced in pars intermedia explants (median 0.59 of control, range 0.19- 0.97). Although the pattern of the response was similar in all three tissue explants (that is ACTH increased in all of the PD and none of the PI explants) the increase in ACTH in the pars distalis explants was not statistically significant because of the variability in the response.

Using RT-PCR, TRH receptor mRNA was found to be strongly expressed in both the pars intermedia and pars distalis, while GnRH mRNA was only strongly expressed in the pars distalis (FIG 4). Real-time PCR was used to more quantitatively assess the ratio of TRH receptor mRNA to GnRH receptor mRNA in isolated pars intermedia and pars distalis samples. GnRH receptor mRNA was identified in all pars intermedia samples, but the expression of TRH receptor mRNA relative to GnRH receptor mRNA expression was 2.7 ± 0.7 times greater in the pars intermedia than the pars distalis ($P<0.05$). This confirms the expression of TRH receptor mRNA in the pars intermediate at levels greater than expected by pars distalis contamination alone. No apparent difference in mRNA expression was observed between the samples from normal and PPID horses, although there were too few samples to adequately compare expression levels. Sequence analysis (FIG 5) confirmed high homology between the amplified product and published sequences from dog (96%), sheep (94%), cow (94%), human (93%), rat (91%), and mouse (91%).

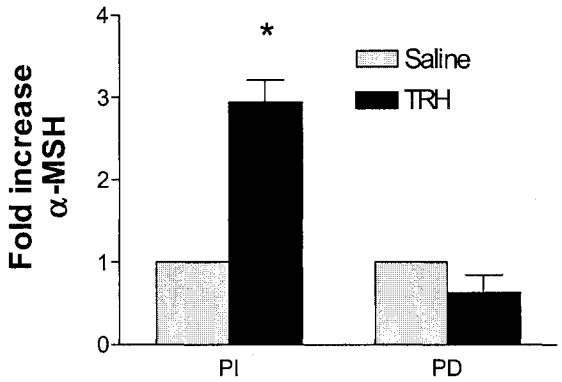

Figure 3. Alpha-MSH response to TRH in pars intermedia and pars distalis tissue explants (n=3). *$P< 0.05$

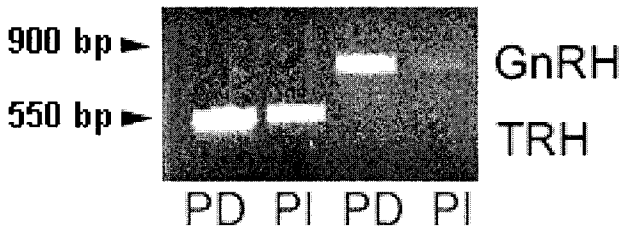

Figure 4. RT-PCR.

TRH receptor 1 was expressed in both pars distalis tissue (Lane 1) and pars intermedia tissue (Lane 2) resulting in amplification of a 550 basepair product. GnRH receptor on the other hand, was expressed in the pars distalis (Lane 3, 900 basepair product), but only a faint band was present in the pars intermedia tissue (Lane 4).

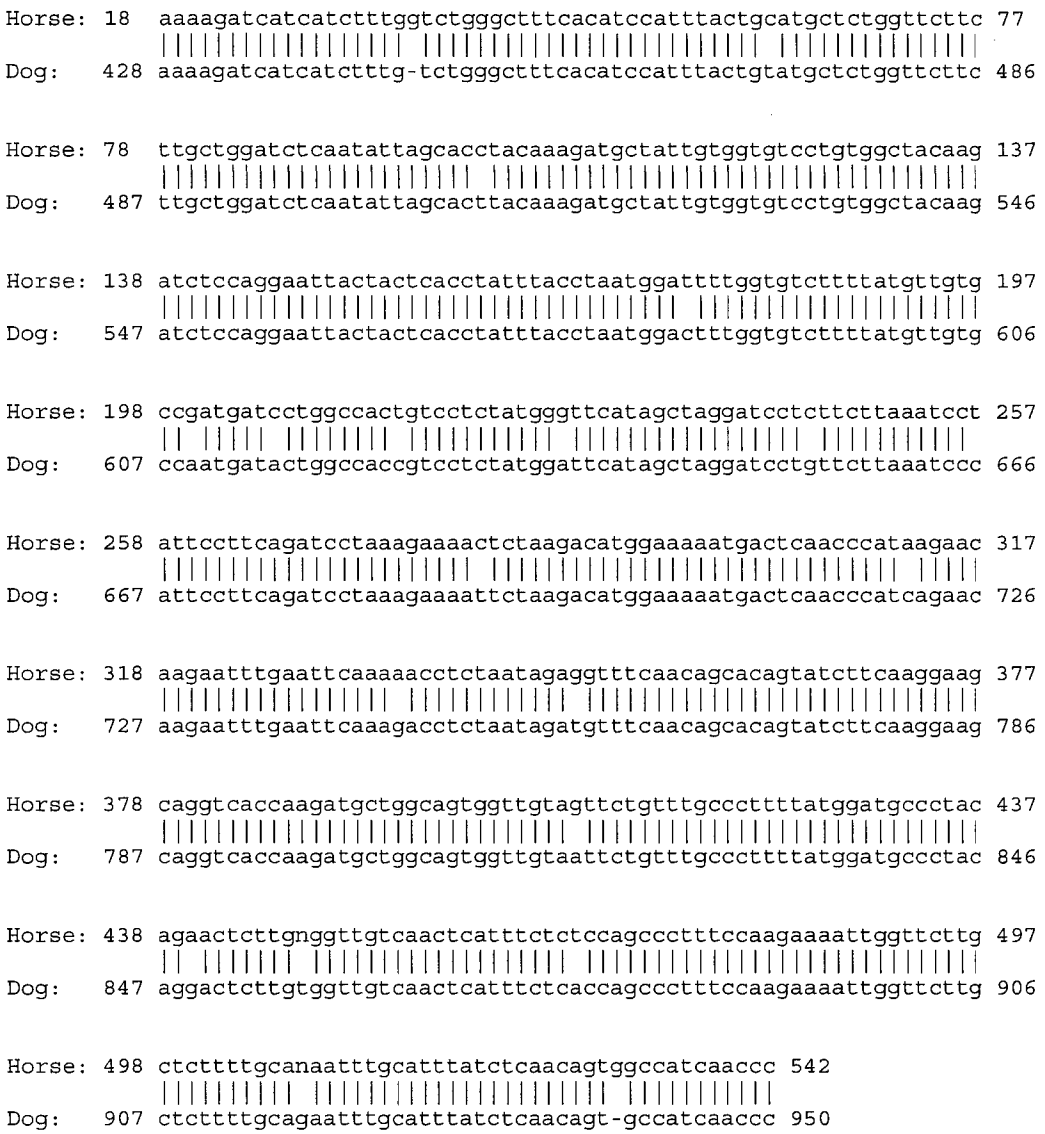

Figure 5. TRH receptor 1 sequence from the horse (5' to 3') compared to the dog (http://www.ncbi.nlm.nih.gov/BLAST gi | 57095411| ref | XM 539128).

The equine TRH-R sequence is well conserved compared to dog (96%), sheep (94%), cow (94%), human (93%), rat (91%), and mouse (91%).

3.4 DISCUSSION

Our findings support the hypothesis that TRH is able to directly stimulate the release of POMC-derived peptides from the pars intermedia. POMC is a hormone precursor protein that is expressed in both melanotropes in the pars intermedia and corticotropes in the pars distalis (Raffin-Sanson et al., 2003). ACTH and α-MSH are both products of POMC processing. Corticotropes lack prohormone convertase II, the enzyme necessary to cleave ACTH, therefore the major product of POMC processing in the pars distalis is ACTH (Raffin-Sanson et al., 2003). In the pars intermedia, α-MSH, a product of ACTH cleavage, is produced (Raffin-Sanson et al., 2003). We used this cell-specific POMC processing to determine if TRH was able to stimulate the release of POMC-derived peptides from the PI.

In healthy horses, we observed an increase in plasma α-MSH concentration following TRH administration (FIG 1). Using pituitary explants, TRH was shown to directly stimulate α-MSH release from pars intermedia tissue but not from pars distalis tissue (FIG 3) similar to what has been observed in pituitary tissue explants from frogs and pigs (Tonon et al., 1983; Trouslard et al., 1989). These findings suggest TRH is a melanotropic releasing factor in the normal horse pars intermedia, stimulating release of α-MSH.

Plasma α-MSH also increased following TRH administration in horses with PPID, showing this response was conserved with disease. The magnitude of this response did not differ in horses with PPID compared to the normal horses (618% ± 149% versus 1046% ± 492% of control, P=0.43; healthy and PPID, respectively), although the absolute α-MSH

concentration was greater in horses with PPID, both before and 30 minutes after TRH administration (FIG 1). Therefore, the ability of TRH to stimulate the PI is a conserved response in horses with PPID rather than a pathological response.

Thyrotropin releasing hormone is known to mediate its cellular effects by interacting with a cell membrane associated, G-coupled TRH receptor. To date, two receptors (TRH R1 and TRH R2) have been identified (Gershengorn and Osman, 1996; O'Dowd et al., 2000). *In situ* studies in rodents have found TRH R1 distributed in the pituitary and the central nervous system, whereas TRH2 is in the central nervous system only. Therefore, if TRH has a direct action on the equine pars intermedia, TRH R1 expression would be expected in melanotropes. We were able to successfully amplify TRH RI mRNA from the pars intermedia and confirm the correct mRNA had been amplified by comparing its sequence to TRH receptor nucleotide sequence from other species.

Amplification of TRH receptor mRNA in pars intermedia samples suggests that the receptor is expressed in the melanotropes found there. However, it might be the result of contamination of the pars intermedia sample with pars distalis tissue due to inaccuracy of dissection. Clean dissection of the pars intermedia, without pars distalis contamination, is difficult in the normal horse. Therefore, to assess the accuracy of dissection, the presence of GnRH receptor mRNA was used as a marker of pars distalis tissue. GnRH receptor is expressed in the pars distalis, but not the pars intermedia. If the TRH receptor mRNA was present in the pars intermedia samples due to contamination with pars distalis tissue, then the ratio of TRH receptor mRNA to GnRH receptor mRNA should be similar in both samples from the same horse.

Routine PCR revealed markedly less GnRH receptor mRNA in the pars intermedia (FIG 4). Real-time PCR was used to more quantitatively assess the expression of TRH receptor mRNA relative to GnRH receptor mRNA in isolated pars intermedia and pars distalis samples. The ratio of TRHR mRNA expression to GnRHR mRNA was greater in the pars intermedia tissue than in the pars distalis tissue sample from the same horse, supporting the contention that TRH receptor expression in the pars intermedia was not solely attributable to pars distalis contamination.

Ideally, protein expression of TRH receptor in the pars intermedia would be shown in addition to mRNA. However, commercially available TRH receptor antibodies (TRH-R1 goat polyclonals # sc-11570 and # 11574, Santa Cruz Technologies, CA; TRH-R1 rabbit polyclonal, #LS-A1200, Lifespan Biosciences, Seattle, WA) failed to recognize the equine receptor in Western blot analysis or immunohistochemistry.

The basis of the proposed equine TRH stimulation test is that it leads to an increase in plasma cortisol, presumably as a result of direct stimulation of ACTH release by TRH, although this was not directly tested (Beech and Garcia, 1985). In the present study, plasma ACTH concentrations increased following TRH administration in healthy and PPID horses. In healthy horses, this was likely predominantly from the pars distalis, as *in vitro* ACTH was released from pars distalis but not pars intermedia tissue isolated from normal horses. In horses with PPID however, the source of the ACTH is likely the PI rather than the PD. Horses with PPID do not suppress when treated with dexamethasone, suggesting that ACTH is no longer arising from the pars distalis, as the PD is normally under feedback regulation (Dybdal et al., 1994). Immunoreactive ACTH has been detected in the PI of normal and PPID-affected horses (Orth and Nicholson, 1982; Heinrichs et al.,

1990; Boujon et al., 1993) and it can be released from the pars intermedia following stimulation in other species (Kraicer, 1977; Kemppainen et al., 1989). The increased release of immunoreactive ACTH from the pars intermedia could occur as a result of hyperplasia, leading to sufficient ACTH of PI origin being released to cause a change in systemic concentrations. Alternatively, it has been suggested that ACTH is not completely processed in the melanotropes of horses with PPID, potentially due to limited activity of prohormone convertase II relative to prohormone convertase I (Wilson et al., 1982; Orth and Nicholson, 1982). This would lead to a greater than normal content of ACTH in the PI that would be available for release upon TRH stimulation. The ACTH contained in the PI has also been reported to not be as bioactive as ACTH released from the pars distalis (Orth and Nicholson, 1982). This is consistent with the observation that cortisol was not elevated to the extent expected by the increased ACTH in horses with PPID. Thus, our data, while not proof, are consistent with the PI being the source of the ACTH released in horses with PPID prior to and following TRH stimulation.

The expression of the TRH receptor in pars intermedia tissue, as well as the ability of TRH to elicit α-MSH release *in vivo* and *in vitro* supports the conclusion that TRH has a direct stimulatory effect on the pars intermedia. Receptor expression and *in vivo* plasma α-MSH increase following TRH administration in horses with PPID suggests this response is preserved in animals with disease. This is in contrast to humans with Cushing's disease or Nelson's syndrome where aberrant receptor expression or function is believed to be responsible for the abnormal cortisol release that occurs following TRH administration.

The overnight dexamethasone suppression test has been considered the "gold standard" method of ante mortem PPID diagnosis. In the unaffected horse, intramuscular

administration of dexamethasone decreases the release of ACTH from the pars distalis, resulting in a serum cortisol concentration of less than 1 pg/ dl 19 hours later (Dybdal et al., 1994). Horses with PPID fail to suppress serum cortisol concentration, presumably due to ACTH production from the pars intermedia. Originally this test was reported to have a sensitivity and specificity of 100% (Dybdal et al., 1994). However, a recent report suggests the reliability of the test has been over-estimated (Meisner et al., 2003). In addition, seasonal variations have been documented, with ponies and horses failing to suppress in the fall (Donaldson et al., 2005). Although it has not been critically assessed, a loss of glucocorticoid suppression by dexamethasone may be a late event in the disease progression, and the high sensitivity originally reported may reflect a case selection bias towards horses with advanced disease. Other limitations of the dexamethasone suppression test include the need for multiple day sampling and the potential exacerbation of laminitis in horses with current or historical laminitis (Dybdal et al., 1994; Schott, 2002). Therefore, alternative diagnostic tests are being sought.

The TRH stimulation test is one alternative test that has been evaluated. An increase in plasma cortisol concentration 15-90 minutes following TRH administration is consistent with the diagnosis of PPID (Beech and Garcia, 1985). Although the magnitude of increase expected with disease has not been rigorously assessed, a minimum increase of 30% (Schott, 2002) to 50% (McFarlane and Cribb, 2004) has been proposed as consistent with disease. Although the experiments reported here were not designed to evaluate the ability of the TRH stimulation test to diagnosis PPID, our results indicate the test is unlikely to be useful. In our study, all horses with PPID had an increase in cortisol following TRH administration (Fig 2). However, unlike the previous study (Beech and

Garcia, 1985), many of the normal horses did as well. Ten of the 16 normal horses had a cortisol increase of greater than 30%. Seven of these had an increase of greater than 50%. These horses would have been falsely diagnosed as having disease, indicating TRH stimulation is of limited use as a diagnostic test for PPID. The cause of the disparity in TRH response in normal horses observed in the current and previous study is not apparent as a similar dose of TRH was used in each study. It is possible season may influence TRH response as it does dexamethasone suppression, endogenous ACTH and α-MSH concentration (McFarlane et al., 2004; Donaldson et al., 2005). Season was not taken into account in either study (Beech and Garcia, 1985). Further work is necessary to determine if the TRH stimulation test is more discriminating when performed during specific seasons.

The data presented shows TRH can directly stimulate the equine PI to release POMC-derived peptides in healthy and PPID affected horses, suggesting this may be the mechanism of TRH-stimulated cortisol release. The physiologic role of TRH in regulation of release of POMC-derived peptides from the equine PI needs to be explored. Performance of the TRH stimulation test needs to be validated using a larger number of horses during specific seasons to assess its usefulness as a discriminating diagnostic test. Interpretation of diagnostic results should be performed with attention to clinical findings, as all diagnostic tests for PPID likely have limitations.

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3.6 SIGNIFICANCE OF FINDINGS

We demonstrated that thyrotropin releasing hormone (TRH) is a releasing factor at the pars intermedia melanotrope and concluded the increase in plasma cortisol observed following TRH administration results through this physiological pathway.
This is the first report of a releasing factor active at equine melanotrope.

Furthermore, we found the TRH stimulation test was not able to discriminate horses with PPID from normal horses (Figure 2, page 93). Therefore, this diagnostic method was not used in future studies in this thesis to identify horses with PPID.

CHAPTER 4:

Discordance in histological assessment of the pituitary pars intermedia in aged horses.

In press as:

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4.1 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID, equine Cushing's disease) is the most common endocrine disease of horses and ponies. Horses with PPID are at high risk for life threatening complications, including laminitis, secondary infections and metabolic derangements (Schott, 2002). Therefore, early and accurate diagnosis of this condition is critical. Ante mortem diagnosis of PPID currently relies on testing hypothalamic-pituitary-adrenal axis responsiveness or measurement of endogenous plasma concentrations of proopiomelanocortin (POMC) derived peptides, such as ACTH.
The overnight dexamethasone suppression test (DST) is considered the "gold standard" method of ante mortem PPID diagnosis. In the unaffected horse, intramuscular administration of dexamethasone decreases release of ACTH from the pars distalis, resulting in a serum cortisol concentration of less than 1 μg/ dl (27.59 nmol/L) 19 hours after dexamethasone (Dybdal et al., 1994). Horses with PPID fail to suppress serum cortisol concentration due to ACTH production from the pars intermedia. Originally this test was reported to have a sensitivity and specificity of 100% (Dybdal et al., 1994). However, a recent report suggests the reliability of the test has been over-estimated (Miesner et al., 2003). In addition, seasonal variation in response to dexamethasone has been documented (Donaldson et al., 2005). Clinically normal ponies and horses had a normal DST in spring but when the same animals were tested in the fall, they failed to suppress. Although it has not been critically assessed, a loss of feedback inhibition by glucocorticoid may be a late event in the disease progression, and the high sensitivity originally reported may reflect a case selection bias towards horses with advanced disease.

Other limitations of the DST include the need for multiple day sampling and the potential exacerbation of laminitis in horses with current or historical laminitis (Dybdal et al., 1994; Schott, 2002).

Due to the limitations of the overnight DST, other ante mortem tests such as endogenous ACTH or α-melanocyte stimulating hormone plasma concentration (Couetil et al., 1996; Horowitz et al., 2003; McFarlane et al., 2004), thyrotropin releasing hormone response test (Beech and Garcia, 1985; McFarlane and Cribb, 2004) or combined hormone response tests (Dybdal et al., 1994; van der Kolk et al., 1995; Eiler et al., 1997; Andrews et al., 2005) have been the subject of recent, more critical evaluation. Emphasis has been placed on their potential for early diagnosis. Sensitivity and specificity of the diagnostic tests have been established by comparing results to clinical signs and postmortem diagnosis (Dybdal et al., 1994, Couetil et al., 1996; McFarlane et al., 2004; Beech and Garcia, 1985; Eiler et al., 1997; van der Kolk et al., 1995; Andrews et al., 2005). In aged horses with advanced disease, the presence of clinical signs including hirsutism, laminitis, muscle atrophy, abnormal fat distribution, polydipsa and polyuria, hyperhidrosis, and secondary infections may be sufficient for diagnosis. In horses with early disease however, the clinical signs are often difficult to distinguish from the changes observed with normal aging. Therefore, heavy emphasis is placed on postmortem diagnosis of PPID for establishing accuracy of ante mortem diagnostic testing protocols. The purpose of this study was to evaluate the concordance among pathologists in the histological assessment of pars intermedia pathology in pituitary sections from aged horses.

