Clinical expression of perennial ryegrass (lolitrem-B) intoxication in New Zealand horses

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Abstract

Perennial ryegrass staggers (PRGS) is a neurological mycotoxicosis caused by the ingestion of lolitrem-B. In this study, seven horses split into two separate groups were exposed to lolitrem-B by feeding them perennial ryegrass seed and hay containing 2 ppm lolitrem-B. Paired data was collected prior to and after two weeks exposure to lolitrem-B including video-documented neurological examination, clinical examination, brainstem auditory evoked (BAEP) and magnetic motor evoked (mMEP) potentials, blood and cerebrospinal fluid, and a frusemide challenge.

All horses developed tremor when exposed to lolitrem-B. The degree of tremor varied between individual horses and also depended on the level of activity, increasing during feeding and exercise. Using an ophthalmoscope a subtle, rapid (~5 Hz) tremor of the eyeball was detected in six of the seven horses. Subtle signs of ataxia were observed during handling, and motor dysfunction was exaggerated when blindfolded. Ataxia primarily involved a truncal sway and irregular, but predictable, limb placement that compensated for the lateralisation of the center of gravity. Results indicate that lolitrem-B may lengthen the peak V latency of BAEP traces. mMEPs also showed a lengthening in take-off latency and peak latency. The frusemide challenge revealed that renal K^+ secretion was impaired significantly ($p = 0.003$) during the first 15 minutes after frusemide administration. During the treatment period resting heart rate increased significantly ($p = 0.018$) but stayed within normal values. No relevant changes were observed in respiration rate, rectal temperature, gastrointestinal auscultation or complete blood count, while changes in serum biochemistry require validation. No change was detected in urine lolitrem-B levels and although plasma lolitrem-B increased during the treatment period, levels did not correlate with the severity of clinical signs displayed.

This study provides a clearer appreciation of the clinical signs and variability of perennial ryegrass intoxication in horses. The clinical effects of lolitrem-B intoxication in horses primarily involve action-related tremors and symmetrical vestibular ataxia. Results from the frusemide challenge indicate that lolitrem-B disrupts renal large-conductance Ca^{2+}-activated K^+ channels, indicating a potential diagnostic avenue. Further research is required to establish the significance of increased mMEP and BAEP latencies.

Preface

The purpose of the study was to describe the clinical effects of lolitrem-B intoxication in horses in relation to those reported in ruminant species and to the function of BK channels. The effects of lolitrem-B were investigated in organ systems where BK channels are reported to play prominent roles. However, the scope was limited to include tests that are applicable to veterinary practice.

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To Aaron, my husband

Thank you for your understanding,
encouragement and patience.

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List of Abbreviations

The following abbreviations are used within the main text and are defined when first used in each chapter.

Ach	Acetylcholine
AP(s)	Action potential(s)
BAEP(s)	Brainstem auditory evoked potential(s)
BK channel	Large-conductance Ca^{2+}-activated K^+ channel
$BK_{\alpha}^{-/-}$	Mice lacking the gene that encodes the α-subunit of the BK channel
BK-$\beta_1^{-/-}$	Mice lacking the gene that encodes the β_1-subunit of the BK channel
BM	Basilar membrane
$[Ca^2]_i$	Concentration of intracellular Ca^{2+}
CAP(s)	Compound action potential(s)
CbTX	Charybdotoxin
CCD	Cortical collecting duct
CNS	Central nervous system
CNT	Connecting tubule
DRK channel	Delayed rectifying K^+ channel
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENa channel	Amiloride-sensitive epithelial Na^+ channel
EPSPs	Excitatory postsynaptic potentials
(f)AHP	(Fast) afterhyperpolarisation
FEK^+	Fractional excretion of K^+
$FENa^+$	Fractional excretion of Na^+
FMKS	Flow mediated K^+ secretion
GP	General proprioceptive
IbTX	Iberiotoxin
IHC(s)	Inner hair cell(s)
IK	Intermediate-conductance Ca^{2+}-activated K^+ channel
LMN(s)	Lower motor neuron(s)
mMEP(s)	Magnetic motor evoked potential(s)
$[Na^+]_i$	Intracellular concentration of sodium ions
OHC(s)	Outer hair cell(s)
PC(s)	Principal cell(s)
PRG	Perennial ryegrass
PRGS	Perennial ryegrass staggers
RMP	Resting membrane potential