4.2 METHODS AND MATERIALS

Seven diplomates of the American College of Veterinary Pathology were recruited for participation in the study. Pathologists were selected for having a stated interest in endocrine disease and/or being at an institute where equine endocrine research is actively conducted. Each pathologist received a set of slides consisting of a single section from 10 individual horse pituitaries, and a description of the animals' signalment and clinical signs (Table 1). Pathologists were instructed to describe the histological findings for each section as they would a case presenting to their pathology service. In addition, pathologists were asked to give an opinion as to whether the section was diagnostic for PPID. Pathologists were unaware of the results of the endocrine diagnostic test at the time of evaluation.

Seven consecutive, 5 μm thick formalin-fixed, paraffin embedded pituitary sagittal sections were cut from ten aged horses. Each section was mounted on a separate glass slide treated with 3-aminopropyltriethoxysilane diluted in acetone and stained with hematoxylin and eosin. Animals were selected for the presence of mild to moderate clinical signs consistent with PPID (Table 1). Nine of the 10 horses had an overnight DST prior to euthanasia (Table 2). One horse (horse 1) had received triamcinolone acetate for treatment of airway disease several weeks prior to presentation and results of the DST were not useful. A second horse (horse 9) presented in distress and was euthanized prior to DST.

Case	Age	Gender	Breed	Clinical signs
1	18	G	Pony X	COPD (treated periodically with triamcinolone), severe muscle atrophy, persistent guard hairs
2	20	F	QH	History laminitis, mild-moderate muscle atrophy, metritis
3	22	G	TB	Good body condition, good haircoat
4	24	F	Standardbred	Mild muscle atrophy
5	25	G	QH	Mild muscle atrophy of topline, DJD
6	30	G	QH	Thin, possible narcolepsy, ± lethargy
7	15	G	Pony/ QH X	Overweight, cresty neck, history laminitis
8	22	F	App	Generalized weight loss, moderate muscle atrophy, some persistent guard hairs, periorbital squamous cell carcinoma with conjunctivitis
9	32	G	TB X	Severe muscle atrophy, COPD (untreated), tachycardia, jugular pulses
10	30	F	Standardbred X	Persistent guard hairs, severe DJD

TABLE 1: Signalment and clinical signs of study horses

Abbreviations: F=female; G=gelding; X=cross; TB=thoroughbred; QH= quarter horse; APP=Appaloosa; COPD=chronic obstructive pulmonary disease; DJD=degenerative joint disease

Case	Month of testing	MSH (pmol/L)	DST
1	Dec	7.3	Not diagnostic
2	Jan	26.8 *	Negative
3	May	4.9	Negative
4	Jan	3.9	Negative
5	July	13.9	Negative
6	Feb	10.7	Positive
7	May	19.3 *	Negative
8	Sep	97.3	Negative
9	July	122.3 *	Not done
10	Jan	6.7	Negative

TABLE 2: Ante mortem diagnostic test results and date of testing

* denotes animals positive for PPID using seasonally specific α–MSH reference values (see methods)

α–melanocyte stimulating hormone (MSH) plasma concentration was measured in all ten horses using a radioimmunoassay previously validated for use in horses (Table 2) (McFarlane et al., 2004). Plasma concentration of > 91 pmol/L was previously reported to be diagnostic of PPID (Horowitz et al., 2003). This reference value was established with 63 horses, (38 unaffected and 25 affected horses) using the DST as the gold standard. Sensitivity and specificity was reported to be 88% and 84.2%, respectively. However, seasonal variation in α-MSH concentration and DST was not considered when determining this reference range. In fact, all the control horses were tested in the fall, while the affected horses were tested throughout the year (personal communication, J Watson).

Therefore, seasonally specific reference ranges (Chapter 2.6, page 96) were used in this study. Non-fall plasma concentrations of > 19 pmol/L and fall concentrations > 83 pmol/L in horses and > 194.5 pmol/L in ponies were considered consistent with a diagnosis of PPID.

Results of histological evaluation were analyzed for agreement among pathologists by establishing multi-rater kappa values using statistical software (SAS, Version 8.2, SAS Institute, Cary, NC). Agreement of diagnosis by histology with ante mortem diagnostic test results (either α-MSH or DST) was compared.

4.3 RESULTS

The horses in this study had a mean age of 23.8 years, with a range from 15 to 32 years. The group consisted of 4 mares and 6 geldings of various light breeds including ponies (Table 1). The most common clinical signs suggestive of PPID were muscle atrophy or weight loss (7/10), persistent guard hairs (2/10), and laminitis (2/10). One of the laminitic animals was a grossly obese pony, the other laminitic horse had chronic metritis. None of the horses exhibited hirsutism.

All seven of the pathologists provided written reports evaluating each pituitary section. Five of the pathologists commented they were limited by either irregular plane of section or the lack of multiple sections for each horse. Five of the pathologists made the comment that PPID was a clinical diagnosis only. Four limited their evaluation of the sections to histological descriptions, providing no final diagnosis. Therefore, a finding of pars intermedia adenoma, adenomatous hyperplasia, nodular hyperplasia or microadenoma(ta) was considered consistent with PPID for the purpose of statistical analysis. Table 3 summarizes some of the common observations of the pathologists.

Kappa values were calculated to assess inter-rater agreement. Perfect agreement among raters would have a kappa value equal to 1; no agreement would have a kappa value of 0. The kappa value for inter-rater agreement for interpretation of all ten sections was 0.34 (SEM=0.069, P <0.0001). A significant kappa value suggests agreement was greater than that expected due to chance alone. Agreement was also assessed for interpretation of sections from horses without overt disease. A kappa value of 0.04 (SEM=0.08, P=0.29) was calculated for inter-rater agreement in interpretation of eight sections, all except

Column	2	3	4	5	6	7
Horse	PPID	hyperplasia	adenoma	adenomatous	hypertrophy	normal
1	e	b c e g		c d e	c e g	f
2	d e f	a b c e f	d f g	a b c e f	a c	
3	e	b c e			e g	a d f
4	d f	b c e f		d f	g	a
5	e	b c g		b e	c g	d f
6	d e	b c	g	b d e	c	f
7	d e	b c e	g	c d e	c	f
8		b e g			c g	b d f g
9	d e f	a b c d f	g	a b c d e f	a c g	
10	d	b e g	d	d	g	b c f

TABLE 3: Summary of pathologists (a-g) descriptions of sections

Pathologists who specified histological findings were consistent with PPID are listed in column 2. Columns 3-6 list pathologists who described pars intermedia "hyperplasia", "adenoma", "adenomatous hyperplasia" or melanotrope "hypertrophy", respectively. Pathologists who stated the histological findings of the sections were consistent with that of a normal aged horse are listed in column 7.

sections from horses 2 and 9. This suggests agreement was no greater than what would be expected due to chance alone when pituitaries from aged horses without overt disease were evaluated.

Plasma α-MSH concentration in this study ranged from 3.9 to 122.3 pmol/L with a mean of 31.3 pmol/L. Using a reference value of 91 pmol/L (Horowitz et al., 2003) two horses (horses 8 and 9) had concentrations consistent with a diagnosis of PPID. Using seasonally specific cut-off values, horses 2, 7 and 9 would be considered positive, and horse 8 would be considered negative. Only horse 6 was diagnosed with PPID using overnight dexamethasone suppression. Therefore, four horses had an ante mortem diagnosis of PPID (Table 2) by either DST or increased α-MSH plasma concentration. Overall, histological assessment by the seven pathologists was in agreement with ante mortem diagnostic test results 79% of the time (Table 4).

Horse	Antemortem diagnosis: PPID*	Histologic assessment: PPID#	% concordance
1	**negative**	**3/7**	**57.1**
2	**positive**	**7/7**	**100**
3	**negative**	**1/7**	**85.7**
4	**negative**	**2/7**	**71.4**
5	**negative**	**2/7**	**71.4**
6	**positive**	**4/7**	**57.1**
7	**positive**	**4/7**	**57.1**
8	**negative**	**0/7**	**100**
9	**positive**	**7/7**	**100**
10	**negative**	**1/7**	**85.7**

TABLE 4: Comparison of ante mortem diagnostic test results and postmortem diagnosis

KEY: * Ante mortem diagnosis was determined by either an abnormal DST or plasma α-MSH concentration.

Proportion of pathologists who considered histological findings in pituitary section consistent with PPID.

% concordance refers to the percent of pathologists whose histological diagnosis were in agreement with ante mortem diagnostic test results for that horse.

4.4 DISCUSSION

Diagnosis of equine pituitary pars intermedia dysfunction (PPID) is not straightforward. PPID is a progressive, degenerative condition and therefore identification of a discrete starting point for the disease is likely to be difficult. Early disease recognition is obscured by the overlap between phenotypic changes accompanying age and disease. Histological changes of the equine pituitary in the healthy aged animal are not well described and likely overlap with lesions observed in early disease. In a recent study of 100 healthy horses, pituitary pathology (cysts, hyperplasia, microadenomas or adenomas) and size (weight, length, width and height) were reported to increase with age (van der Kolk et al., 2004). Changes in the appearance of the pars intermedia when physiologically activated, such as seasonally in the fall, have not been investigated. Evidence of the pathologic changes that accompany disease progression in individual animals is currently lacking.

In reading the comments provided by the pathologists, it was apparent that there were no clear pathologic criteria for diagnosing PPID. One pathologist stated diagnosis was reserved for horses with gross enlargement of the pars intermedia resulting in compression of the hypothalamus, pars distalis and/ or pars nervosa, such that functionality of these regions would be compromised. These horses would be expected to have overt clinical signs, such as hirsuitism. Others considered the disease to encompass adenomas that did not compress adjacent lobes, while some included diffuse hyperplasia and focal microadenomas. Clinical signs might be expected to be more subtle in these cases and likely the result of an increase in plasma concentration of POMC-derived peptides. A

concise definition of the histological findings consistent with PPID is needed to facilitate accurate communication between pathologists and clinicians.

In histological evaluation of two of the pituitary glands (horses 2 and 9) there was 100% agreement among the pathologists (Figure 1). Both of these horses were considered positive for PPID using plasma α-MSH concentration with seasonally specific reference ranges. This suggests consensus may occur more readily in horses with more overt disease. There was strong agreement among pathologists that three of the horses (horses 3, 8 and 10) did not have evidence of PPID (Figure 1). Horses 3 and 10 were among those with the lowest plasma α-MSH concentration. It might be that a disease-free pituitary is more easily recognized in horses less actively secreting POMC peptides. The least agreement among pathologists tended to occur when evaluating horses that had intermediate α-MSH concentrations (Tables 2 and 4). This may reflect the difficulty in differentiating early pathological changes from normal changes associated with physiologic stimulation. As one pathologist queried, "When does pituitary function become dysfunction? (when does) reactive hypertrophy and hyperplasia (become) pathologic?"

It may be that diagnosis of PPID should not be made without ante mortem endocrine testing and overt phenotypic changes consistent with disease. If this is the case, using postmortem histology as a gold standard for evaluation of ante mortem diagnostic tests is inappropriate. Identification of "early disease" status will need to be addressed in a novel manner as the currently available diagnostic tests are not adequately validated for use in early disease. Accurate diagnosis of early disease may not prove possible due to the progressive nature of PPID.

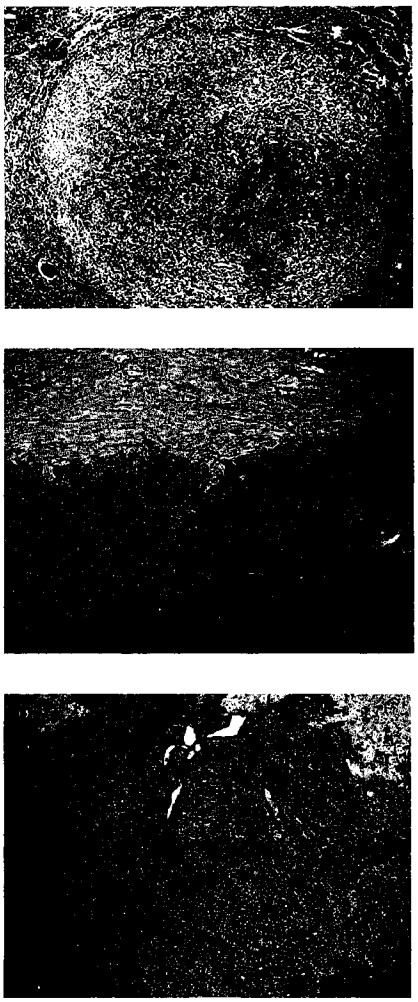

Figure 1. Hematoxylin and eosin stained pituitary sections from three horses

Panel A (horse 2) shows a well demarcated microadenoma within the pars intermedia and pigment deposition in the pars nervosa. There was 100% agreement among the 7 pathologists that the section had histological changes consistent with PPID. Ante mortem testing in this horse revealed increased plasma α-MSH concentration consistent with PPID, but a normal DST. Panel B (horse 3) shows a pituitary from a horse with normal ante mortem diagnostic test results. Six out of seven pathologists considered this section to be normal for an aged horse. Panel C (horse 1) shows a pituitary from a horse with moderate pars intermedia hyperplasia, nests of spindle shaped cells and occasional hypertrophied round cells. The border between the pars intermedia and pars nervosa is ill defined. Plasma α-MSH concentration was intermediate in this horse although well below the cut off value for diagnosis of PPID. There was no consensus among pathologists in histological evaluation of this section. Magnification for all three panels, BAR = 250 μm.

The results of this study highlight the need for further definition of the histological changes that occur in the horse pituitary with normal aging, with seasonal physiologic stimulation and with PPID. In addition, until the histological changes that accompany early disease are clarified, ante mortem diagnostic tests validated using the gold standard of postmortem histology should be viewed cautiously. Authors of such studies must provide a clear description of the histological criteria they used to define PPID to avoid confusion. It is likely using currently available diagnostic testing methods, early disease may go undetected or be incorrectly diagnosed. Therefore, careful consideration of clinical signs and the progression of those signs as well as repeated endocrine function testing would be prudent in aged horses in which early PPID is suspected.

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4.6 SIGNIFICANCE OF FINDINGS

The lack of agreement among pathologists as to what histological findings are consistent with early signs of pituitary pars intermedia dysfunction (PPID) is important as histology is often used as the "gold standard" of diagnosis. In this thesis, diagnosis of PPID was often confirmed using postmortem histology of the pituitary. Based on the findings of Chapter 4, diagnosis relying solely on early histological changes (hyperplasia, hypertrophy, rosette formation, etc) should be avoided to reduce the potential for misdiagnosis of physiological activity with pars intermedia dysregulation or disease. To avoid misdiagnosis or miscommunication the following actions were taken in this thesis:

1) Histological findings were interpreted as compatible with PPID only if adenomatous hyperplasia or adenoma were present.
2) A clear description of the histological criteria used for diagnosis was provided in all chapters where this method was used.
3) All animals were selected by the presence or absence of clinical signs of PPID. Ante mortem tests (plasma α-melanocyte stimulating hormone concentration or overnight dexamethasone suppression test) were performed whenever possible to support the clinical diagnosis. In terminal animals, postmortem histological evaluation was used to confirm diagnosis. Animals in which the clinical signs and diagnostic testing were not consistent were not included in the study.

CHAPTER 5:

Nitration and increased α-synuclein expression associated with dopaminergic neurodegeneration in equine pituitary pars intermedia dysfunction

Published as:

McFarlane D, Dybdal N, Donaldson MT, Miller L, Cribb AE 2005 Nitration and increased α-synuclein expression associated with dopaminergic neurodegeneration in equine pituitary pars intermedia dysfunction. J Neuroendocrinol 17:73-80

5.1 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID, Equine Cushing's disease) is one of the most common diseases of horses and ponies 15 years and older (Brosnahan and Paradis, 2003). It is a naturally occurring, progressive condition characterized by hirsutism, muscle wasting, abnormal fat distribution, glucose intolerance, lethargy, infertility, and polydipsia / polyuria (van der Kolk et al., 1993; Love, 1993). Affected animals have an increased susceptibility to both bacterial and parasitic infections, with sinusitis, dermatitis, and endoparasitism being common (Hillyer et al., 1992; van der Kolk et al., 1993; Couetil et al., 1996). Further, affected horses may develop laminitis, a severe inflammatory disease of the foot that often necessitates euthanasia (Heinrichs et al., 1990; Hillyer et al., 1992). Despite clinical recognition of PPID for over 70 years, the pathophysiology remains poorly understood (Pallaske, 1932).

Post mortem examination of horses with PPID reveals enlargement of the pituitary gland due to hypertrophy, hyperplasia, and adenoma formation in the pars intermedia. In one study of 19 affected horses, the pituitary was reported to be 2- 3 times normal weight (Heinrichs et al., 1990). The pars intermedia of the horse is comprised of a single cell type, the melanotrope, that produces proopiomelanocortin (POMC). POMC expression is under inhibitory control by dopamine, released in the pars intermedia from local nerve terminals (Kemppainen et al., 1989; Kemppainen and Peterson, 1999). Retrograde tracing studies performed in cats and rodents demonstrate pars intermedia dopaminergic neurons originate in the periventricular nucleus of the hypothalamus, adjacent to the third ventricle (Luppi et al., 1986; Goudreau et al., 1995). The neurons project through the infundibulum

terminating in the pars intermedia. Dopamine released from these neurons inhibits the secretion of POMC-derived peptides through interaction at dopamine D2 receptors on the melanotropes (Cote et al., 1982). Horses affected with PPID have a marked increase in circulating concentration of POMC-derived peptides, α-melanocyte stimulating hormone, β-endorphin and corticotropin-like intermediate lobe peptide (CLIP) (Orth et al., 1982). Adrenocorticotropin hormone is also increased, although more modestly. The increase in pars intermedia POMC-dervived peptides is believed to lead to the development of clinical signs through an as yet poorly defined sequence of events.

The mechanism of pars intermedia hypertrophy, hyperplasia, and adenoma formation in PPID is not clear. It is possible the lesion is a spontaneously occurring primary pituitary tumor. Alternatively the pars intermedia enlargement may result from loss of hypothalamic dopaminergic inhibition. In neonatal rats, the development of dopaminergic innervation of the pars intermedia coincides with a decrease in melanotrope proliferation (Gary and Chronwall, 1992). If dopaminergic innervation is chemically disrupted, the rate of melanotrope proliferation is greater. Dopamine D2 receptor knockout mice develop pars intermedia hypertrophy and hyperplasia similar to PPID (Saiardi and Borrelli, 1998). In horses with PPID, Millington et al. (1988) demonstrated an 88% decrease in dopamine and dopamine metabolite concentrations in the pars intermedia compared to age-matched control horses. Orth et al. (1982) showed administration of dopamine or dopamine agonists to horses with PPID resulted in a decrease in plasma concentration of POMC peptides. Administration of the dopamine agonist pergolide to affected horses results in a decrease in plasma concentration of POMC-derived peptides, improvement of clinical signs and improvement in biochemical abnormalities associated

with disease (Schott et al., 2001; Donaldson et al., 2002). These results support the hypothesis that loss of dopaminergic inhibition is a key step in the pathogenesis of PPID.

Loss of dopamine in the pars intermedia could result from either a functional loss of dopamine synthesis or degeneration of the periventricular dopaminergic neurons. Dopaminergic cell body or nerve terminal degeneration could occur secondary to oxidative stress, particularly in the presence of increased α-synuclein expression. Oxidative stress with nitration of abundant α-synuclein has been implicated in the development of Parkinson's disease (Gilgun-Sherki et al., 2001). Chronic exposure to oxidants in excess of an animal's antioxidant capacity results in accumulation of functionally impaired cellular components, ultimately resulting in cell death. Measurement of these altered cellular components is a useful marker of historical exposure to oxidative stress (Frank et al., 2000).

We hypothesized that equine pituitary pars intermedia dysfunction results from loss of dopaminergic inhibition of the pars intermedia secondary to degeneration or damage of the periventricular hypophyseal dopaminergic nerve terminals ("dopaminergic neurodegeneration"). Furthermore, we hypothesized that chronic oxidative stress coupled with increased α–synuclein expression is the cause of dopaminergic neurodegeneration in PPID, which is similar to that postulated to occur in Parkinson's disease.

5.2 METHODS AND MATERIALS

Animals

All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care. Three study groups (n = 12 per group) of horses were used for pituitary tyrosine hydroxylase and 3-nitrotyrosine examination: (1) young ($\leq$15 yrs old) unaffected horses, (2) old (> 15 yrs old) unaffected horses, and (3) horses with PPID. The age of horses with PPID was not significantly different from aged controls (young, 5.4 ± 1.4; aged, 20.67 ± 1.3; PPID, 19.33 ± 1.9 years; PPID vs. aged, $P = 0.6$). The breeds of horses with PPID included 4 ponies, 2 quarter horses, 2 Arabians, 1 Appaloosa, 1 Morgan, 1 warmblood and 1 mixed breed. The breeds of the combined two control groups included 13 standardbreds, 5 quarter horses, 2 thoroughbreds, 1 warmblood, 1 Appaloosa, and 2 mixed breeds. There were 10 geldings, 2 mares in the affected group, and 10 geldings, 9 mares, 5 stallions in the controls. 75% of the affected horses were reported to have laminitis (active or historical), 58% hirsutism, 42% weight loss, 33% obesity, 17% excessive superorbital fat and 8% lethargy. Polydipsa and polyuria were not reported to occur in any of the affected horses.

Horses with PPID were selected based on clinical signs. Cases were confirmed at post mortem by histological evaluation of hematoxylin and eosin stained pituitary sections. Disease was defined by the presence of pars intermedia adenomatous hyperplasia or an adenoma. There were no physical examination or historical findings of PPID in control horses. Horses were excluded from the control groups if they were in the late stages of pregnancy, lactating, under a year of age, or had a history of laminitis.

Two groups (n = 12 per group) of horses were used for immunohistochemical examination of the hypothalamus for tyrosine hydroxylase: (i) unaffected and (ii) horses with PPID. Three groups of horses were used for the 3-nitrotyrosine hypothalamus assay: (i) affected (n = 5); (ii) PPID negative, oxidative stress positive (n = 5); and (iii) unaffected, oxidative stress negative (n = 10). Oxidative stress positive horses were defined as having greater than 30% of the pars intermedia fields of view positive for 3-nitrotyrosine. Oxidative stress negative horses had less than 5% of the pars intermedia fields of view positive for 3-nitrotyrosine. Three groups of horses (n = 8 per group) were used for α-synuclein analysis of the pituitary: (i) affected, (ii) PPID negative, oxidative stress positive, and (iii) unaffected, oxidative stress negative.

Tyrosine hydroxylase immunohistochemistry

To assess dopaminergic nerve terminal and cell body density, immunohistochemical analysis was performed. Formalin-fixed, paraffin-embedded, 5 μm thick pituitary sections from affected and control horses were mounted as pairs on glass slides treated with 3-aminopropyltriethoxysilane diluted in acetone. Hypothalamic sections (5 μm thick) were mounted individually on slides. Slides were deparaffinized in xylene then hydrated by a graded ethanol series. Hypothalamic sections were unmasked by incubation for 10 min in 0.01M sodium citrate (pH 6.0) at 95° C. All sections were incubated for 5 min in 3% hydrogen peroxide to inactivate endogenous peroxidases, and digested in 1 mg/ml trypsin for 10 min at 37° C. Sections were incubated for 20 min at 25° C in 5% goat serum to reduce non-specific staining, then for 36 - 48 h at 4 ° C with rabbit polyclonal tyrosine hydroxylase antiserum (Affiniti Research Products, England) at a 1 : 500 dilution.

Sections were incubated in biotinylated goat anti-rabbit secondary antibody (15 μg/ml) for 1 h at 25° C. Sections were then incubated in an avidin – biotinylated enzyme complex for 30 min at 25° C (Vectastain Elite ABC Kit, Vector Laboratories) followed by the enzyme substrate, diaminobenzidine for 8 min at 25° C. Sections were dehydrated in a graded series of ethanol, cleared with xylene then mounted with coverslips. Negative controls included normal horse pituitary sections with the primary antibody omitted or replaced with non-specific rabbit IgG.

Following immunohistochemistry, six fields within the pars intermedia were selected in a random and masked fashion. A positive tyrosine hydroxylase (TH) signal was counted using a computer imaging program (Bioquant TCW98, V3.50.6 MST © 1993-1998, R & M Biometrics Inc, Nashville, TN, USA). The average count for the six fields was calculated for each horse and results were expressed as TH signal / field. Results from the three study groups were compared using one-way analysis of variance. For hypothalamic sections, periventricular cell bodies were identified as being adjacent to the third ventricle (within 1.5 mm), ventral to the paraventricular nucleus, and caudal to the arcuate nucleus. Each block was cut a minimum of three times and the slide with the greatest number of tyrosine hydroxylase positive cell bodies was selected for counting. The identity of the slides was masked and the tyrosine hydroxylase positive cell bodies were counted. All slides were counted twice, on separate days and the average counts from the two groups compared.

Tyrosine hydroxylase western blot analysis

Pituitary tissue from 16 horses, including 2 with PPID, was collected within 15 min of death. Pars intermedia tissue was dissected, washed 3 times in ice cold phosphate buffered saline (PBS), flash frozen in liquid nitrogen, then stored at -80° C until assayed. Samples were homogenized in 500 µl PBS, followed by centrifugation at 350g for 20 min at 4° C. Protein content of the supernatant was determined (Biorad DC Protein Assay, Biorad Laboratory, Inc., Carlsbad, CA, USA). Equal amounts of protein (25µg) were separated by SDS-PAGE on a 4- 20% gradient gel (Readygel, BioRad) and transferred to nitrocellulose membrane. Western blot analysis was performed using ECL detection system (Ambersham, Piscataway, NJ) with rabbit tyrosine hydroxylase polyclonal antibody (1 : 2000, Sigma, St Louis, MO, USA). Accuracy of dissection of pars intermedia from pars distalis tissue was assessed by Western blot analysis, using prolactin (rabbit polyclonal antibody, sc-7805, 1 : 250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a marker for the pars distalis. Staining intensity of tyrosine hydroxylase for the seven most accurately dissected samples were standardized to β–actin concentration and results compared with immunohistochemistry.

3-nitrotyrosine immunohistochemistry

The presence of oxidative stress in the pituitary and hypothalamus was similarly assessed by immunohistochemistry, with the following modifications. Primary antibody incubation was performed with 3- nitrotyrosine monoclonal antibody (Cayman Chemicals, Ann Arbor, MI, USA) at a 1 : 100 dilution followed by incubation with a biotinylated goat anti-mouse IgG secondary antibody then avidin-biotin complex. For pituitary sections, the chromagen

Nova Red was used instead of diaminobenzadine and slides were counterstained with hematoxylin for 10 s before dehydrating and applying a coverslip. This resulted in better visualization of the borders between the pituitary lobes, as well as other anatomical landmarks, and aided in counting the pars intermedia fields of view. Negative controls included normal horse pituitary sections with the primary antibody omitted or with the primary antibody pre-incubated with 3-nitrotyrosine (10mM for 12 h at 4° C).

Following immunohistochemistry, all fields of view within the pars intermedia were examined for each horse section analyzed. Coordinates (x,y) were used to systematically examine the sections. Fields encompassing both the pars intermedia and an adjacent lobe were counted if the pars intermedia comprised greater than 75% of the field of view. Those fields in which the pars intermedia comprised between 25- 75% were counted as a half field. Fields were graded as either positive or negative for 3-nitrotyrosine. A positive field had a minimum of two immunoreactive cells. The percent positive fields was calculated for each horse and results compared by one–way ANOVA. For hypothalamic sections, identity of the slides was masked and 3-nitrotyrosine stained periventricular cell bodies were counted on three separate days. The average of the results was expressed as a percent of the total tyrosine hydroxylase positive periventricular cell bodies counted in the adjacent section.

Synuclein immunohistochemisty

To assess α-synuclein protein expression, immunohistochemistry was performed as described above using rabbit polyclonal α-synuclein antiserum (Affiniti Research Products) at a 1 : 1000 dilution. Negative controls included normal horse pituitary sections

with the primary antibody omitted, replaced with non-specific rabbit IgG, or with the primary antibody pre-incubated with α-synuclein blocking peptide (5 μg/ ml peptide in 1 : 1000 dilution of antibody for 12 h at 4° C). Following immunohistochemistry, the pars intermedia and pars distalis staining densities were determined using a computer imaging program (Scion Image, Beta 4.02, Scion Corporation, Frederick, MD, USA). Results were expressed as the ratio of pars intermedia density/ pars distalis density.

Nitration of synuclein

Immunohistochemistry of two consecutive, 5μm thick pituitary sections was performed using α-synuclein antibody (1 : 1000, rabbit polyclonal, Affiniti Research Products) for the first and 3-nitrotyrosine antibody (1 : 100, monoclonal, Cayman Chemicals) for the second slide. Following incubation with the appropriate secondary antibody, tissues were incubated with chromagens (Nova Red or Texas Red for 3-nitrotyrosine, diaminobenzidine or fluorescein for α-synuclein, Vector Laboratories). Computer imaging software (Adobe Photoshop CS, Seattle, WA, USA) was used to merge the images to assess similarity of staining pattern.

Nitration of synuclein was further confirmed by Western blotting. Pituitary pars intermedia protein was collected as described above. Equal amounts of protein (100 μg) from two horses (one with PPID, one with oxidative stress, but PPID negative) was separated by SDS-PAGE on a 4- 20% gradient gel (Readygel, BioRad) and transferred to nitrocellulose membrane. Western blot analysis was performed using ECL detection system (Ambersham). Samples were run in duplicate on the same gel. The blot was cut in two. Half was blotted for α-synuclein (1 : 1000, rabbit polyclonal, Sigma) and half for 3-

nitrotyrosine (1 : 250, monoclonal, Cayman Chemicals). First, specificity of the antibodies was confirmed by pre-incubating primary antibodies with blocking peptide (α-synuclein) or 3-nitrotyrosine for 12 h at 4°C. The same blots were then probed using primary antibody alone to confirm immunoreactivity of the 20 kDa band.

Statistical Analysis

Statistical calculations were performed using commercial statistical software (Minitab Statistical Software, 13.32 ™, State College, PA, USA; GraphPad Prism®, V3.03, San Diego, CA, USA). Normality of the data was confirmed using the Kolmogorov-Smirnov test. All results are expressed as mean ± SEM. For three-point comparisons (pituitary groups) ANOVA was used, with Tukey's multiple comparison test used for post-hoc analysis. Students t-test was used for two-point comparisons. Comparison of western blot and immunohistochemistry results was performed by calculating the Pearson product moment correlation coefficient. $P < 0.05$ was considered statistically significant.

5.3 RESULTS

Tyrosine hydroxylase immunohistochemistry

Tyrosine hydroxylase staining was significantly decreased in the pars intermedia of horses with PPID compared to both the aged and young controls (28.68 ± 6.51, 133.5 ± 10.2, 145.1 ± 12.4 TH signal/ field, respectively; $P< 0.001$) (Figures 1A and 1B). No difference was observed between the young and aged controls. Horses with PPID had significantly fewer tyrosine hydroxylase positive periventricular cell bodies compared to the controls (102 ± 9.9 vs. 151.5 ± 10 TH positive cell bodies/ section; $P< 0.01$).

Western blots

A 55-kDa, tyrosine hydroxylase immunoreactive band was identified in the horse pituitary tissue (Figure 1C). To assess correlation with immunohistochemistry quantification of pars intermedia TH, the samples with the most accurate dissection of the pars intermedia were selected. All samples showed prolactin immunoreactivity, indicating some degree of pars distalis contamination (Figure 1C). The intensity of the prolactin band was measured in all 16 samples using computer imaging software (Kodak Image 1D, Scientific Imaging Systems, Eastman Kodak, New Haven, CT, USA). The seven samples with the lowest prolactin band net intensity (less than 7000) were selected for further analysis. Measured density of the 55-kDa band was significantly correlated to the score calculated from tyrosine hydroxylase immunohistochemistry for these seven samples (Figure 1D) ($r = 0.84$, $P < 0.05$).

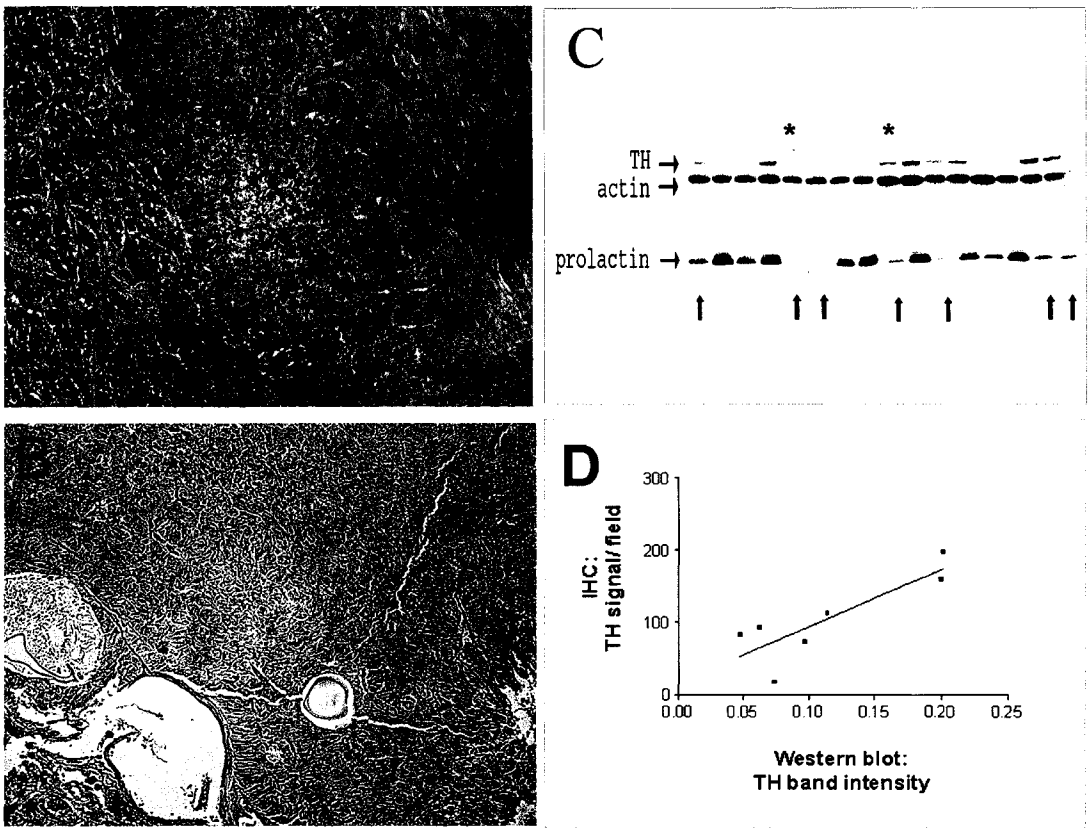

Figure 1: Pars intermedia tyrosine hydroxylase immunoreactivity

(A) Immunohistochemistry of pituitary section from normal, 8-year old horse. Pars intermedia shows strong immunoreactivity for tyrosine hydroxylase. Arrowheads mark the border of pars distalis and pars intermedia. Arrows mark the border of pars nervosa and pars intermedia. (B) Immunohistochemistry of pituitary section of horse with pars intermedia dysfunction (PPID). Marked hyperplasia of the pars intermedia is apparent when compared to the normal horse (A) shown at the same magnification. Pars distalis and pars nervosa are not evident in this field. No tyrosine hydroxylase staining is seen in this section. A small amount of endogenous pigment is present in the lower left corner. Scale bar = 200μm. (C) Western blot analysis of pars intermedia tissue. Arrows indicate lanes of samples (7) used for tyrosine hydroxylase quantification and comparison to immunohistochemistry results. Asterisks indicate PPID horses. (D) Tyrosine hydroxylase quantification by immunohistochemistry is positively correlated to quantification by Western blot analysis in samples with minimal pars distalis contamination. ($r=0.84$, $P < 0.05$)

3-nitrotyrosine immunohistochemistry

Horses with PPID had a greater number of total fields of view in the pars intermedia compared to controls [57.1 ± 8 (PPID), 18 ± 4 (young), 22 ± 3 (aged) fields; $P < 0.001$]. This is expected as the number of fields of view parallels the total area and PPID is characterized by hyperplasia of the pars intermedia. As shown in Figure 2, horses with PPID had a significantly greater percent of fields positive for the presence of 3-nitrotyrosine compared to both the young (48 ± 6% versus 3 ± 2%; $P < 0.001$) and aged controls (48 ± 6% versus 20 ± 7%; $P < 0.001$). In addition, the 3-nitrotyrosine staining in the pars intermedia of the aged controls was significantly greater than that observed in the young control group ($P < 0.05$). In contrast, there was no difference in the percent of periventricular cell bodies positive for 3-nitrotyrosine observed between the affected, oxidative stress horses and control horses (45 ± 1.0%, 46 ± 18%, 37 ± 8%, $P = 0.8$).

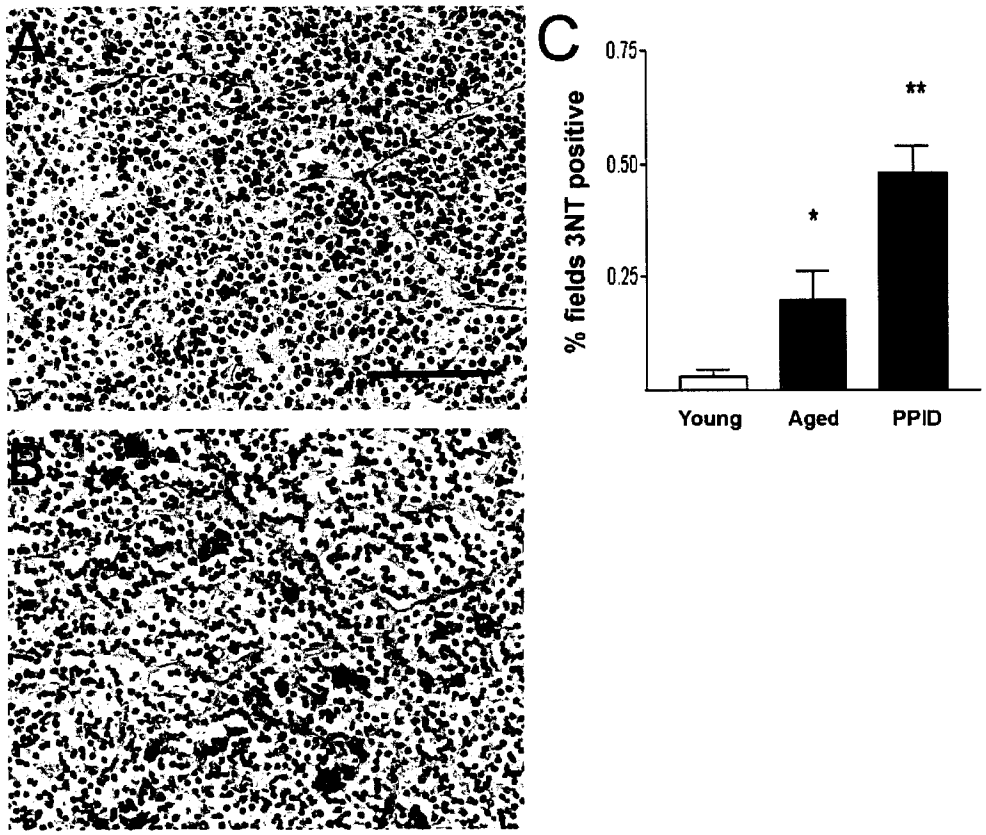

Figure 2. Pars intermedia 3-nitrotyrosine immunoreactivity

(A) Immunohistochemistry for 3-nitrotyrosine in the pars intermedia of normal, 3-year old horse. No positive staining is observed. (B) Immunohistochemistry of pars intermedia of horse with pars intermedia dysfunction (PPID). Positive 3-nitrotyrosine staining is evident throughout the field. Scale bar = 100μm. (C) The percent of 3-nitrotyrosine positive fields is significantly greater for PPID, than aged or young controls (48 ± 6%, 20 ± 7%, 3 ± 2%; $^{**}P < 0.001$). There is also a greater percent of positive fields in the aged control horses compared to the young horses. ($^{*}P < 0.05$)

Alpha synuclein expression

Horses with PPID had a greater ratio of α-synuclein expression in the pars intermedia relative to pars distalis compared to unaffected horses (ratio: 1.54 ± 0.08 versus 0.91 ± 0.04, $P < 0.001$). Oxidatively stressed, non-diseased horses had a greater ratio compared to unaffected horses (ratio: 1.3 ± 0.11 versus 0.91 ± 0.04, $P< 0.05$) (Figure 3). Although the mean ratio of pars intermedia/ pars distalis α-synuclein in horses with PPID was greater than the non-diseased horses with oxidative stress, the difference was not statistically significant ($P = 0.08$). 3-nitrotyrosine and α–synuclein staining showed a similar pattern that colocalized when adjacent slides were merged using a computer imaging program, suggesting α-synuclein protein nitration (Figure 4). Western blotting demonstrated marked 3-nitrotyrosine immunoreactivity of a 20-kDa protein that migrated similarly to α–synuclein (Figure 4).