ROMK	Renal outer medullary K^+ channels
SK	Slow-conductance Ca^{2+}-activated K^+ channel
TEA	Tetraethylammonium
UMN(s)	Upper motor neuron(s)
VGC channel	Voltage-gated Ca^{2+} channel
VGNa channel	Voltage-gated Na^+ channel
VHCII	Type II vestibular hair cells
WT	Wild-type
ΔFEK^+_{15}	The difference in the fractional excretion of K^+ between 0 and 15 min after frusemide administration
$\Delta FENa^+_{15}$	The difference in the fractional excretion of Na^+ between 0 and 15 min after frusemide administration
ΔAld_{30}	The difference in plasma aldosterone between 0 and 30 min after frusemide administration
μSOLT	Familial microsaccadic eye oscillations and limb tremor

Commentary on the DVD clips from neurological examinations.

The following is a commentary on the DVD movie clips, which are included in the DVD in the pocket of the back cover of this thesis. Readers are encouraged to refer to these as supplements to the descriptions provided in chapter 5.

DVD 1: Muscle fasciculations

Fasciculations are small involuntary muscle twitches that that do not effect movement of a body part or segment and were observed as fine movement of the hair over the shoulder and pectoral muscles.

- **Horse #1:** After 9 days exposure to lolitrem-B, horse #1 showed a subtle fascicular tremor over the shoulder region while eating.
- **Horse #7:** Fasciculations predominantly involved the triceps and pectoral muscles after 5 days of lolitrem-B exposure. On day 12, fasciculations were prominent immediately after trotting and cantering on the lunge.

DVD 2: Limb tremor and spasms

- **Horse #2:** This horse demonstrated severe spasms of both forelimbs while eating, which increased in severity from a slight tremor on day 6 to severe spasms on day 9. If food were removed tremors would ease and when food was replaced the severity of tremor would again increase. Due to the amplitude of flexor spasm the heel bulbs would frequently leave the ground or the entire limb would be lifted during flexion and replaced again during the extension phase. Tremor of the right and left limb was reciprocal, in that as the right limb flexed the left limb would extend. However, an irregular sequence of right and left tremor was observed, rather than a 1:1 ratio. In this movie clip, forelimb tremor is most frequent in the left limb, which is bearing the least weight. Slowing the movie down allows an appreciation of the reciprocal motion and the order of joint flexion from distal to proximal, which gives an appearance of a ripple ascending the limb. Although tremor was most severe while eating, horse #2 also showed forelimb tremor during gait analysis particularly during pauses in movement or changes of direction.
- **Horse #1:** After 9 days of exposure to lolitrem-B, horse #1 displayed tremor in both pelvic and thoracic limbs. Pelvic limb tremor was subtle but occurred in both right and left limbs and was particularly noticeable at the fetlock when the heels were lifted as the horse pivoted or when the limb was lifted. As in the previous movie clip, this demonstrates that the tremor is not associated with

weight bearing. In the movie clip, the heels of the left forelimb remain grounded while the fetlock and carpus are flexed. During gait analysis, severe reciprocating left and right tremor of the thoracic limbs occurred, particularly when blindfolded.

- **Horse #3:** This movie clip was taken after 5 days exposure to lolitrem-B. In addition to slight tremor of the trunk, horse #3 showed tremor of the right forelimb that involved flexion of the carpus and fetlock, most obvious towards the end of the movie clip. The closer view displays tremor of the left forelimb.
- **Horse #5:** Recorded after 5 days of exposure, horse #5 showed tremor of the right forelimb and pectoral muscles immediately after lunging.
- **Horse #7:** On day 10 of the treatment period horse #7 showed a right forelimb tremor while at rest. Limb tremor was also observed when horse #7 was made to stand stationary after rapid movements.