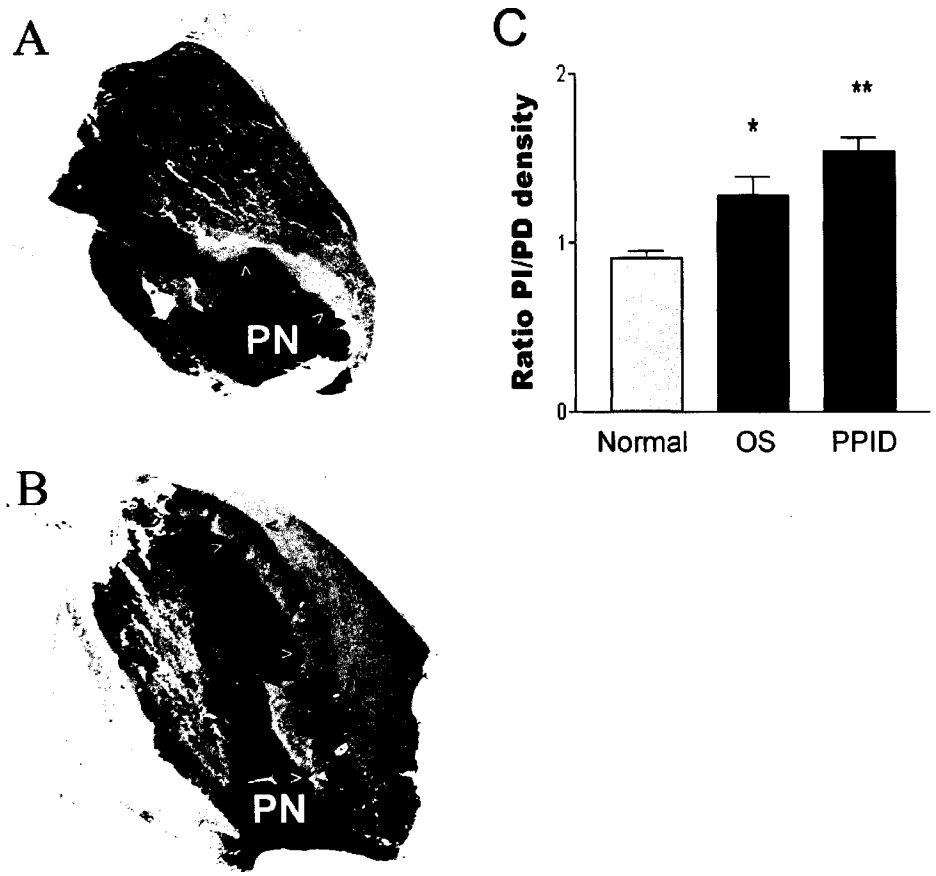

Figure 3: Pars intermedia α-synuclein immunoreactivity

(A) Immunohistochemistry for α-synuclein of normal horse pituitary. White arrowheads mark border of pars intermedia and pars nervosa (PN), black arrowheads mark border of pars intermedia and pars distalis (PD). (B) Immunohistochemistry for α-synuclein of pituitary pars intermedia dysfunction (PPID) horse. (C) The ratio of α-synuclein in the pars intermedia / pars distalis is greater in horses with PPID compared to normal horses and greater in horses with pars intermedia oxidative stress (OS) compared to normal. Ratios: 1.54 ± 0.08 (PPID) versus 0.91 ± 0.04 (normal), $^{**}P < 0.001$; 1.3 ± 0.11 (OS) versus 0.91 ± 0.04 (normal), $^{*}P < 0.05$.

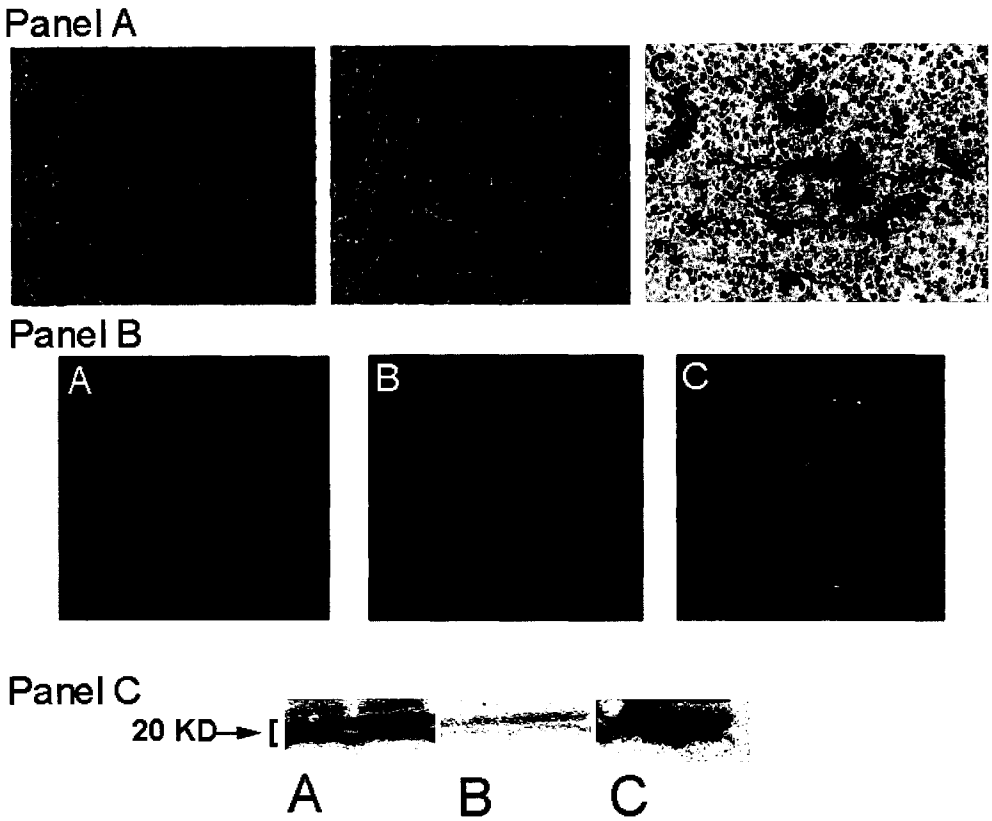

Figure 4: Pars intermedia colocalization of α-synuclein and 3-nitrotyrosine

(Panel A, A) Immunohistochemistry for α-synuclein in pituitary from an oxidatively stressed, pituitary pars intermedia dysfunction (PPID) negative horse (chromagen, DAB). (Panel A, B) Immunohistochemistry for 3-nitrotyrosine of sequential tissue from same horse (chromagen, Nova Red). (Panel A, C) α-synuclein and 3-nitrotyrosine images color modified (DAB to yellow, Nova Red to pink) and merged, showing similar localization of immunoreactivity within the pars intermedia. (Panel B, A) Immunohistochemistry for α-synuclein in pituitary from a PPID horse (fluorogen, fluorescein). (Panel B, B) Immunohistochemistry for 3-nitrotyrosine of sequential tissue from same horse (fluorogen, Texas Red). (Panel B, C) α-synuclein and 3-nitrotyrosine images merged, showing similar localization of immunoreactivity within the pars intermedia. (Panel C) Western blot analysis showing α-synuclein doublet band at 20-kDa (A, 5-min exposure) that is blocked by pre-incubating antibody with blocking peptide (B, 10-min exposure). 3-nitrotyrosine antibody binds the same 20-kDa proteins (C, 40 min exposure).

5.4 DISCUSSION

The clinical progression of PPID is characteristic of a degenerative disease. It is an insidious, progressive disease of aged animals from which there is no recovery. The marked reduction in dopamine and dopamine metabolite concentration observed in the pars intermedia of diseased horses, as well as the response of affected horses to dopamine agonist therapy, suggests a loss of dopamine-producing neurons with disease (Millington et al., 1988; Schott et al., 2001; Donaldson et al., 2002). In this study, immunohistochemical evaluation of pituitary and hypothalamic tissue was used to assess the presence of dopaminergic neurons in horses with or without PPID.

Tyrosine hydroxylase, the rate-limiting enzyme in dopamine biosynthesis, is a commonly used marker of dopaminergic neurons. Using immunohistochemistry, we examined the pituitary pars intermedia of affected and unaffected horses for the presence of tyrosine hydroxylase. In horses with PPID, tyrosine hydroxylase staining in the PI was reduced to 20% of the controls. This is similar to the percent reduction in dopamine reported by Millington et al. (1988). The reduction of both tyrosine hydroxylase and its product dopamine in the pars intermedia of affected horses supports a loss of functional dopaminergic nerve terminals in diseased horses.

Loss of functional neurons may be confirmed by demonstrating a concurrent loss of cell bodies in animals with reduced number of nerve terminals. Examination of sections from the hypothalamus for the presence of periventricular cell bodies revealed 50% fewer tyrosine hydroxlase immunoreactive cell bodies in affected horses compared to non-diseased animals. Although not conclusive, this suggests an actual loss of periventricular dopaminergic neurons occurs with PPID. Attempts to confirm a loss of cell bodies in

affected horses using Nissl staining was unsuccessful because it was not possible to differentiate the periventricular cell bodies using Nissl staining from the abundant non-tyrosine hydroxylase positive cell bodies present in this region.

Comparison of young and aged horses was necessary to determine the impact of aging on dopaminergic neurodegeneration in the absence of concurrent PPID. An age-related decrease in tyrosine hydroxylase immunoreactive neurons has been shown to occur in other species in the absence of disease (Luo and Roth, 2000; McCormack et al., 2004). This was not observed in the horses of this study. In humans, tyrosine hydroxylase immunoreactive, dopaminergic neurons decrease linearly at the rate of 5-14% per decade of life (Kaasinen et al., 2000). In primates, aging was accompanied by a loss in tyrosine hydroxylase immunoreactive neurons in the substania nigra, a decrease in dopamine concentration and an increased vulnerability to the dopaminergic neurotoxin, MPTP. However, the total number of dopaminergic neurons did not decrease as a function of aging (McCormack et al., 2004). The failure to appreciate an age-related neuronal loss in our study may be due to the aged control group being too young. In a recent epidemiological study of 467 horses 20 years or older, 15% were $\geq$ 30 years and the oldest horse was 45 years (Brosnahan and Paradis, 2003). The aged group in this study was selected to match the age of the horses with PPID (19 years), and averaged only 21 years. Evaluation of a larger number of older horses may provide insight as to whether age-related dopaminergic neuronal cell loss occurs in horses.

Having shown a loss of dopaminergic neurons in the pars intermedia of horses with PPID, we next sought to determine whether oxidative stress was involved in the mechanism of the neurodegeneration. Degeneration of dopaminergic neurons in other

diseases has been associated with oxidative stress. Oxidative stress occurs when cellular components are damaged as a result of exposure to free radicals. Free radicals are created as a normal consequence of aerobic metabolism; thus, exposure occurs in all living organisms over time. This chronic exposure to oxygen-derived radicals is believed to play a critical role in the process of cellular damage and death that is associated with aging.

Chronic oxidative damage can be documented by the presence of oxidatively modified lipids, proteins or DNA (Frank et al., 2000). In this study immunohistochemistry was used to detect 3-nitrotyrosine in formalin-fixed tissues. 3-nitrotyrosine results from the nitration of tyrosine by peroxynitrite, the product of nitric oxide reacting with superoxide anion. 3-nitrotyrosine has been shown to accumulate in tissues of animals with diseases associated with oxidative stress (Kojda and Harrison, 1999). Oxidative stress markers, including 3-nitrotyrosine, also accumulate in tissues as a function of aging (Beckman and Ames, 1998). Consistent with what has been reported in other species, a six-fold increase in the presence of 3-nitrotyrosine in the pars intermedia of normal aged horses was observed compared to young horses. This is the first report of an age-related accumulation of a marker of chronic oxidative stress in horses.

Chronic oxidative stress is considered to be an inciting factor in the development of neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease and multiple sclerosis (Calabrese et al., 2000; Gilgun-Sherki et al., 2001). Markers of oxidative stress including 3-nitrotyrosine have been demonstrated to accumulate in patients with each of these diseases (Calabrese et al., 2000). Dopaminergic neurons are particularly vulnerable to oxidative damage, due to the production of reactive oxygen species during neurotransmitter metabolism. Hydrogen

peroxide is a by-product of enzymatic metabolism of dopamine by monoamine oxidase B. Dopamine autooxidizes to form quinones, semiquinones and other reactive oxygen species. The presence of oxidative stress in the dopaminergic nerve terminals of the pars intermedia of horses with PPID has not been previously reported. Horses with PPID had a greater than two-fold increase in nitrotyrosine staining compared to non-affected aged horses and greater than 15-fold increase compared to young horses. Because severely damaged cells may die, the actual extent of oxidative damage may even greater. Oxidative stress was not observed in the periventricular cell bodies, suggesting a greater exposure or vulnerability to oxidative damage in the nerve terminals.

There are several factors that alone or together may contribute to oxidative damage to neurons. In Parkinson's disease, mutations in α–synuclein and parkin, proteins integral to nerve terminal function, have been linked to an early onset, familial form of the disease (Polymeropoulos et al., 1997; Kitada et al., 1998). Nerve terminals that express excessive α–synuclein are at greater risk of nerve terminal oxidative damage and neurodegeneration (Saha et al., 2000; Kirik et al., 2002; Lauwers et al., 2003). Over-expression of α–synuclein has been demonstrated to occur as a result of the presence of multiple functional copies of the gene or a promoter polymorphism that increases gene transcription (Chiba-Falek and Nussbaum, 2001; Singleton et al., 2003; Holzmann et al., 2003). Both of these genetic variations are associated with an increased risk of Parkinson's disease (Farrer et al., 2001; Singleton et al., 2003; Holzmann et al., 2003; Tan et al., 2003).

To evaluate whether α-synuclein has a role in development of PPID, protein expression was assessed using immunohistochemistry of pituitary tissue from affected horses, non-diseased horses with significant pars intermedia oxidative stress and normal

horses. Horses with PPID had significantly more α-synuclein observed in the pars intermedia than unaffected horses (Figure 4). In addition, horses with evidence of marked pars intermedia oxidative stress that were free of disease had more α-synuclein in the pars intermedia than normal horses ($P < 0.05$). The finding of aged horses with normal tyrosine hydroxylase, increased 3-nitrotyrosine and increased α-synuclein expression in the pars intermedia suggests that oxidative stress and α–synuclein overexpression may precede neurodegeneration and disease. Longitudinal studies are necessary to determine a timecourse of pathologic events in PPID to confirm oxidative stress and α–synuclein overexpression are causes and not consequences of the disease.

α–synuclein has also been shown to be a target for nitration by peroxynitrite. When α–synuclein is nitrated, it aggregates forming a non-soluble protein (Giasson et al., 2000). The accumulation of this non-soluble form of α–synuclein leads to the formation of inclusion bodies, known as Lewy bodies, a pathological hallmark of Parkinson's (Giasson et al., 2000). Accumulation of nitrated α-synuclein has been reported in Lewy bodies and Lewy neurites from brains with different synucleinopathies (Giasson et al., 2000). Using sequential tissue sections, we were able to demonstrate 3-nitrotyrosine and α-synuclein were colocalized in the pars intermedia, suggesting nitration of α-synuclein occurs in PPID as in Parkinson's disease. Colocalization of 3-nitrotyrosine and α-synuclein was also demonstrated by Western blot analysis (Figure 4).

The results of this study demonstrate a role for neurodegeneration, oxidative stress and α-synuclein expression in the pathophysiology of PPID. Further work is necessary to determine the factors that predispose a horse to oxidative damage of the pars intermedia dopaminergic nerve terminals. A better understanding of the factors leading to disease

may permit earlier recognition and treatment of PPID, thereby avoiding the life-threatening complications. PPID may also provide a useful and unique animal model for investigation of other dopaminergic neurodegenerative diseases.

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5.6 SIGNIFICANCE OF FINDINGS

Our findings of periventricular dopaminergic neurodegeneration in horses with pituitary pars intermedia dysfunction (PPID) are consistent with the previous evidence that PPID results from a loss of dopaminergic inhibition. This is the first report that oxidative damage of the pars intermedia may have a role in development of PPID. To further investigate the role of oxidative stress in the pathogenesis of PPID, we sought to determine the factors that predisposed individual horses to pars intermedia oxidative stress. Potential factors that alone or in combination may contribute to oxidative stress and subsequent neurodegeneration include:

1. Excessive exposure to environmental oxidants
2. Excessive endogenous production of reactive oxygen species due to
    a. mitochondrial dysfunction
    b. inflammation
    c. systemic metabolic derangement
3. Impaired antioxidant capacity (systemic or local)
4. Increased vulnerability of the neurons to cell death following stress

We proposed that horses develop oxidative damage of the pars intermedia and subsequently PPID secondary to impaired antioxidant capacity (systemic or pars intermedia). Chapters 6 and 7 investigate this hypothesis.

Alpha-synuclein expression was increased in the pars intermedia of horses with oxidative stress and PPID. Accumulation of α-synuclein, particularly nitrated α-synuclein,

is known to be neurotoxic. Our findings suggests a role for increased vulnerability of the periventricular neurons in development of PPID. It is likely an interaction between damaging cellular events and neuronal vulnerability culminates in degeneration of the periventricular neurons and thus PPID. Therefore, subtle differences in any component of the antioxidant system, while insufficient to cause disease alone, may be important when present in combination with other risk factors.

CHAPTER 6

Systemic and pituitary pars intermedia antioxidant capacity associated with pars intermedia oxidative stress and dysfunction in horses

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6.1 INTRODUCTION

Equine pituitary pars intermedia dysfunction (PPID) is a spontaneous, progressive disease that affects aged horses and ponies. Although all breeds of horses may be affected, ponies and Morgan horses appear to be at greater risk (Schott, 2002). Animals with this condition have hypertrophy, hyperplasia and adenoma formation of the pars intermedia of the pituitary, leading to overexpression of proopiomelanocortin (POMC). POMC in the pars intermedia is processed to the peptides α- melanocyte stimulating hormone (α-MSH), β- endorphin and corticotropin-like intermediate lobe peptide. A small amount of adrenocorticotropin (ACTH) is also produced. Over-expression of these POMC-derived peptides leads to clinical signs of disease by a poorly defined mechanism.