DVD 3: Vermiform tremor at the flank and over the ribs

- Horse #5 demonstrated flank tremor, which appeared as irregular pulsations or punches from structures beneath the skin. Over the ribs the movement of the overlying skin was more undulating and wave-like, with ripples of muscle contractions.

DVD 4: Initial signs of ataxia

Initial signs of ataxia observed included a truncal sway at rest and a cautious gait at a walk with a wide-based placement of limbs and dishing of the limbs underneath the body during the protraction phase, particularly when blindfolded or with elevation of the head.

- **Horse #7:** The subtle, multidirectional sway of the trunk was best observed by watching the changing angle of the fetlock joints. Sway was noted at rest, when eating and before the onset of movement. Tremor of the right forelimb can also be seen on the movie clip from day 12.
- **Horse #6:** This horse scored 1/5 on day 14 of exposure and showed a very subtle craniocaudal sway, which was first observed on day 9.
- **Horse #3:** When blindfolded, horse #3 demonstrated a lateral truncal sway after turning. Ipsilateral limbs bore weight in synchrony not only changing angulations at the joints but also lifting as weight was transferred to the contralateral limbs. The right thoracic and pelvic limbs were observed to lift together. Horse #3 stabilised its stance by wide and forward placement of the left pelvic limb.

- **Horse #1:** Prior to lolitrem-B exposure, limbs were placed directly underneath the body. After 5 days of exposure to lolitrem-B, limb placement was lateral to the point of the shoulder and during the protraction phase the limb swung underneath the body in a curved path to be placed lateral once more. The wide-based gait slowed as the severity of PRGS progressed and resembled a waddle with thoracic and pelvic limbs moving in near synchrony. There was also a reduction in flexion of the joints during protraction i.e. hypometria.
- **Horse #5:** A wide based stance was adopted at rest and while eating. Blindfolding exaggerated the wide placement of limbs at a walk.

DVD 5: Blindfolding

- **Horse #2:** Prior to lolitrem-B exposure, horse #2 walked confidently when blindfolded and showed regular foot placement when walking in a straight line or turning. After 5 days exposure to lolitrem-B, blindfolding exposed an ataxic gait. When walking in a straight line with minimal prompts from the handler horse #2 was unable to maintain his line of direction. The trunk would sway, drift or lean to either side. The direction of the truncal sway was followed by irregular foot placements that would compensate for the lateral drift of the center of mass. Note the wide-based stance that was adopted at the end of movement. During circling, movement was regular when vision was not obscured. However, when blindfolded there was a delayed movement of the hindlimbs and the truncal leaned towards the inside of the circle. The outside hindlimb was brought under the body, compensating for the lean of the trunk; however, this resulted in the outside hindlimb interfering with the dorsal hoof of the inside hindlimb, which was subsequently rapidly moved to a wide position. Wide placement of the inside forelimb follows and the horse was stabilised by the wide-based stance. After 9 days of exposure, a mild ataxia is evident without blindfolding, with slight irregularities and jerkiness to movement and a tendency for the outside limb to circumduct during tight circling. However, ataxia was profoundly exaggerated by blindfolding. Horse #2 demonstrates a hesitancy and forelimb tremor at the onset of movement. While turning tightly on the left rein the trunk leaned inwards and the right fore and hindlimbs were suspended for a time. This was followed by circumduction of the outside hindlimb. Stumbling on the inside forelimb was followed by parallel placement of the outside forelimb and reciprocating tremor of both thoracic limbs, during which the inward and cranial lean of the trunk was exacerbated causing

imbalance. Hurried, lurching forelimb movement regained balance while the hindlimbs bounded forward in a wide-based, bunny-hopping gait.