The etiology of PPID is not known, but a loss of dopamine in the pars intermedia appears to be critical to the development of disease (Millington et al., 1988). Dopamine inhibits pars intermedia expression and its loss results in increased expression and plasma concentration of POMC-derived peptides. Administration of dopamine or dopamine agonists to affected horses results in a decrease in plasma concentration of POMC-derived peptides, improvement of clinical signs and improvement in biochemical abnormalities associated with disease (Orth et al., 1982; Schott et al., 2001; Donaldson et al., 2002). The concentration of dopamine and dopamine metabolites in the pars intermedia of horses with disease is decreased 9-fold compared to age-matched controls (Millington et al., 1988). Recently we reported a loss of the periventricular dopaminergic neurons in affected horses compared to age-matched and young control horses (McFarlane et al., 2005).

The inciting factor(s) that initiate the neuronal loss observed with PPID are unknown, but evidence from our laboratory suggests oxidative stress may have a role (McFarlane et al., 2005). Immunohistological examination of pituitary tissue demonstrated an increase in 3-nitrotyrosine, a marker of oxidative stress, in diseased horses (McFarlane et al., 2005). 3-nitrotyrosine has been demonstrated to accumulate in human patients with neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Calabrese et al., 2000; Luo and Roth, 2000; Gilgun-Sherki et al., 2001). Accumulation of 3-nitrotyrosine also occurs with healthy aging (Finkel and Holbrook, 2000). An age-associated increase in 3-nitrotyrosine was observed in the pars intermedia of horses (McFarlane et al., 2005).

Several potential factors may predispose an animal to neuronal loss secondary to oxidative stress, including environmental (e.g. pesticides, chemical toxins) and genetic factors. Production of endogenous free radicals occurs as a product of metabolism and inflammation. Therefore, mitochondrial dysfunction, abnormal systemic metabolism or chronic inflammation can expose an individual to a greater than normal concentration of free radicals. Another risk factor for oxidative stress is a deficiency in antioxidant capacity. Antioxidant capacity results from a combination of enzymatic and non-enzymatic activities that neutralize free radicals, preventing them from causing further cellular damage. A genetic deficiency in an antioxidant enzyme or a dietary lack of antioxidants may contribute to oxidative stress. Finally, in some individuals, neurons may be more susceptible to free radical damage. In humans with familial Parkinson's disease, a mutation in the nerve terminal protein α-synuclein leads to its cytoplasmic accumulation resulting in cell death of the nigrostriatal dopaminergic neurons (Farrer et al., 2001).

Nitrated α-synuclein accumulates more readily and is more neurotoxic than native, unaltered α-synuclein (Giasson et al., 2000). We recently reported horses with PPID have increased nitrated α-synuclein in the dopaminergic nerve terminals of the pars intermedia compared to healthy animals (McFarlane et al., 2005). It is likely that oxidant exposure, antioxidant deficiency and susceptibility of neurons all interact in the development of PPID.

We hypothesized that horses with PPID have a deficiency in systemic antioxidant capacity. To test this theory, we measured red blood cell total glutathione concentration, glutathione peroxidase activity and total superoxide dismutase activity. In addition, we measured the concentration of 3-nitrotyrosine, an oxidative stress marker, in plasma, adrenal gland and substantia nigral tissue. To assess the role of local antioxidant capacity in oxidative damage of the pars intermedia, total glutathione concentration, total superoxide dismutase, manganese superoxide dismutase and glutathione peroxidase activities were compared to the 3-nitrotyrosine accumulation in pars intermedia tissue.

6.2 METHODS AND MATERIALS

I. Peripheral antioxidant study

Study Population: All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care, following approval by the UPEI Animal Care Committee. Twenty horses with clinical signs of PPID were recruited from the referral population of AVC. Horses were confirmed to have PPID by high endogenous plasma α-melanocyte stimulating hormone concentration (n=19) using seasonally specific reference range values (Chapter 2.6, Figure 1) and/or post mortem histology (n=5). Twenty control horses were selected from the same farm when possible. If a control was not available from the same location, an age-matched control was selected from a similar environment. Control horses had no clinical evidence of PPID and a plasma α- MSH concentration <45 pmol/L (Figure 1). This concentration is below the previously reported mean value (59.6 pmol/L) for normal horses (Horowitz et al., 2003), which at the time of animal recruitment was the only available reference range. Using the newly established seasonally specific reference ranges (Chapter 2.6), three control animals had plasma α- MSH concentrations slightly above the upper limit of the normal reference range.

Sample collection: Whole blood was collected by jugular venipuncture into evacuated glass tubes containing EDTA as an anticoagulant. Samples were maintained on ice until processed within 4 hours of collection. Samples were centrifuged at 800*g* for 10 minutes at 4°C. Plasma was removed to a polypropylene tube and frozen in multiple aliquots at -80°C until assayed. Red blood cells were diluted 50% in 0.9% saline and frozen at

-80°C until assayed (Marshall et al., 2002).

Measurement of α-MSH concentration: Equine α-melanocyte stimulating hormone was measured using a commercially available radioimmunoassay (Euria-α–MSH RIA, American Laboratory Products Company, Windham, NH) designed for use with human plasma and validated for use in the horse as previously described (McFarlane et al., 2004).

Blood antioxidant assays: Hemolysates were prepared by mixing equine packed red blood cells with ice-cold distilled water at a 1:9 dilution. The lysate was centrifuged at 800 *g* at 4°C for 20 minutes. Red blood cell total superoxide dismutase activity was determined in microtiter plates using a previously described method (Peskin and Winterbourn, 2000). Briefly, hemolysates were cleared by adding 200 µl of 95% ethanol and 130 µl of chloroform to 800 µl of lysate. Samples were mixed thoroughly, then spun at 10,000 *g* for 5 min at 4°C. The cleared supernatant was diluted 1:5 in phosphate buffer (pH 8.0) for assay. 10 µl of the sample was added to triplicate wells containing 230 µl of PBS, 10 µl of 3 mM EDTA, and 10 µl of 3mM xanthine. To start the reaction, 20 µl of 58 mU/ml xanthine oxidase and 20 µl of 0.75 mM WST-1 (Dojindo Laboratories, Japan) were added to each well. Change in absorbance at 438 nm over 30 minutes at 25°C was measured. Bovine superoxide dismutase was used as a control. Red blood cell glutathione peroxidase activity was determined by the rate of disappearance of NADPH measured by absorbance at 340 nm as described (Tappel, 1978) and modified for use in a microtiter plate (Smith and Levander, 2002). Hemolysates were diluted 1:10 in 50 mM Tris HCl, 5 mM EDTA with 1 mg/ml BSA. 10 µl of sample was added to triplicate wells containing 155 µl of 0.4

mM NADPH, 2mM glutathione, 0.175 U glutathione reductase in 50 mM Tris HCl, 5 mM EDTA pH 7.6. 10 µl of 2 mg/ml cumene hydroperoxide was added to each well to start the reaction. Plates were read at 340 nm over 10 minutes at 25°C. Bovine glutathione peroxidase was used as a control. Total red blood cell glutathione concentration was determined in microtiter plates by the technique of Tietze (1969) modified as follows. Hemolysates were diluted 1:20 in 100 mM NaPO4 buffer pH 8.0 with 1 mM EDTA. 50 µl of samples were pipetted in triplicate to microtiter plate wells. 100 µl of reaction mix (100 mM NaPO4 buffer pH 8.0 with 1 mM EDTA, 0.32 mM DNTB, 0.32 mM NADPH and 1.25 U/ml GSH reductase) was added to each well and change in absorbance at 415 nm was measured for 5 minutes. Hemoglobin concentration in lysates was determined by adding 20 µl of lysate to 5 mls of Drabkin's reagent, incubating for 15 minutes at room temperature and measuring optical density at 540 nm. Hemoglobin concentration of samples was calculated by comparing to a known concentration of ovine methemoglobin.

II. Systemic oxidative stress study

Plasma 3-nitrotyrosine: Plasma 3-nitrotyrosine was determined in the same twenty pairs of horses using a commercially available ELISA (Hycult Biotechnology b.v, Netherlands). Plasma 3-nitrotyrosine was measured on two separate days and an average concentration calculated and converted to categorical data (detectable (>2nM) or non-detectable concentration) for statistical comparision.

Immunohistochemistry: Formalin-fixed paraffin-embedded tissue was obtained from 20 horses from which pituitary, adrenal and substantia nigra (SN) tissue was available. Five

of the horses were reported to have had clinical signs of PPID. Disease was confirmed at necropsy by the presence of adenomatous hyperplasia or adenoma of the pars intermedia.

Formalin-fixed, paraffin-embedded tissue was mounted on glass slides treated with 3-aminopropyltriethoxysilane diluted in acetone. Slides were deparaffinized in xylene then hydrated by a graded ethanol series. Slides were incubated for 5 minutes in 3% hydrogen peroxide to inactivate endogenous peroxidases, and digested in 1 mg/ml trypsin for 10 minutes at 37°C. Slides were incubated for 20 minutes at 25°C in 5% goat serum to reduce non-specific staining. Primary antibody incubation was performed with 3-nitrotyrosine monoclonal antibody (Cayman Chemicals, Ann Arbor, MI) at a 1:100 dilution for 12- 24 hours at 4°C, followed by incubation with a biotinylated goat anti-mouse IgG secondary antibody (15 ug/ml) for 1 hour at 25°C. Slides were then incubated in an avidin-biotinylated enzyme complex for 30 minutes at 25°C (Vectastain Elite ABC Kit, Vector Laboratories, Burlington, ON) followed by diaminobenzidine for 8 minutes at 25°C. Slides were dehydrated in a graded series of ethanol, cleared with xylene then mounted with coverslips. For pituitary sections, the chromagen Nova Red was used instead of diaminobenzadine and slides were counterstained with hematoxylin for 10 s before dehydrating and applying a coverslip. This resulted in better visualization of the borders between the pituitary lobes, as well as other anatomical landmarks, and aided in counting the pars intermedia fields of view. Negative controls included positive horse pituitary sections with the primary antibody omitted or preincubated for 24 hours at 4°C with 3-nitrotyrosine (Cayman Chemicals, Ann Arbor, MI). Following immunohistochemistry, the identity of the slides was masked for evaluation. All fields of view within the section were

examined for positively stained cells. The percent of fields (pars intermedia) or cell bodies (substantia nigra) positive for 3-nitrotyrosine was calculated and results compared.

III. Local antioxidant study

Sample collection: Pituitary pars intermedia tissue was collected from 16 additional horses donated to the Atlantic Veterinary College for euthanasia. Two horses had clinical signs of PPID which were confirmed at necropsy. Pars intermedia tissue was dissected within 15 minutes of death, washed 3X in PBS and flash frozen in liquid nitrogen. Samples were stored at -80°C until assayed.

Tissue antioxidant assays: Pituitary samples were extracted by homogenization in 500 µl PBS, followed by centrifugation at 350g for 20 minutes at 4°C. Protein content of the supernatant was measured (Biorad DC Protein Assay, Hercules, CA) and antioxidant assays performed as described above. Manganese superoxide dismutase activity was determined by inhibiting copper-zinc superoxide dismutase by adding 10 µl of 150 mM sodium cyanide to the total superoxide dismutase reaction described above. All results were standardized to the protein concentration of the sample.

Statistics: All calculations were performed using commercial statistical software (Minitab Inc, Version 13.32, State College, PA; GraphPad Software, Version Prism 3.03, San Diego, CA). Normality of the data was confirmed using the Kolmogorov-Smirnov test. All results are expressed as mean ± standard error. Red blood cell antioxidant enzyme activities were compared using a paired t-test. Results of plasma 3-nitrotyrosine was

converted to categorical data (detectable or non-detectable concentration) and compared by chi-square analysis. Pearson coefficient of correlation was used to compare pars intermedia to substantia nigra oxidative stress, pars intermedia oxidative stress to antioxidant enzyme activities, and age to antioxidant activity. A P value of less than 0.05 was considered significant.

6.3 RESULTS

I. Peripheral antioxidant study

Enzyme study groups: Breed and gender of the horses included in this study are presented in Table 1. The control horses were slightly younger than the horses with disease (19.4 ± 1.8 vs 25.8 ± 2; P=0.02). This was primarily due to a wide age difference in 2 pairs, when the only available on-farm cohort was a much younger adult (7 versus 34 years and 3 versus 39 years). Statistical comparisons performed for each assay with and without the two age-divergent pairs were not different. In addition, three control animals had α–MSH concentrations above the seasonally specific reference range cut-off values. These reference values were not established at the time of blood sample collection and analysis. Statistical comparisons performed for each assay with and without these 3 pairs were not different. Therefore, results of the complete group (20 pairs) have been presented.

Diagnosis: MSH concentration was markedly higher in the affected group compared to controls (175.5 ±21.2 vs. 17.5 ± 2.1). Nine control horses and 7 PPID horses had post-mortem histological confirmation of disease status subsequent to the study (Figure 1).

Antioxidant assays: There was no significant difference in red blood cell glutathione peroxidase or superoxide dismutase enzyme activity between the control horses and those with PPID (Figure 2). There was no difference in total glutathione concentration between the control and diseased horses (Figure 2). Red blood cell antioxidant activity was not related to age (data not shown).

Breed	Controls	PPID
Pony	3	9
Standardbred	7	0
Quarter Horse	4	6
Warmblood	1	1
Thoroughbred	0	1
Morgan	1	2
Arabian	1	1
Other breeds	3	0
Gender		
Mares	13	9
Stallions	1	0
Geldings	6	11

Table 1: Distribution of gender and breeds for horses used in peripheral antioxidant study

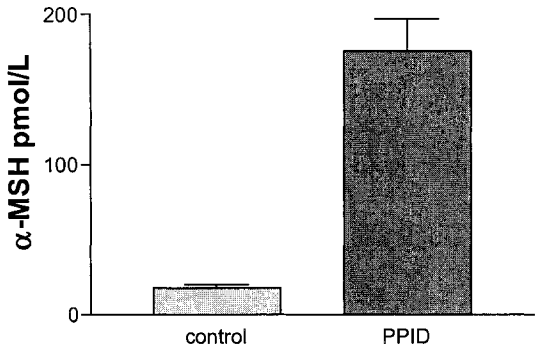

Figure 1: Plasma alpha-melanocyte stimulating hormone (α-MSH) concentration in horses used in peripheral antioxidant study (n= 20 pairs)

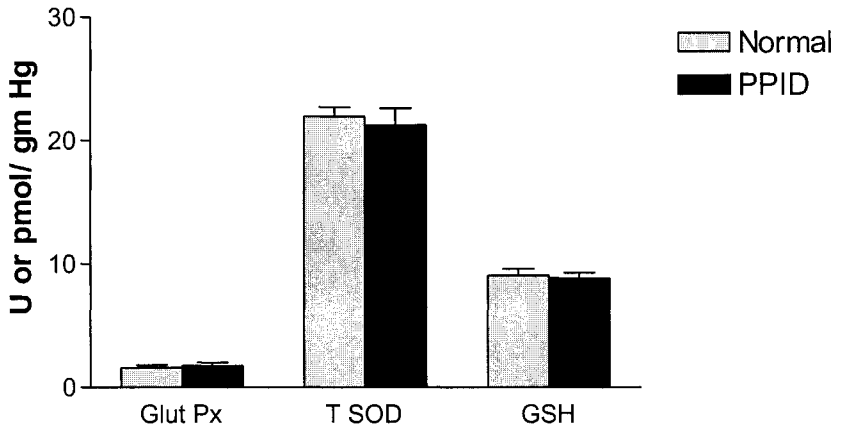

Figure 2: Red blood cell antioxidant enzyme activity in horses with PPID and healthy horses

Enzyme activity and total glutathione (GSH) concentration in red blood cell lysates from horses with PPID (n=20) and controls (n=20) were measured. Results are expressed in units of enzyme activity/gm of hemoglobin (glutathione peroxidase (Glut Px), total superoxide dismutase (TSOD) or nmol / gm of hemoglobin (GSH). There is no statistical difference between the two groups for any of the assays ($P<0.05$).

II. Systemic oxidative stress study

Plasma: The number of horses with detectable plasma 3-nitrotyrosine concentration was not different between horses with and without disease (Chi square, P=0.49) (Table 2).

Immunohistochemistry: There was no specific binding of 3-nitrotyrosine antibody observed in the adrenal glands from any of the horses. There was minimal immunoreactivity in the cell bodies of the substantia nigra (≤7% positive cells). No correlation was found between the percent of 3-nitrotyrosine positive cell bodies in the substantia nigra and oxidative stress in the pars intermedia (r=0.35, P=0.11). No correlation was found between the percent of 3-nitrotyrosine positive cell bodies in the substantia nigra and oxidative stress in the pars intermedia comparing only animals with (r= - 0.26, n = 5, P=0.67) or without (r=0.36, n= 15, P=0.15) PPID.

	3NT (nM)	Controls	PPID
	not detectable	15	13
Detectable	<100	3	4
	100-500	2	0
	> 500	0	3

Table 2: Plasma 3-nitrotyrosine concentration in PPID and normal horses

III. Local antioxidant study

Pituitary Antioxidant Activity: Glutathione peroxidase activity increased as pars intermedia 3-nitrotyrosine (oxidative stress) increased (r=0.51, *P*=0.04, Figure 3). There was no correlation between total glutathione, total superoxide dismutase or manganese superoxide dismutase activity and oxidative stress in the pars intermedia (Figure 3). Manganese superoxide dismutase activity in the pars intermedia decreased significantly with age (r= -0.55, *P*= 0.02, Figure 4). There was no affect of age on pars intermedia total glutathione concentration, glutathione peroxidase or total superoxide dismutase activity (Figure 4).

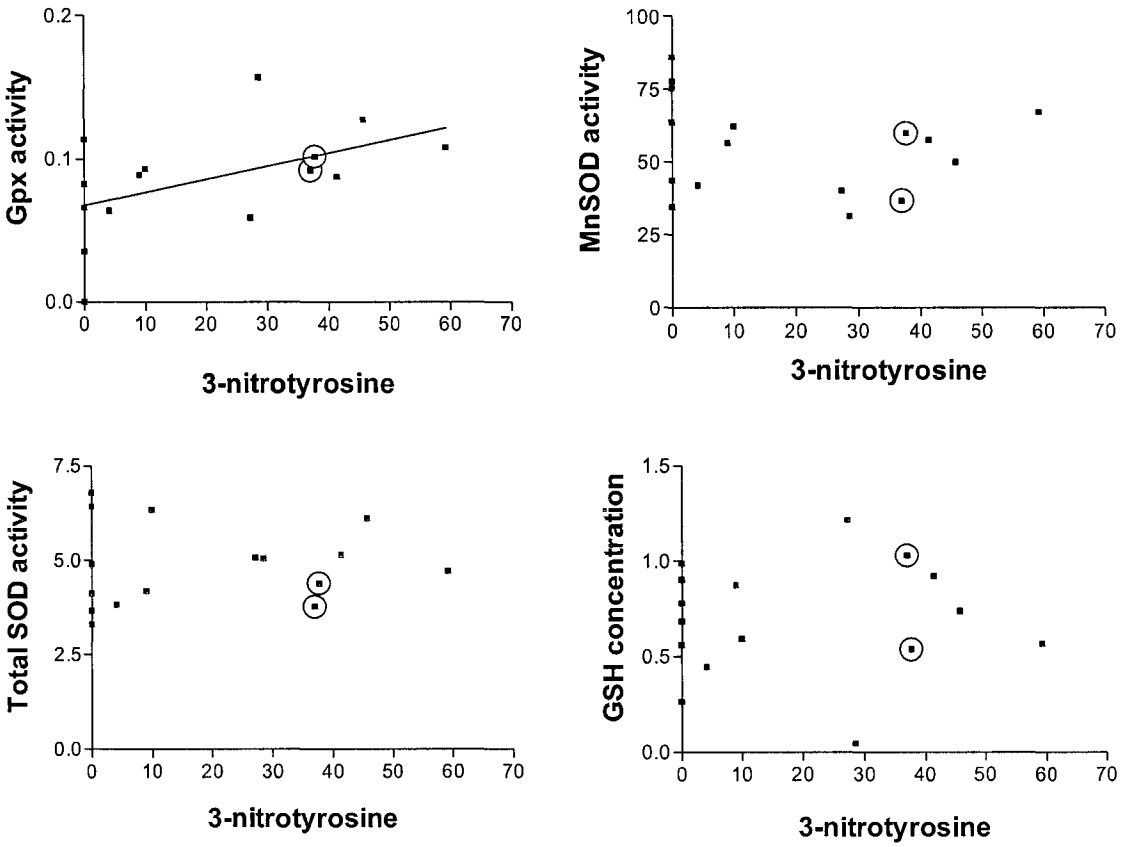

Figure 3: Pars intermedia antioxidant activity compared to oxidative stress

Glutathione peroxidase activity increased as the percent of pars intermedia 3-nitrotyrosine increased (r = 0.51, *P*= 0.04). Pars intermedia manganese superoxide dismutase activity, total superoxide dismutase activity and total glutathione concentration did not correlate to pars intermedia oxidative stress (r = -0.2, *P*=0.45; r = -0.5, *P*=0.8; r = 0.06, *P*=0.84, respectively). Circles indicate horses with pituitary pars intermedia dysfunction.

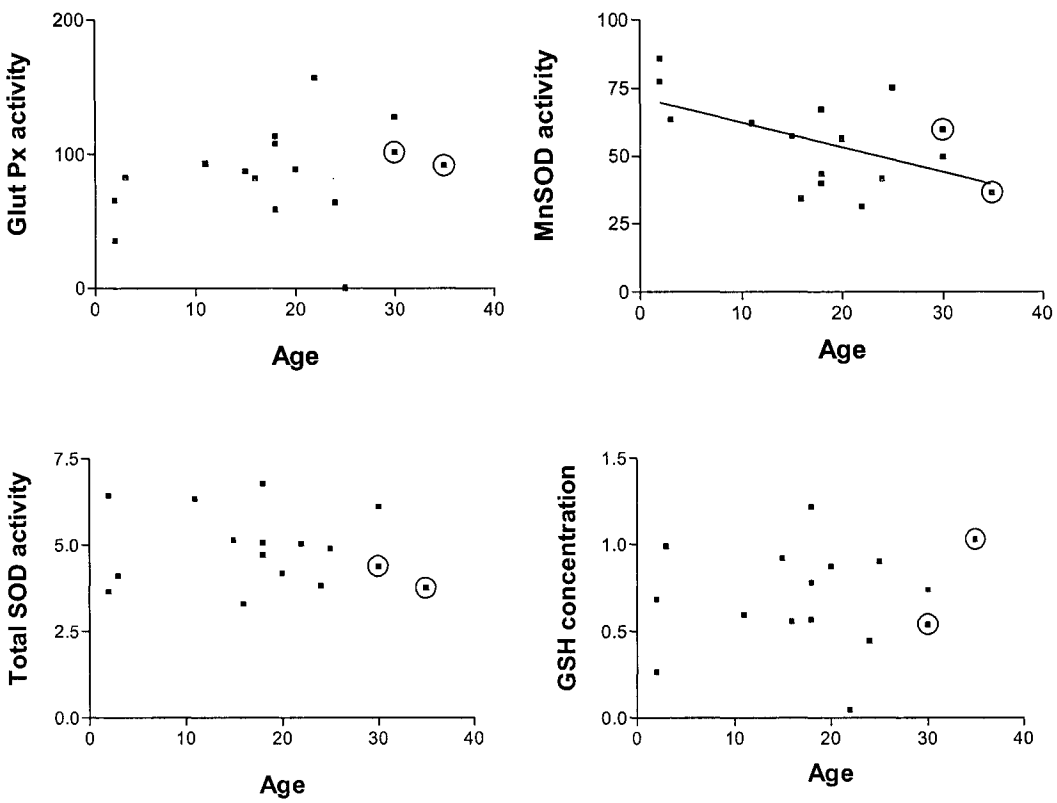

Figure 4: Pars intermedia antioxidant activity compared to age of horse

Pars intermedia manganese superoxide dismutase activity was negatively correlated to age ($r = -0.55$, $P = 0.02$). Pars intermedia glutathione peroxidase activity, total superoxide dismutase activity and total glutathione concentration did not correlate with age ($r = 0.27$, $P = 0.31$; $r = -0.11$, $P = 0.7$; $r = 0.11$, $P = 0.7$, respectively). Circles indicate horses with pituitary pars intermedia dysfunction.

6.4 DISCUSSION

It is well established that both environmental and genetic factors may contribute to an individual's risk of disease. The breed specific increased prevalence of PPID in ponies and Morgan horses suggests there may be genetic as well as environmental factors that influence risk of PPID. Studies performed in our laboratory suggest a role for oxidative stress in the pathogenesis of PPID. 3-nitrotyrosine was demonstrated to be increased in the pars intermedia of affected horses compared to age-matched and young, non-affected horses (McFarlane et al., 2005). 3-nitrotyrosine, the product of nitration of tyrosine by the reactive intermediate peroxynitrite, is a marker of oxidative stress and has been shown to accumulate in tissues of animals with diseases associated with oxidative stress (Kojda and Harrison, 1999). Horses with PPID had a 2-fold increase in 3-nitrotyrosine compared to non-affected aged horses and a 15-fold increase compared to young horses (McFarlane et al., 2005).

Oxidative stress can result from exposure to exogenous or endogenous reactive oxygen species (ROS) or poor antioxidant capacity. Therefore, it is important to consider the impact of environment factors when assessing risk of developing a disease associated with oxidative damage. Epidemiological studies report an increased risk of Parkinson's disease, a condition associated with oxidative stress and degeneration of the nigrostriatal dopaminergic neurons, with increased exposure to pesticides and herbicides (Priyadarshi et al., 2001). In animal models for PD, exposure to agricultural chemicals increases both markers of oxidative stress and neurodegeneration (Gillette and Bloomquist, 2003). Although it has not been investigated, geographic and farm factors might influence risk of PPID. For example, pesticides, herbicides and heavy metals in water, soils or pasture

could be a source of exogenous oxidants for grazing animals. Selenium deficiency, which occurs in horses fed crops grown in selenium poor soil, can result in decreased antioxidant capacity in part due to reduced glutathione peroxidase activity. A protein- poor diet or heavy parasite burden might also affect an animal's synthesis and homeostasis of glutathione and other antioxidant proteins. Therefore, to minimize the potential impact of environmental factors contributing to free radical exposure, we prioritized selection of control horses from the same farm as affected horses. When this was not possible, we picked an age-matched animal from a similar environment.

While all breeds of horses may develop PPID, ponies and Morgan horses are at greater risk (Schott, 2002). We therefore hypothesized that horses with PPID might have a genetic defect leading to a systemic decrease in antioxidant capacity. We chose to measure superoxide dismutase and glutathione peroxidase in the peripheral blood because systemic deficiencies of these two enzymes have been reported to be associated with dopaminergic neuronal vulnerability and degeneration in animal models and in human cases of Parkinson's disease (Kalra et al., 1992). In addition, because catalase is confined to peroxisomes, it is unlikely to have a major role in protecting neurons from exogenous or endogenous free radical exposure (Schulz et al., 2000). In our study, we did not find any difference in superoxide dismutase or glutathione peroxidase activity in the peripheral red blood cells of PPID affected horses compared to normal horses from the same environment (Figure 2). A polymorphism can also occur in the non-coding region of a gene, and result in a decrease in the inducible expression of the enzyme or an alteration in the tissue specificity of its expression. In this case, *in vitro* assays of peripheral blood would be

unlikely to reveal altered enzyme activities. Therefore, we measured antioxidant activity in pituitary pars intermedia tissue.

Antioxidant activity was compared to 3-nitrotyrosine accumulation in the pars intermedia from 16 horses. In addition to the antioxidants measured in the peripheral blood, we chose to also measure manganese superoxide dismutase in the pituitary tissue, as it is an inducible enzyme whose activity has been reported to be decreased in the substantia nigra of Parkinson's patients (Schulz et al., 2000). Total and manganese superoxide dismutase activity was not correlated to oxidative stress in the pars intermedia (Figure 3). However, glutathione peroxidase activity increased in proportion to the amount of 3-nitrotyrosine in the pars intermedia (Figure 3). These data suggest the accumulation of 3-nitrotyrosine in the pars intermedia is not a function of a deficiency in constitutive superoxide dismutase or glutathione peroxidase expression. In fact, there appears to be a local upregulation of glutathione peroxidase activity in the presence of oxidative stress. Induction of glutathione peroxidase and manganese superoxide dismutase has been reported in both cell culture and *in vivo* systems in response to oxidative stressors such as hydrogen peroxide or paraquat (Krall et al., 1988; Bianchi et al., 2002; Kunikowska and Jenner, 2003). It is possible the lack of increase in manganese superoxide dismutase activity in association with oxidative damage observed in this study reflects a failure of this enzyme to be appropriately induced by the presence of ROS.

Another important cellular antioxidant is the tri-peptide, glutathione. Glutathione has the ability to accept electrons, becoming glutathione disulfide. Together, these two comprise the redox system that predominantly determines antioxidant capacity of cells. Glutathione is manufactured within the cell, using dietary sources of amino acids through

the action of two cytosolic enzymes. Deficiencies in glutathione have been demonstrated to occur in the substantia nigra in Parkinson's disease, prior to any clinical manifestations of disease (Schulz et al., 2000). Therefore we measured total glutathione concentration in the peripheral blood as well as in the pars intermedia. There was no difference in glutathione concentration in red blood cells from PPID horses compared to normal horses. In addition, glutathione concentration was not correlated to oxidative stress in the pars intermedia.

The antioxidant system is complex, and involves a large number of enzymes and non-enzymatic antioxidants beyond what was specifically measured in this study. Therefore we assessed total plasma, adrenal and substantia nigra 3-nitrotyrosine as a marker of systemic free radical exposure or antioxidant failure. There was no difference in 3-nitrotyrosine accumulation in any tissue examined other than the pituitary, suggesting PPID is not the result of systemic oxidative stress.

In addition to contributing to disease development, oxidative stress is believed to play a role in the process of normal aging. Accumulation of oxidative damage is proposed to contribute to age-associated cellular and systemic organ failure (Finkel and Holbrook, 2000). In both invertebrate and rodent models of aging, genetic manipulation to increase antioxidant capacity results in a decrease in incidence of age-related disease as well as clinical manifestions of advanced age such as frailty (Martin et al, 1996; Guarente and Kenyon, 2000; Aigaki et al., 2002). In a previous study, we demonstrated an age-associated accumulation of 3-nitrotyrosine in the pars intermedia of horses (McFarlane et al., 2005). Here we report an age-associated decrease in manganese superoxide dismutase activity in the pars intermedia of horses. There was no association between age and

peripheral blood activity of any antioxidant measured. It is attractive to hypothesize that the age-related decline of manganese superoxide dismutase activity results in a chronic accumulation of pars intermedia oxidative damage, thereby contributing to increased risk of PPID with age. However, we found no association between pars intermedia manganese superoxide dismutase activity and 3-nitrotryosine accumulation in horses. Further studies, with larger numbers of animals are necessary to adequately determine the role of age–associated decline in antioxidant capacity with development of PPID.

The experiments described in this report were designed to answer three questions. First, is there a systemic deficiency in antioxidant capacity in horses with PPID? No evidence of systemic accumulation of oxidative stress markers or deficiencies in antioxidant capacity was observed. Second, is there a local deficiency in antioxidant capacity that leads to oxidative stress in the pars intermedia of horses? In the small number of horses and limited number of antioxidants examined, there was no decrease in antioxidant capacity associated with 3-nitrotyrosine accumulation in the pars intermedia. On the other hand, glutathione peroxidase activity increased with oxidative stress. It is possible that manganese superoxide dismutase should have also been induced, and the lack of response may have contributed to oxidative damage. Finally, is there evidence of an age-related decline in systemic or local antioxidant capacity in the horse? While systemic antioxidant capacity was maintained during aging, pars intermedia manganese superoxide dismutase activity declined in an age-dependant manner and therefore may contribute to the increased risk of PPID observed with advanced age.

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6.6 SIGNIFICANCE OF FINDINGS

These data suggest a deficiency in antioxidant capacity (systemic or pars intermedia) alone is unlikely to be a major predisposing factor for pituitary pars intermedia dysfunction (PPID). However, manganese superoxide dismutase may have a role in the development of disease in the presence of other risk factors. The age-associated decrease in enzyme activity could contribute to the increased risk of PPID in geriatric horses. In addition, manganese superoxide dismutase induction was not observed in response to pars intermedia oxidative stress in our study. This suggests a deficiency in enzyme induction might contribute to chronic cellular injury in those animals in which induction is most impaired and/or exposure to oxidants the greatest.

To further explore the role of manganese superoxide dismutase in risk of PPID, a genomic study was performed (Chapter 7). The equine manganese superoxide dismutase gene was examined for the presence of polymorphisms and the association of these genetic variants with risk of oxidative stress and/ or PPID was assessed in a large population of horses.

CHAPTER 7:

Manganese superoxide dismutase genetic polymorphism in horses and its association with pituitary pars intermedia oxidative stress and dysfunction.

7.1 INTRODUCTION

Cellular damage secondary to exposure to reactive oxygen species (ROS) has been implicated in development of a number of age-related diseases, as well as aging itself (Reviewed in Kohen and Nystra, 2002; Bokov et al., 2004). Oxidative damage may result secondary to excessive exposure to ROS or due to impairment in the antioxidant system. The antioxidant system responsible for detoxifying reactive intermediates is comprised of enzymes, such as glutathione peroxidase and superoxide dismutase, and non-enzymatic antioxidants, such as glutathione.

Recently, we reported an accumulation of the oxidative stress marker, 3-nitrotyrosine in the pars intermedia of aged healthy horses and in horses with pituitary pars intermedia dysfunction (PPID) (McFarlane et al., 2005). Concurrent with an increase in 3-nitrotyrosine, we observed a proportional increase in activity of the antioxidant enzyme glutathione peroxidase (Chapter 6). The physiologically appropriate induction of glutathione peroxidase activity in response to pars intermedia oxidative stress suggests impairment of this enzyme is unlikely a major risk factor for development of PPID. However, manganese superoxide dismutase (MnSOD) activity was not increased in the pars intermedia of horses with oxidative damage. A decrease in pars intermedia MnSOD activity was correlated to the age of the horse. Based on these data, we proposed that deficiencies in pars intermedia MnSOD activity and/or inducibility could contribute to increased risk of oxidative stress and/or PPID.

Protein activity may be decreased due to alterations in protein structure that reduce but do not abolish enzyme function. Alterations in structure are usually the result of

genetic mutations (also known as variants or polymorphisms) in the coding region for that protein. Enzyme activity may also be impaired due to a decrease in gene expression. Variation in gene expression may result from mutations in non-coding, regulatory regions of a gene. Polymorphisms may increase risk of disease when present alone, or in combination with other polymorphisms or other environmental risk factors. We hypothesized that genetic polymorphisms exist in the equine MnSOD gene and are associated with increased risk of oxidative stress and/or PPID.

7.2 METHODS AND MATERIALS

Animals: All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care, following approval by the UPEI Animal Care Committee. Horses with pituitary pars intermedia dysfunction (PPID) were selected based on the presence of clinical signs including hirsutism, abnormal shedding, laminitis, polydipsia/ polyuria, bilateral epaxial or gluteal muscle mass atrophy, chronic sinusitis or dermatitis, abnormal fat deposits or lethargy. PPID status in study animals was confirmed either by postmortem examination (adenomatous hyperplasia) or high endogenous plasma α-melanocyte stimulating hormone (α–MSH) concentration (Boujon et al., 1993; McFarlane et al., 2004). Seasonally specific reference ranges were used for diagnosis of PPID using endogenous plasma α-MSH concentration. Plasma samples collected in the winter, spring, or summer were considered consistent with disease if > 19 pmol/L, samples collected from horses in the fall (September 1- November 30) were consistent with disease if > 70 pmol/L, those collected from ponies in the fall were consistent if > 195 pmol/L (Chapter 2.6).

Genotype versus PPID or PPID high risk breeds

Animals: To assess the impact of genotype on risk of disease, DNA was collected from 172 client-owned horses recruited from the referral population of the Atlantic Veterinary College (102 healthy aged horses (15 or older) and 70 with PPID). DNA from 208 animals (141 horses, 19 Morgan horses and 48 ponies) was used to assess the association between genotype and breed.

DNA and RNA isolation: Whole blood was collected by jugular venipuncture into evacuated glass tubes containing EDTA as an anticoagulant. Samples were maintained on ice until processed. Samples were centrifuged at 800*g* for 10 minutes at 4°C. The plasma and buffy coat were removed to separate polypropylene tubes for α-MSH measurement and nucleic acid extraction. In samples for RNA extraction, the buffy coat layer was diluted 1:9 in lysis solution (17 mM NH_4Cl, 10 mM $KHCO_3$) and incubated at 25°C for 10 minutes. Samples were centrifuged at 300 *g* for 10 minutes at 4°C. Total RNA was extracted from the pellet of white blood cells using TRIzol™ (Molecular Research Center, Cincinnati, OH). RNA was treated with DNase (Ambion, Austin, Texas) to remove any genomic DNA contamination and reverse transcribed into cDNA using random primers (Thermoscript RT PCR kit, Invitrogen, Carlsbad, CA). Genomic DNA was extracted from buffy coats using DNeasy Tissue purification system (Qiagen, Inc, Mississauga, Ontario).

PCR and sequencing: Polymerase chain reaction (PCR) was performed using primers (Table 1) selected based on published sequence (Ishida et al., 1999) or sequence determined in our laboratory for equine MnSOD and reported in this manuscript. Standard PCR reactions were performed in a thermal cycler (MJ DNA engine, Watertown, MA) using 40 cycles of annealing at 56°C for 20 seconds, extension at 76°C for 1 min, and denaturation at 94°C for 20 seconds. PCR controls included a sample in which RNA was omitted in the reverse transcription (RT) reaction, a sample in which the reverse transcription enzyme was omitted, and a sample where the cDNA was replaced with water. The resulting PCR products were gel purified (cDNA PCR product, n = 8; genomic DNA PCR product, n =7) and sequenced in the forward and reverse direction (Guelph Molecular

SuperCentre, Ontario, CA, ABI Prism 3100). Comparison to published sequences (http://www.ncbi.nlm.nih.gov/BLAST) confirmed the PCR products as MnSOD.

Restriction fragment length polymorphism analysis (RFLP): RFLP was performed following PCR of genomic DNA using full length MnSOD primers (Primers 1 & 2, Table 1). PCR products were digested for 2 hours at 37° C using restriction enzyme Sdu I (Fermentas, Burlington, ON). Resulting products were separated on a 15% TBE acrylamide gel for 3 hours and stained for 10 minutes in 0.5 μg/ml ethidium bromide in 0.5 TBE.