- **Horse #1:** Prior to exposure, horse #1 had a confident and regular gait when blindfolded, whereas after 5 days exposure movement contained irregularities and involved increased movement of the trunk. During backing the trunk leaned to the right and when the left hindlimb was raised imbalance occurred but rapid movement of the limbs stabilised the horse. On day 9 exposure, horse #1 leant inward and cranial as it was led in a circle to the left resulting in imbalance and lurching of the body as rapid forelimb movement regained balance. During complex maneuvers, movement stammered and was awkward and jerky but limb placement was appropriate for the prompts given by the handler.
- **Horse #3:** This movie clip compares gait when the horse was and was not blindfolded on day 12 exposure.
- **Horse #7:** Pre-exposure, this horse did not maintain a straight line when walking blindfold; however, movement is not irregular and begins with turning of the neck, which the limbs follow without excessive sway of the trunk. When instructed to halt after turning, the horse did so without repositioning its limbs. Likewise on day 12 exposure when the horse was not blindfolded. However, when blindfolded and instructed to halt, the horse would sway and take stuttering steps to reposition limbs in a wide-based stance. He also showed interference of the hindlimbs and circumduction of the outside hindlimb during complex maneuvers.
- **Horse #5:** After 14 days of exposure to lolitrem-B there is a dramatic difference between gait when the horse was and was not blindfolded. This clip exemplifies how blindfolding exaggerated ataxia resulting in a slowed gait, increased sway of the trunk, irregular but predictable limb placement and a tendency to place limbs wide.

DVD 6: Awkward stance

After abrupt cessation of movement, horses would stand in awkward, abnormal positions and would only correct limb placement after a delay or when the next movement was initiated.

- **Horse #2:** On day 5 of lolitrem-B exposure horse #2 stood with forelimbs crossed and did not reposition limbs until forward movement was initiated.
- **Horse #4:** After turning in a circle the horse #4 stood with hindlimbs crossed.

- **Horse #2:** After lurching forward, limbs were placed wide with the right thoracic limb placed abnormally caudal. The forelimb was only repositioned square with the other forelimb after a delay.

DVD 7: The serpentine maneuver

The purpose of the serpentine maneuver was to test the ability of the horse to rapidly change the direction of movement of a limb while it is protracted. These movie clips demonstrate that when horses were given minimal direction from the handler irregular movement was observed. However, when the horse was lead in a serpentine manner, horses responded appropriately — changing the direction of limb placement according to the ordered movement of the head and neck.

- **Horse #2:** Day 5 exposure
- **Horse #5:** Day 14 exposure

DVD 8: Lower motor neuron weakness was not displayed

Horses did not demonstrate weakness during the tail pull, the tail and halter pull while circling, or thoracic limb hopping.

- **Horse #1:** During forelimb hopping, a weak horse has a tendency to tremble or collapse on the limb bearing weight. Horse #1 on day 9 demonstrates that an ability to bear weight well and resist pushing by the handler. Horses also exerted strong voluntary pull against the lateral tension applied during the tail pull. Horse #1, which was graded 3/5 on day 9 of lolitrem-B exposure demonstrates an ability to resist the tail pull.
- **Horse #7:** Pulling the tail while the patient is stationary initiates an extensor reflex in the hindlimb. This reflex is poor when there is a lower motor lesion at the level of L_{3-5}. Horse #7 demonstrates a strong resistance to pull of the tail while standing still.

DVD 9: Allodynia

Horses showed increased response to a slap or threatening gesture at the withers.

- **Horse #1:** Day 9 exposure
- **Horse #3:** Day 12 exposure

Horses often startled or hesitated at gateways

- **Horse #2:** Day 9 exposure

Chapter 1: Introduction

1.1: Problem Statement

Perennial ryegrass staggers (PRGS) is purported to be a common disease affecting New Zealand horses. However, there is a lack of documented evidence about the disease in this species. Anecdotal evidence suggests that equids are particularly sensitive to the causative mycotoxin, lolitrem-B. In addition to signs of neuromuscular dysfunction, numerous vague behavioural problems are allegedly due to lolitrem-B intoxication, but these are unsubstantiated. Presently, diagnosis of PRGS in horses is at best speculative as the clinical effects of lolitrem-B are ill-defined and effective diagnostic tools have not been validated.