	Name	Sequence (5'-3')	Fig 1, color
1	MnSODF1	gacggtcaccatgttgtgc	red
2	MnSODR1	ggtatactgtggtacttcgag	red
3	MnGenF430	gcaattgcgggacgccaactttg	purple
4	MnPubF430	gacgccatcaaacgtgactt	green

TABLE 1: Equine magnanese superoxide dismutase PCR primers

Genotype versus enzyme activity: Manganese superoxide dismutase activity was measured in pituitary pars intermedia tissue from 16 horses donated to the Atlantic Veterinary College for euthanasia. Genomic DNA was collected and genotype determined as described above. The pituitary was removed within 15 minutes of euthanasia, and pars intermedia tissue dissected and flash frozen in liquid nitrogen. Samples were stored at -80°C until assayed. Pars intermedia was homogenized in 500 µl PBS, followed by centrifugation at 350 *g* for 20 minutes at 4°C. Protein content of the supernatant was determined (Biorad DC Protein Assay, Hercules, CA). Pars intermedia manganese superoxide dismutase activity was measured in microtiter plates using a previously described method (Peskin and Winterbourn, 2000). 10 µl of the sample was added to triplicate wells containing 230 µl of PBS (pH 8.0), 10 µl of 3 mM EDTA, 10 µl of 3mM xanthine and 10 µl of 150 mM sodium cyanide. To start the reaction, 20 µl of 58 mU/ml xanthine oxidase and 20 µl of 0.75 mM WST-1 (Dojindo Laboratiories, Japan) were added to each well. Change in absorbance at 438 nm over 30 minutes at 25°C was measured. Bovine superoxide dismutase was used as a control. Results were standardized to the protein concentration of the sample and enzyme activity compared to genotype.

Genotype versus pars intermedia oxidative stress: The association between genotype and pars intermedia oxidative stress was assessed in 38 mature horses (≥14 years). The presence of oxidative stress in the pars intermedia was assessed by immunohistochemistry of formalin-fixed, paraffin-embedded pituitary tissue. Sections were incubated for 24 hours at 4°C with 3- nitrotyrosine monoclonal antibody (Cayman Chemicals, Ann Arbor, MI) at a 1: 100 dilution followed by incubation at 25 °C for one hour with a biotinylated

goat anti-mouse IgG secondary antibody. Sections were then incubated in an avidin-biotinylated enzyme complex for 30 minutes at 25°C (Vectastain Elite ABC Kit, Vector Laboratories) followed by the enzyme substrate Nova Red, for 8 minutes at 25°C. Slides were counterstained with hematoxylin for 10 seconds prior to dehydrating and applying a coverslip. Negative controls included normal horse pituitary sections with the primary antibody omitted or with the primary antibody pre-incubated with 3-nitrotyrosine (10mM for 12 hours at 4°C). Following immunohistochemistry, all fields of view within the pars intermedia were examined for each horse section analyzed and graded as either positive or negative for 3-nitrotyrosine. The percent positive fields was calculated for each horse and compared to the genotype.

Statistics: All statistical calculations were performed using commercial statistical software (Minitab Statistical Software, 13.32 ™, State College, PA, USA; GraphPad Prism®, V3.03, San Diego, CA, USA). Frequency of alleles in animals with disease or at risk breeds was compared using Chi square test. For three-point comparisons (genotype versus enzyme activity, genotype versus oxidative stress) analysis of variance was used, with Newman-Keuls multiple comparison test used for post-hoc analysis. $P < 0.05$ was considered statistically significant.

7.3 RESULTS

The cDNA sequence of MnSOD was obtained in eight horses (6 healthy, 2 PPID; mean age: 16.4 ± 12.5 years; 6 mares, 2 geldings; 5 ponies, 3 Standardbreds). There was 100 % consensus among the eight sequences obtained. Sequence obtained from cDNA from all 8 horses showed a 1-basepair substitution (base 415, adenosine to guanosine) compared to the previously published cDNA sequence (Gen Bank AB0011693, Figure 1). This nucleotide substitution results in a change in the predicted amino acid sequence, changing a lysine (amino acid 115) to glutamic acid. Our sequence data is consistent with a partial MnSOD gene sequence in the horse (GenBank: AY246751) and sequence data from humans (GenBank: NM000636).

To facilitate rapid detection of polymorphisms in a large number of horses, we examined genomic DNA for the presence of restriction fragment length polymorphisms (RFPL). Using the restriction enzyme Sdu I, a RFLP was observed in the amplified genomic MnSOD. Horses were subsequently referred to as having the low migrating homozygous (L/L), high migrating homozygous (H/H) or heterozygous (L/H) genotypes (Figure 2).

To elucidate the nature of the variant, PCR products amplified from genomic DNA from 7 horses (5 healthy, 2 PPID; mean age: 21.4 ± 8.6 years; 4 mares, 3 geldings; 4 ponies, 3 light breed horses) representing all three genotypes were sequenced (Figure 3). Results from sequencing showed the presence of a 9-basepair deletion at bases 450-459 in genomic DNA sequence (all horses) compared to the cDNA sequence. In addition, in horses with the L/L genotype, there was a second 9-basepair deletion at bases 150-159.

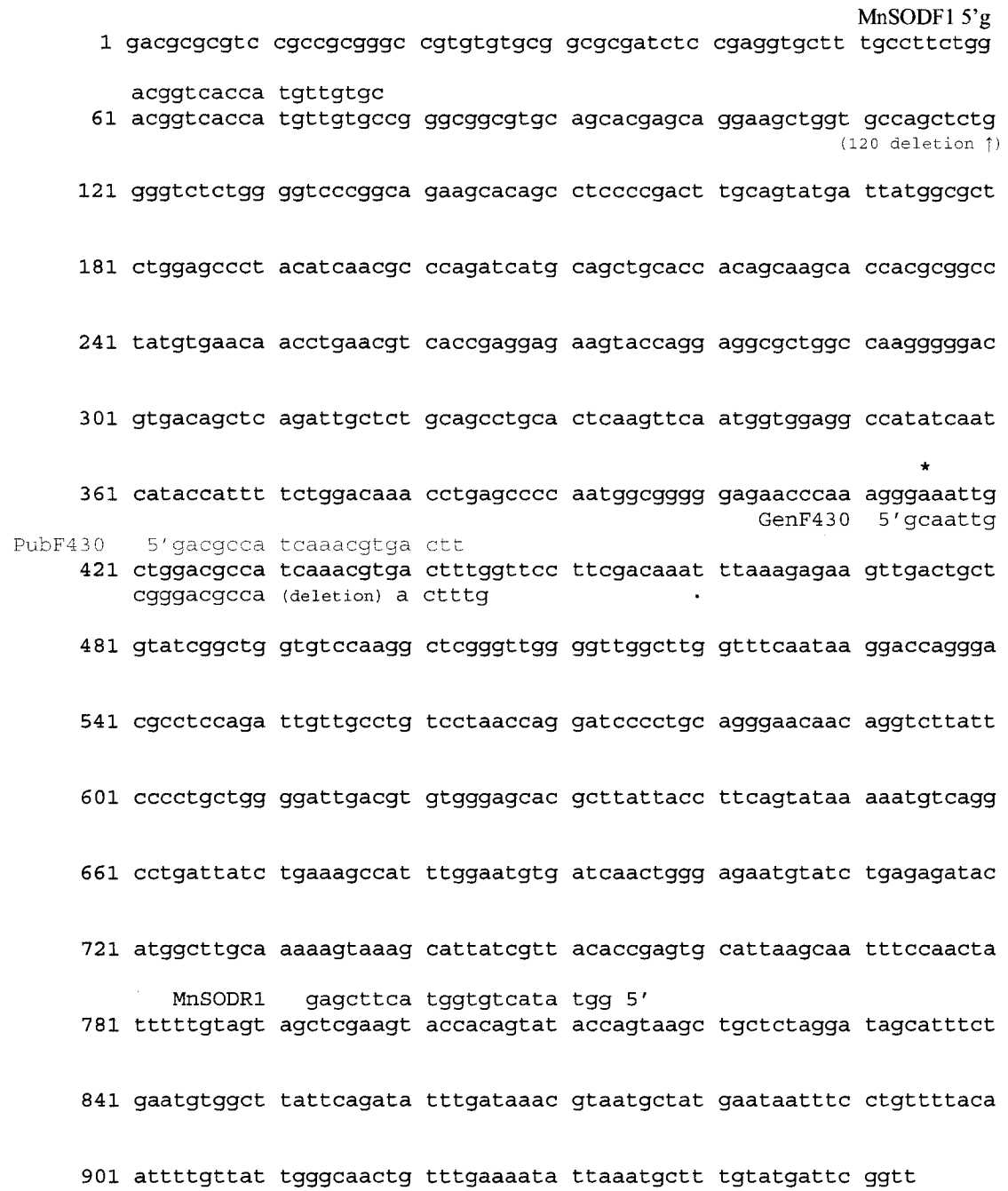


Figure 1: Published sequence of equine manganese superoxide dismutase (Gen Bank AB0011693 and location of primers (Table 1) used in study.

Asterisk- A single nucleotide substitution (A to C) was present in all horses sequenced in this study compared to published sequence. Deletions found in genomic sequence are indicated in blue.

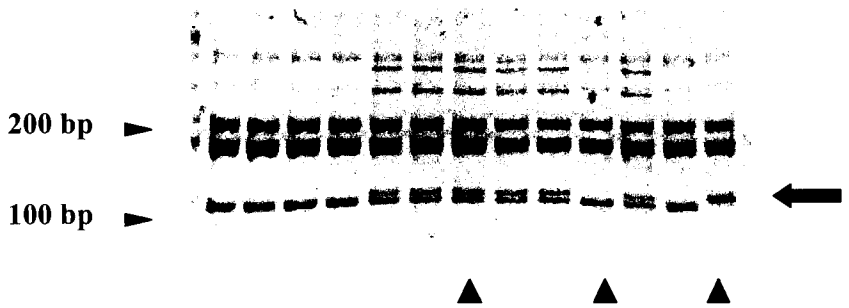


Figure 2: Restriction fragment length polymorphism in equine MnSOD.

Genomic DNA from horses was amplified for the MnSOD gene, digested with SduI and separated on a 15% polyacrylamide gel. Black arrow: band of interest. Blue arrowhead, L/H genotype; red arrowhead, L/L genotype; green arrowhead, H/H genotype.

With the exception of this deletion, there was 100% homology in the genomic DNA sequence data from all seven horses. However, the sequence of the genomic DNA differed from the cDNA sequence by 12-basepair substitutions. This suggested the sequence obtained from genomic DNA was not from the same gene as the cDNA sequence data. Therefore, it was considered likely that there are two MnSOD genes in the horse. The outcome of the 12-basepair variations was a change in the predicted amino acid sequence in 10 amino acids (Figure 4). In addition, the protein sequence predicted by the genomic MnSOD gene would be 3 ("H" allele) or 6 ("L" allele) amino acids shorter than the cDNA gene due to the deletions. To determine if the genomic MnSOD gene was transcribed, primers were designed that spanned the deletion at basepairs 430-439 (Figure 1). Primers that included the nucleotides 430-439 amplified the cDNA but not the genomic DNA. Conversely, "deletion primers" that included sequences from either side of the deletion but not the deletion itself, amplified both the genomic DNA and cDNA. PCR in both cases resulted in an appropriate size product (Figure 5).

The frequency of the "H" or "L" allele in horses with PPID was evaluated in 102 normal, aged horses (≥15 years) and 70 PPID horses (Figure 6). No difference was found in the frequency of either allele ($P = 0.6$) or genotype (H/H, L/L, H/L) associated with disease. The relationship between frequency of the "H" or "L" allele and breed was assessed in 208 horses. Breeds at high risk of PPID (ponies and Morgan horses) were compared to all other breeds (low risk). The "L" allele was more common in ponies ($P< 0.0001$) compared to horses of low risk breeds (Figure 6). The "L" allele was also more common in Morgan horses compared to low risk horses, however this difference was not significant, likely due to the small sample size (n = 19, P=0.19). There was no association between allelic frequency and disease within either high risk or low risk breeds.

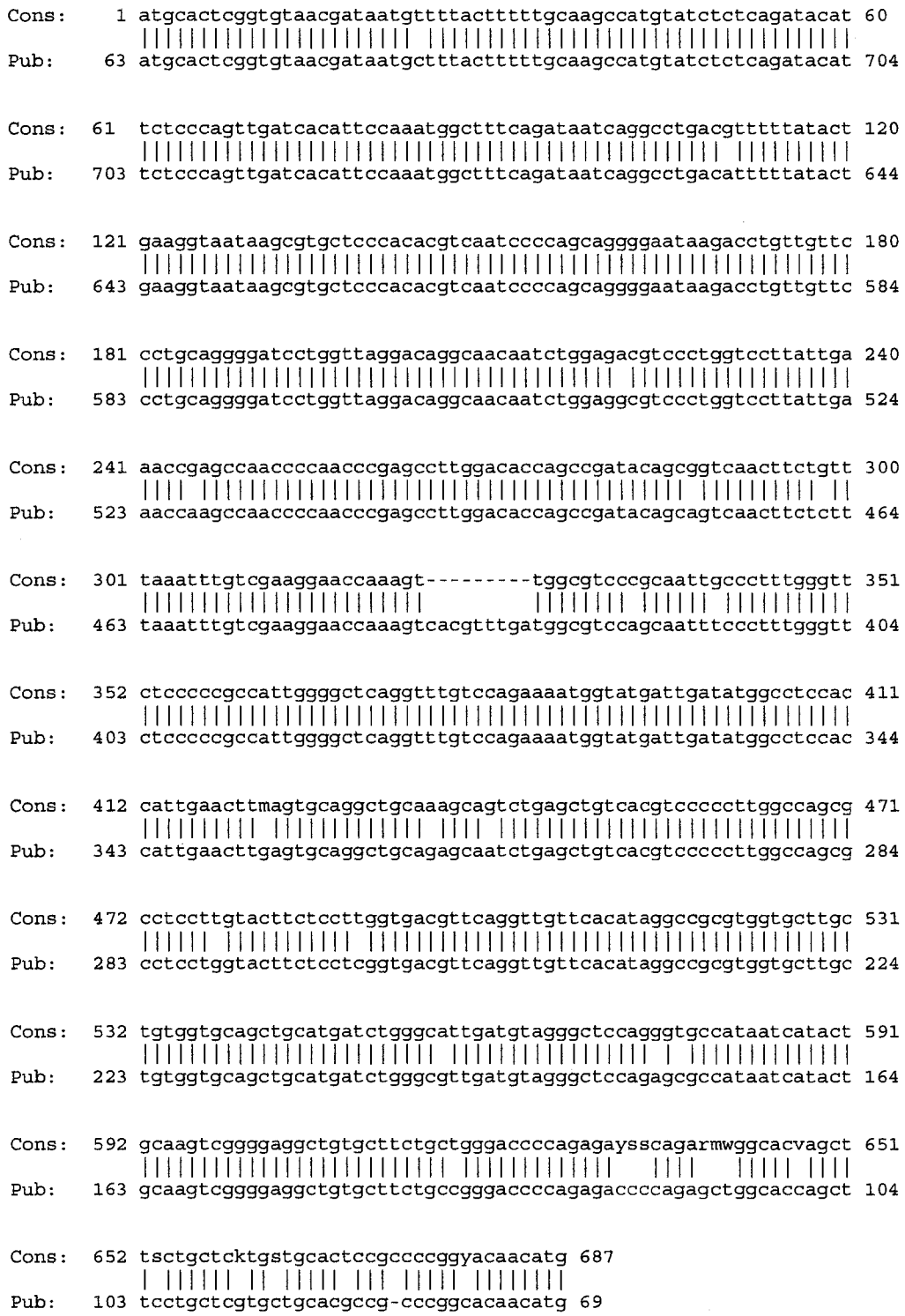


Figure 3. Consensus DNA sequence for genomic MnSOD (cons) aligned with published cDNA sequence (pub). There are 12 nucleotides that differ and a 9-basepair deletion at nucleotide 430-439. The 9 nucleotides shown in red are also deleted in the "L" genomic MnSOD allele.

```
"L"  consensus      --------------------TXSMLXRAECSTSRKLVPALG---SQQKHSLPDLQYDYGT
"H"  consensus      --------------------RSPMLCRAECSTSRKLVPALGSLGSQQKHSLPDLQYDYGT
cDNA consensus      DARPPRAVCAARSPRCFAFWTVTMLCRAACSTSRKLVPALGSLGSRQKHSLPDLQYDYGA
cDNA published      ----------------------MLCRAACSTSRKLVPALGSLGSRQKHSLPDLQYDYGA
                                           ** ** ************   *@*************:

"L"  consensus      LEPYINAQIMQLHHSKHHAAYVNNLNVTKEKYKEALAKGDVTAQTALQPALKFNGGGHIN
"H"  consensus      LEPYINAQIMQLHHSKHHAAYVNNLNVTKEKYKEALAKGDVTAQTALQPALKFNGGGHIN
cDNA consensus      LEPYINAQIMQLHHSKHHAAYVNNLNVTEEKYQEALAKGDVTAQIALQPALKFNGGGHIN
cDNA published      LEPYINAQIMQLHHSKHHAAYVNNLNVTEEKYQEALAKGDVTAQIALQPALKFNGGGHIN
                    ****************************:***:*********** ***************

"L"  consensus      HTIFWTNLSPNGGGEPKGQLRDAN---FGSFDKFKQKLTAVSAGVQGSGWGWLGFNKDQG
"H"  consensus      HTIFWTNLSPNGGGEPKGQLRDAN---FGSFDKFKQKLTAVSAGVQGSGWGWLGFNKDQG
cDNA consensus      HTIFWTNLSPNGGGEPKGELLDAIKRDFGSFDKFKEKLTAVSAGVQGSGWGWLGFNKDQG
cDNA published      HTIFWTNLSPNGGGEPKGKLLDAIKRDFGSFDKFKEKLTAVSAGVQGSGWGWLGFNKDQG
                    ******************:* **    ********:************************

"L"  consensus      RLQIVACPNQDPLQGTTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVSERY
"H"  consensus      RLQIVACPNQDPLQGTTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVSERY
cDNA consensus      RLQIVACPNQDPLQGTTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVSERY
cDNA published      RLQIVACPNQDPLQGTTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINWENVSERY
                    ************************************************************

"L"  consensus      MACKKSTOP
"H"  consensus      MACKKSTOP
cDNA consensus      MACKKSTOP
cDNA published      MACKKSTOP
                    *****
```

FIGURE 4: Deduced amino acid sequence of equine MnSOD

Consensus nucleotide sequences were obtained for the "L" allele and "H" allele of the genomic DNA as well as for cDNA sequences obtained in our study by aligning sequence data using genomic computer software (Hall, 1999). Amino acid sequences were predicted using proteomic computer software (Gasteiger et al., 2003) * Conserved in all sequences. @ Arginine residue within the mitochondrial target sequence that is conserved in other species and equine cDNA MnSOD.

Panel A **Panel B**

Figure 5: PCR using primers with and without the 9-basepair deletion at nucleotide 430-439.

Panel (A) Amplification of genomic DNA (A-D, 4 horses) or cDNA (E) using the deletion primer set [MnGen430F (without nucleotides 430-439) and MnSODR, Table 1] results in the expected 400 basepair product. Genomic DNA (F-I, 4 horses) does not amplify using the non-deletion primer set [MnPub430F (includes nucleotide 430-439) and MnSODR, Table 1], whereas cDNA (J) produces a 400 basepair product. Panel (B) Amplification of cDNA using the deletion primer set was confirmed in two additional horses. Dark molecular weight marker (arrowhead) is 500 basepairs.

Manganese superoxide dismutase enzyme *in vitro* activity in pars intermedia tissue was not associated with genotype, although tissue was available in only 16 horses (P=0.56, Figure 7a). Pituitary pars intermedia 3-nitrotyrosine concentration was significantly less in horses with the H/H genotype as compared to the L/H genotype (n=38, P=0.03, Figure 7b). Although the 3-nitrotyrosine concentration was less in horses with the H/H genotype compared to the L/L genotype, this difference wasn't statistically significant due to the large variability within the groups.

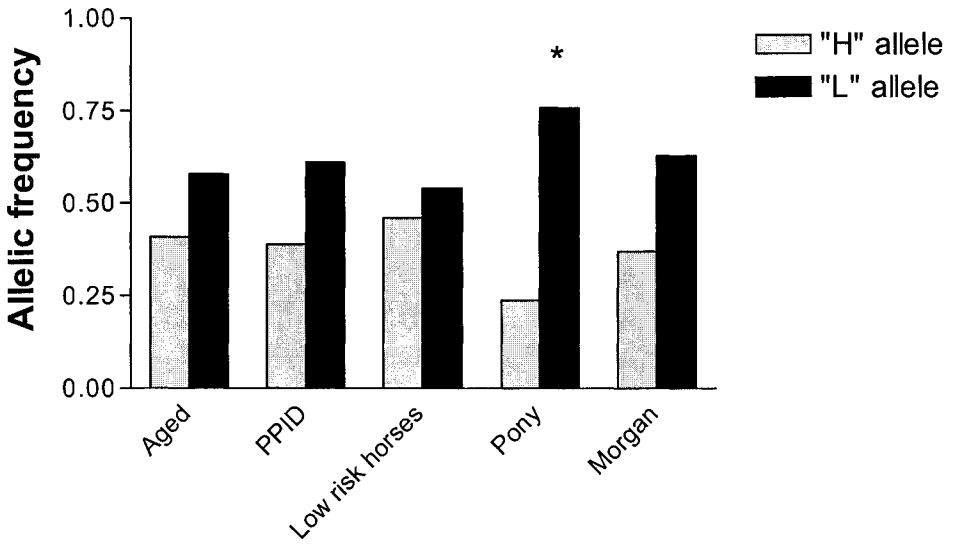
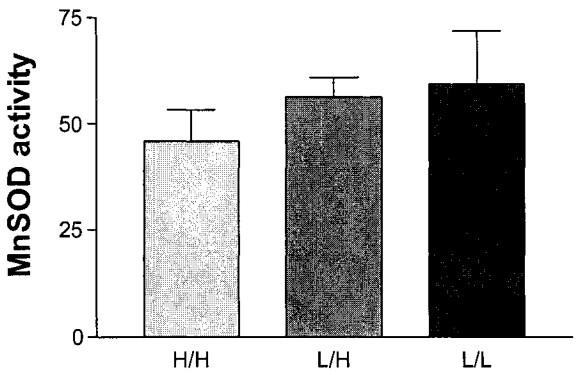


Figure 6: Frequency of the "H" or "L" MnSOD allele in equids

Frequency of the "H" or "L" MnSOD allele in aged healthy equids (n=102), equids with PPID (n=70), low risk breed horses (n=141), ponies (n=48) and Morgan horses (n=19). Low risk breeds were defined as all breeds except ponies and Morgan horses. There was no difference in allelic frequency or genotype (not shown) in horses with PPID compared to healthy aged horses. The "L" allele was more frequent in ponies compared to horses of low risk breeds. * $P < 0.0001$

Panel A



Panel B

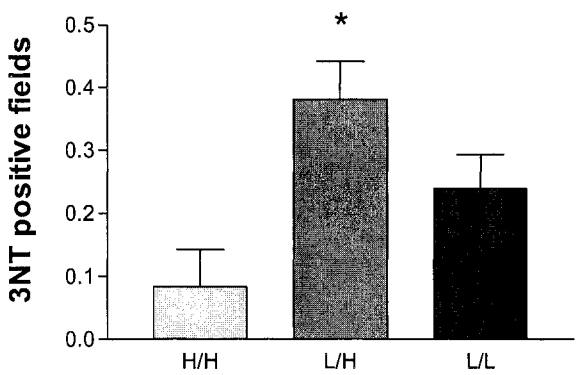


Figure 7: Manganese superoxide dismutase activity or oxidative stress among genotypes

A) There was no difference in pars intermedia MnSOD enzyme activity (U/g protein) *in vitro* among genotypes (n=16). B) Pars intermedia oxidative stress (3-nitrotyrosine, 3NT) was decreased in horses with the H/H genotype (n=5) compared to L/L (n=15) or L/H (n=18) although this difference was only significant in the L/H genotype (P=0.03).

7.4 DISCUSSION

Manganese superoxide dismutase (MnSOD) is a critical enzyme in antioxidant defense. MnSOD is encoded in the nucleus (*SOD2*) and then migrates to the mitochondrial matrix where it forms a homotetramer that catalyzes the conversion of superoxide to hydrogen peroxide (Wan et al., 1994). Expression of MnSOD is inducible by a variety of conditions that cause oxidative stress. Examples of inducers of MnSOD include lipopolysaccharide, interleukin 1, interferon-γ, mitochondrial redox state, paraquat and X-irradiation (Krall et al., 1988; Niwa et al., 1993; White and Tsan, 2001; Tsan et al., 2001; Bianchi et al., 2002).

Deficiency in MnSOD activity has been associated with increased risk of neurodegeneration. MnSOD knock-out mice die within the first 3-18 days after birth, with evidence of neurodegeneration and dilated cardiomyopathy associated with mitochondrial injury (Lebovitz et al., 1996; Van Remmen et al., 2001). Knockout mice can be partially rescued by administration of MnSOD analogs (Hinerfeld et al., 2004). *SOD2* knockout heterozygote (*SOD2-/SOD+*) mice show increased superoxide radical concentrations and are more sensitive to neurotoxins and brain ischemia (Andreassen et al., 2001).

Pituitary pars intermedia dysfunction (PPID) is a spontaneous, progressive disease of aged horses. Horses with PPID have evidence of dopaminergic neurodegeneration and oxidative stress in the pars intermedia (McFarlane et al., 2005). Therefore, we proposed horses with deficient manganese superoxide dismutase activity would be at greater risk to

develop PPID, and that a genetic variant might exist that was associated with decreased enzyme activity and increased risk of disease.

To test this hypothesis, we first examined the equine MnSOD gene for the presence of polymorphisms. Amplification of genomic DNA and cDNA resulted in distinct PCR products, suggesting the horse has two MnSOD genes. The first MnSOD gene amplified preferentially from cDNA and matched published equine cDNA sequence (Ishida et al., 1999). A second MnSOD gene amplified preferentially from genomic DNA and matched a short, partial gene sequence from the horse (Caetano et al., 1999). This second gene differed by 12-nucleotide substitutions and a 9-basepair deletion from the cDNA gene. Sequence data from rat and *C. elegans* indicates the presence of two MnSOD genes while only one gene has been reported in humans, cows and mice (Ho et al., 1991; Wan et al., 1994; DiSilvestre et al., 1995; Hunter et al., 1997). In humans and cows, multiple polyadenylation sites are present, resulting in multiple RNA transcripts of differing sizes (Church, 1990; Wan et al., 1994).

It is possible that the sequence of MnSOD amplified from equine genomic DNA is a pseudogene. Pseudogenes, genes not translated into functional proteins, are not uncommon findings in the genomes of higher animals. Pseudogenes are believed to arise by one of two mechanisms, gene duplication or reverse transcription of processed mRNA. Pseudogenes resulting from gene duplication are unprocessed; they have introns and regulatory regions typical of coding genes, but are non-functional due to an accumulation of disabling mutations (Coin and Durbin, 2004). Processed pseudogenes (reverse transcribed) are typically non-transcribed (silent) regions that differ from coding genes by the absence of introns and the presence of strings of adenosines (poly-A tails) and

flanking target duplications (Coin and Durbin, 2004). Based on these sequence characteristics reminiscent of mRNA, they are believed to be the result of reverse transcription of mRNA from coding genes. Although usually silent, a small percentage of pseudogenes are transcribed. It is estimated 2-3% of human and 0.5- 1% of mouse pseudogenes are expressed (Yano et al., 2004). Recent evidence suggests that expressed pseudogenes may function in regulation of the genes from which they are derived. Pseudogenes that enhance stability of mRNA from their homologous coding gene and pseudogenes that exert a cooperative effect with promoter elements of coding genes have been described (Yano et al., 2004; Troyanovsky and Leube, 1994). In addition, expressed pseudogenes have been shown to function as antisense RNA, inhibiting the expression of coding genes (Korneev et al., 1999).

The sequence of the equine manganese superoxide dismutase gene amplified from genomic DNA in this study did not have a poly-A tail or terminal repeats, although it did lack introns. A poly-A tail and terminal repeats if present, would likely have been outside the primer sequences used to amplify the gene. To determine whether the gene was transcribed we constructed primers that spanned the deleted sequence at amino acids 450-459. One set of primers included the 9-bases that were deleted from the genomic MnSOD gene, and was expected to only amplify cDNA. The second set of primers spanned the deleted sequence but did not include the 9-nucleotide deletion. This set was expected to amplify genomic DNA. If the genomic MnSOD gene was expressed, these primers would also amplify cDNA. Our results showed both sets of primers amplified cDNA, but only the deletion primers amplified genomic DNA. All products amplified were of an appropriate size. Nucleotide sequencing of the amplified PCR product

confirmed each primer set amplified the expected gene. Although it is not possible to determine from these experiments if genomic MnSOD is a pseudogene or coding gene, the presence of mRNA transcripts increases the likelihood that it is functional.

Using the identified restriction fragment length polymorphism (RFLP), horses were genotyped for the two genomic DNA MnSOD alleles, a low migrating ("L", with the 9-basepair deletion at nucleotide 120-129) and high migrating ("H", without the deletion) allele. To evaluate the significance of this polymorphism, we compared allelic frequency with *in vitro* pars intermedia enzyme activity, pars intermedia oxidative stress, breed, and PPID. We found no association between genotype and *in vitro* pars intermedia MnSOD activity (Figure 7a). This data must be interpreted cautiously due to the small number of horses in this experiment (n =16). It is also possible *in vitro* enzyme activity may not reflect *in vivo* activity.

To determine if the polymorphism was associated with oxidative stress *in vivo,* we examined pituitary tissue from 38 mature horses using immunohistochemistry to detect the oxidative stress marker, 3-nitrotyrosine. Oxidative stress was significantly reduced in the pars intermedia of horses with the H/H genotype compared to horses with the L/H genotype but not the L/L genotype (Figure 7b). This suggests the "H" allele might be protective against pars intermedia oxidative stress and thereby reduce risk of PPID. However, no association between genotype and PPID was found in 172 horses, 70 of which had disease. We did, however, find an increased prevalence of the L allele in ponies compared to breeds with a low risk of PPID. In fact, out of 48 ponies examined, there were none with the H/H genotype. While statistically very significant ($P< 0.0001$), the biological importance of this association is unclear. It is interesting to speculate that

the presence of the L allele might predispose ponies to develop oxidative damage more readily in conditions of increased exogenous or endogenous oxidant exposure.

Two polymorphisms in the *SOD2* gene of humans, Ile58Thr and Ala9Val, have been associated with increased incidence of Parkinson's disease (Shimoda-Matsubayashi et al., 1996; Borgstahl et al., 1996; Grasbon-Frodl et al., 1999). The first MnSOD polymorphism (Ile58Thr) results in decreased stability of the protein tetrameric interface (Borgstahl et al., 1996). A decreased ability of the enzyme to form tetramers may result in decreased *in vivo* enzyme activity. This polymorphism was not present in the horse.

A second polymorphism associated with Parkinson's disease (Ala9Val) is found in the mitochondrial target sequence of human MnSOD (Shimoda-Matsubayashi et al., 1996). All known MnSOD genes contain a 5' mitochondrial targeting sequence, that is responsible for transporting the protein to its site of action, the mitochondrial matrix (Hunter et al., 1997). In our sequence data this was not a site of genetic variation in the horse. However, other conserved mitochondrial target sequences were variable in the horse. Consensus data from the mitochondrial target sequences from other species reveal a conserved lysine (at the start of the mature MnSOD protein) and arginine residue (penultimate to the lysine). In the equine genomic MnSOD sequence reported here, the arginine residue (conserved in the equine cDNA MnSOD gene) was not conserved, but was replaced by glutamine (Figure 4). Also, in the "L" allele, the 9-basepair deletion at nucleotides 120- 129 results in a 3 amino acid deletion within the leader sequence of the protein. If the genomic DNA is translated, which has yet to be determined, it is unclear if the sequence of either or both genotypes would be transported to the mitochondria and appropriately processed to a mature MnSOD protein.

We have presented evidence of a second, expressed MnSOD gene in the horse. Sequencing of this gene has revealed the presence of two alleles, one with a 9-basepair deletion in the mitochondrial target sequence. Horses homozygous for the non-deletion, "H" allele showed a decrease in pituitary pars intermedia 3-nitrotyrosine accumulation. The allele containing the deletion, "L", was more common in equids at high risk for PPID, a disease associated with oxidative stress damage of the pituitary. Further studies are needed to reveal the interactions of genotype with other influencing factors on risk of PPID. In addition, the nature and function of the newly recognized equine genomic MnSOD gene needs to be determined.

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Chapter 8

Significance of Thesis Work and Future Directions

8.1 SIGNIFICANCE OF WORK AND FUTURE DIRECTIONS

Equine pituitary pars intermedia dysfunction (PPID) is a neuroendocrine disease of aged equids that is associated with development of life threatening complications. The pathophysiology of this condition is poorly understood and has not been the subject of investigation since 1988. The overall objective of this thesis was to gain a better understanding of the risk factors and pathogenesis of PPID. Before embarking on experiments to investigate the pathology of PPID, we sought to gain insight into the physiology of the equine pars intermedia and to assess several diagnostic tests for further use in the study. Several important findings were made during the course of this initial work.

First, we discovered that healthy horses and ponies have an increase in plasma α-melanocyte stimulating hormone (α-MSH) in the fall. This is the first evidence of seasonal regulation of equine pars intermedia activity. This finding lead to the study by Donaldson that demonstrated significant seasonal variability in results of commonly used PPID diagnostic testing methods (Donaldson et al., 2005). The outcome of these two studies has resulted in revision in disease testing strategy; it is no longer considered appropriate to test horses for PPID in the fall. This new information also calls into question previous test validation studies in which season was not considered.

The elucidation of the role of thyrotropin releasing hormone (TRH) as a positive releasing factor of the equine pars intermedia melanotrope provides insight into the mechanism of TRH-mediated cortisol release that has been observed in horses with PPID. Prior to this study the mechanism of this response, used in the diagnosis of PPID, was

unexplained. The importance of this finding in furthering our understanding of the pathophysiology of PPID is not yet known. Our study also demonstrated a failure of TRH stimulation to differentiate normal horses from those with PPID. These results suggest the TRH stimulation test is of limited value as a diagnostic method.

Another finding that impacts diagnostic testing was the lack of agreement among pathologists in interpretation of pituitary lesions in early PPID. This study highlights the need for a better description and definition of the pars intermedia changes that may be attributed to early disease, versus seasonal activity and aging. This is particularly important because histology is currently being used as a gold standard in test validation (Andrews et al., 2005) and without a consensus as to what defines PPID, it will be impossible to interpret and compare results between studies.

The primary focus of this thesis was to gain an understanding of the risk factors and early events that lead to the development of PPID. Previous research suggests PPID results from a loss of dopaminergic inhibition of the pars intermedia. Data presented here supports the previous findings and suggests dopamine loss to be the result of dopaminergic neurodegeneration. Furthermore, our evidence indicates oxidative stress of the pars intermedia dopaminergic nerve terminals has a role in the pathology of PPID. Dopaminergic neurodegeneration and oxidative stress are two pathologic hallmarks of Parkinson's disease. Therefore we investigated whether PPID shares other pathologic similarities with Parkinson's disease. Horses with PPID or marked pars intermedia oxidative stress were shown to have increased expression of the nerve terminal protein α-synuclein. This protein is integral in dopaminergic neurotoxicity and cell death in Parkinson's disease. We propose that the pathophysiology of PPID is mechanistically

similar to that of Parkinson's disease. Further studies are needed to elucidate the role of α–synuclein in neurodegeneration in PPID.

The design of the experiments presented in this thesis did not enable us to differentiate whether oxidative stress and increased α–synuclein expression was a cause or result of neurodegeneration and PPID. However, the presence of animals that had oxidative stress, increased α-synuclein protein expression and subtle histological signs of pars intermedia dysfunction (melanotrope hypertrophy, palisades and rosette formation, mild to moderate pars intermedia hyperplasia, pigment deposition in pars nervosa) without PPID (adenomatous hyperplasia or adenoma) or loss of tyrosine hydroxylase immunoreactivity leads us to suspect oxidative stress and α-synuclein induction proceeds PPID. In addition, α-synuclein was increased in horses with 3-nitrotyrosine accumulation compared to normal animals, suggesting α-synuclein may be induced secondary to oxidative stress. Further studies are needed to clarify the order in which the pathologic events occur.

Finding 3-nitrotyrosine, a marker of oxidative damage, accumulated in the pituitary of horses with PPID led us to suspect that affected animals may have a deficiency in antioxidant capacity. Oxidative damage may occur secondary to excess exposure to reactive oxygen species (ROS) or impaired antioxidant capacity. Excessive ROS may be the result of exposure to environmental oxidants, such as agricultural chemicals. Agricultural chemical exposure has been linked to increased risk of Parkinson's disease. Epidemiological data is needed to evaluate the impact of environmental toxins on pituitary function in horses. Endogenous production of excessive ROS is a second cause of oxidative stress. Mitochondrial dysfunction results in

excessive ROS production. This is believed to be an important source of oxidative stress in Parkinson's disease patients and warrants further investigation in the horse with PPID. Other sources of endogenous ROS include inflammation and systemic metabolic disease. Although it has not been critically assessed, many horses that develop PPID have a history of chronic obesity, and breeds that are predisposed to obesity are also predisposed to PPID. Obesity and chronic hyperglycemia are associated with oxidative stress in rodents and humans. Obese horses and horses with PPID often are hyperglycemic and insulin resistant. It is unclear if disturbance of glucose regulation precedes or follows pituitary dysfunction. It is interesting to speculate that obesity, hyperglycemia and insulin resistance may be risk factors for development of PPID.

We investigated the role of antioxidant capacity in the development of PPID. We suspected antioxidant deficiencies might contribute to PPID because it is known to promote development of Parkinson's and other neurodegenerative diseases. In addition, genetic predisposition of PPID (as evidenced by "at risk" breeds) might be explained by a mutation in an antioxidant gene. Enzymatic assays revealed a potential role for manganese superoxide dismutase (MnSOD) in risk for PPID. MnSOD pars intermedia activity decreased as horses aged and induction of MnSOD in response to oxidative stress was lacking. A genomic study was also used to evaluate the contribution of MnSOD to risk of disease. If a gene mutation (known as a polymorphism) occurs more frequently in individuals with a disease, it suggests the mutated gene codes for a protein involved in the disease pathology. Therefore, we looked for polymorphism in the equine MnSOD gene. Our results were unexpected. We found two distinct RNA transcripts for MnSOD gene. The human and cow are reported to have only a single MnSOD gene. The second

equine gene, which we called genomic MnSOD because it amplified more readily from genomic DNA, had a deletion polymorphism. Genotyping of a large number of horses revealed no association between PPID and allelic frequency ["L" (with deletion) versus "H" (without deletion)]. There was however an increased frequency of the "L" allele in breeds with high risk of PPID. In fact, of 48 ponies examined, none had the H/H genotype. Based on simple Mendelian genetics, 14 ponies would have been expected to be H/H. This bias is interesting, but unexplained.

The decrease in 3-nitrotyrosine accumulation in horses with the H/H genotype suggests the presence of this allele maybe protective against pars intermedia oxidative stress. However, more animals of the H/H genotype need to be examined as only 5 horses with this genotype were available for this study.

The results of this thesis suggest equine pituitary pars intermedia dysfunction may share many pathologic similarities with Parkinson's disease. Further characterization of PPID is warranted to determine if it may serve as a naturally occurring animal model of spontaneous dopaminergic neurodegeneration secondary to oxidative stress and α-synuclein overexpression.

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