In ruminant studies, neurological signs dominate the clinical presentation of PRGS, yet the ubiquitous distribution of large-conductance Ca^{2+}-activated K^{+} (BK) channels, to which lolitrem-B binds, would suggest that lolitrem-B intoxication involves multiple organ systems. There is a lack of knowledge of the extent to which lolitrem-B disrupts other organ systems.

1.2: Aim and Limitations

The aim of this trial was to describe the clinical syndrome of lolitrem-B intoxication in New Zealand horses, in relation to clinical signs reported in ruminant species and to the function of BK channels. The description includes the effect of lolitrem-B on the neurological system as well as other organ systems where BK channels are reported to play prominent roles. Tests included in the investigation were limited to those that are applicable to veterinary practice.

1.3: Thesis Structure

The following two chapters examine the literature on PRGS and the proposed molecular mechanisms of the disease. Chapter 2 presents the etiology of PRGS and reviews previous reports of PRGS in ruminants and horses. This chapter highlights that despite the purported high prevalence of PRGS in New Zealand horses, there is little documentation in this species and current methods of diagnosis are speculative. Chapter 3 reviews the recent advances in molecular biology, which have identified the channel involved in lolitrem-B intoxication. The structure and function of BK channels

are reviewed in relation to the reported and theoretical effects of lolitrem-B intoxication. This chapter illustrates that despite the predominance of neurological signs in PRGS, evidence of BK channel involvement in multiple organ systems indicates that lolitrem-B intoxication may interrupt multiple body functions.

Based on the prior two chapters, chapter 4 outlines the hypotheses and research objectives, then develops and justifies the chosen research design. Results are presented and analysed in chapter 5. In this section DVD movie clips are referred to, which are included in the DVD located in the pocket of the back cover of this thesis and a commentary is provided on page XI. Readers are encouraged to refer to these as supplements to the descriptions provided in the text. Results are discussed in chapter 6, and conclusions are drawn in chapter 7.

Chapter 2: Diagnosing perennial ryegrass staggers in horses

The following chapter identifies the lack of evidence regarding the clinical signs of lolitrem-B intoxication in horses and how this impinges on the ability to diagnose perennial ryegrass staggers (PRGS) in horses. The etiology of PRGS will be discussed, followed by a review of previous studies on PRGS in ruminant and equine species, and the limitations of these studies in assisting the diagnosis of lolitrem-B intoxication in horses. This review highlights that the present methods used to diagnose lolitrem-B intoxication in horses are inadequate.

2.1: The etiology of perennial ryegrass staggers

PRGS is a common neurological mycotoxicosis that impairs motor function in herbivores (Prestidge, 1993). It is caused by ingestion of perennial ryegrass (PRG) infected with the endophytic fungus *Neotyphodium lolii* (Fletcher & Harvey, 1981; Prestidge, 1993), which produces tremorgenic indole-diterpenoid compounds, including the principal toxin lolitrem-B (Blythe, Estill, Males, & Craig, 2007; Gallagher, White, & Mortimer, 1981). Molecular studies have revealed that lolitrem-B binds to large-conductance Ca^{2+}-activated K^+ (BK) channels, inhibiting K^+ currents out of cells (Dalziel, Finch, & Dunlop, 2005).

The relationship between the presence of an endophytic fungus and the occurrence of PRGS was established in 1940 (Neill, 1940). However, it was not until the 1980s that evidence of causation by *N. lolii* and lolitrem-B arose in parallel through sheep trials (Fletcher, 1982; Fletcher & Harvey, 1981) and mice bioassays (Gallagher, Smith, Di Menna, & Young, 1982; Gallagher, et al., 1981). Subsequent research has revealed the complexity of the mutualistic relationship between PRG and *N. lolii*.

N. lolii produces a number of alkaloids including peramine, lolitrem-B and ergovaline (Ball, Prestidge, & Sprosen, 1995; Rowan, 1993). Although the latter two compounds cause animal disease, this trio of mycotoxins prevent insect attack (Ball, et al., 1995; Mortimer, Young, & Di Menna, 1984) and confer drought resistance to the plant host (H. Hahn, et al., 2008). Peramine imparts resistance against the Argentine stem weevil *(Listronotus bonariensis*) (Ball, et al., 1995; Mortimer, et al., 1984), the black beetle (*Heteronychus arator*), the cutworm (*Graphania mutans*) and the pasture mealy bug (*Balanococcus poae*) (Ball, et al., 1995). The other alkaloids also contribute to insect resistance; for example, lolitrem-B is toxic to Argentine stem weevil larvae (Ball, Barker, Prestidge, & Lauren, 1997). In return, the endophyte is dependent on the reproductive cycle of the plant. *N. lolii* grows into the forming PRG seed and remains

dormant as resting mycelium, it is then distributed with the seed and when the seed germinates the endophyte mycelium resumes growth and establishes in the new seedling (Easton, 2007). This mutualistic relationship underpins the popularity of PRG as a pasture species in New Zealand. Insect predation of endophyte-free PRG swards, particularly by the Argentine stem weevil, radically decreases the persistence of these swards (Easton & Fletcher, 2007; Rowan, 1993). As a result, most old PRG pastures in New Zealand contain a high level of endophyte (Bluett, Hodgson, Kemp, & Barry, 2001; Easton & Fletcher, 2007), explaining the high prevalence of PRGS in New Zealand. In other parts of the world such as Europe, Australia and the United States, selection pressure for endophyte-associated PRG is not as strong as in New Zealand (Easton & Fletcher, 2007). Nevertheless, PRGS has been reported in Australia, Northern California, Oregon, the United Kingdom and Argentina (Blythe, et al., 2007; Galey, et al., 1991; Holmes, Frame, Frame, Duff, & Lewis, 1999; Hunt, Blythe, & Holtan, 1983; Odriozola, Lopez, Campero, & Placeres, 1993).

The difficulty faced when controlling microbial diseases that involve mutualistic relationships, is the low persistence of cultivars that lack their biological allies (Easton, 2007). However, selective breeding has cultivated novel endophyte-grass combinations that maximise the beneficial mycotoxins (peramine) and minimise those causing animal disease (lolitrem-B and ergovaline). However, re-sowing extensive areas of New Zealand pasture is not feasible and it is difficult to prevent contamination by wild type (WT) cultivars (Easton & Fletcher, 2007). Therefore, increasing knowledge about PRGS in animals and the development of diagnostic tests and prophylactic and therapeutic measures is of considerable interest to those involved in New Zealand grassland farming industries.

2.2: The clinical presentation of perennial ryegrass staggers in ruminants and other herbivores

Cases of PRGS have been reported in cattle, sheep, deer, alpaca and horses (Cunningham & Hartley, 1959; Holmes, et al., 1999; Mackintosh, Orr, Gallagher, & Harvey, 1982). Extensive research on PRGS has been performed in ruminants and demonstrates that ingestion of lolitrem-B at levels greater than 2 ppm causes signs of neuromuscular dysfunction (Blythe, et al., 2007; Di Menna, Mortimer, Prestidge, Hawkes, & Sprosen, 1992; Fisher, et al., 2004; Galey, et al., 1991; Miyazaki, Ishizaki, Ishizaka, Kanbara, & Ishiguro-Takeda, 2004; Tor-Agbidye, Blythe, & Craig, 2001). The onset of clinical signs occurs within 7 – 14 days of ingesting toxic pasture (Galey, et al., 1991; Prestidge, 1993) and involves fine tremor of the head and neck and coarse tremor of limb musculature (Blythe, et al., 2007; Galey, et al., 1991). As severity

increases, mild postural incoordination, head nodding and truncal sway are observed at rest. Animals become hyperaesthetic, in that they display an exaggerated response to external stimuli (Rowan, 1993). Gait is stiff, stilted and ataxic (Galey, et al., 1991). At rest these gait deficits subside (Galey, et al., 1991); however, signs are exacerbated by exercise and stress, and can culminate in a prancing gait, tetany and collapse. Later episodes involve recumbency with opisthotonus and interludes of limb thrashing, without loss of consciousness (Blythe, et al., 2007; Cunningham & Hartley, 1959; Galey, et al., 1991; Mackintosh, et al., 1982; Mayhew, 2007; Tor-Agbidye, et al., 2001), which describes the occurrence of cerebellar fits and will be discussed further in the following chapter. Opisthotonus refers to hyperextension of the neck and head, thoracolumbar lordosis and rigid extension of the limbs that results from tonic contraction of the epaxial and limb muscles (de Lahunta & Glass, 2009). Animals recover from these episodes after a short time if external stimuli are removed (Cunningham & Hartley, 1959).

Lolitrem-B has a basal to apical concentration gradient in PRG tillers (Ball, et al., 1997; Ball, et al., 1995; Gallagher, et al., 1982) and reaches peak concentrations during summer to early autumn (Ball, et al., 1995). This is consistent with the occurrence of PRGS outbreaks during summer and autumn in association with close grazing (Di Menna, et al., 1992; Prestidge, 1993). PRGS is also a problem in late spring when PRG is in flower, as the seed-heads contain high levels of endophyte mycelia and lolitrem-B (Smith & Towers, 2002).

Controlled ruminant studies, in which animals were grazed on pasture containing 2 ppm lolitrem-B or greater, report a morbidity range of 20 – 100% (Di Menna, et al., 1992; Fisher, et al., 2004; Galey, et al., 1991; Tor-Agbidye, et al., 2001), but case reports commonly involve a morbidity in the region of 10% (Cunningham & Hartley, 1959). It is not known whether the range in individual susceptibility for PRGS is due to differences in grazing behaviour, differences in pharmacokinetic handling or receptor binding of lolitrem-B, or differences in neuro-compensatory mechanisms. A genetic component for PRGS resistance has been demonstrated in sheep (Morris, Towers, Amyes, & Wheeler, 1998; Morris, Towers, Wheeler, & Amyes, 1995), suggesting that the latter three are important in determining individual variability. No effective treatment has been developed, but animals generally recover within a week of removal from endophyte-infected pasture (Galey, et al., 1991; Tor-Agbidye, et al., 2001). Mortality is rare (Cunningham & Hartley, 1959); however, sudden death has occurred in a stag during an episode of collapse (Mackintosh, et al., 1982). Gross pathological lesions are usually absent (Cunningham & Hartley, 1959; Rowan, 1993); however,

cerebellar lesions and secondary myonecrosis have been reported (Mackintosh, et al., 1982; Mason, 1968).

Ruminant studies have revealed subclinical effects of lolitrem-B. These include decreased milk production in cows (Blackwell & Keogh, 1999), reduced serum prolactin (Fletcher & Barrell, 1984), decreased reproductive performance (Peterson, Bass, & Byford, 1978, 1984), decreased liveweight gain (Fletcher & Barrell, 1984), increased perianal faecal contamination and ill-thrift (Fletcher, 1993). A study conducted *in vitro* indicated that grazing pasture containing high levels of endophyte influences the response of Thymic-derived lymphocytes to mitogens (McFarlane, Bell, & Fletcher, 1993), but it is unknown if these effects on the immune system are important *in vivo*. Electrophysiological studies show that lolitrem-B has profound effects on gastrointestinal motility (McLeay, Smith, & Munday-Finch, 1999; Smith, McLeay, & Embling, 1997), which may impair digestion and health. Ergovaline, another mycotoxin produced by *N. lolii*, is also reported to reduce milk production, liveweight gain, reproductive performance and serum prolactin levels (Cheeke, 1995; Lean, 2001; Waghorn, Latch, & Rolston, 1994). It is difficult to separate the effects of ergovaline and lolitrem-B as most varieties of *N. lolii* produce both mycotoxins.

These reports from ruminant studies provide information about the epidemiology, clinical signs and pathology associated with PRGS. However, our ability to extrapolate these findings to horses is limited and will be discussed later in this chapter.

2.3: Literature on perennial ryegrass staggers in horses

PRGS is purported to be a common disease of New Zealand horses, but there is limited documentation of the disease in this species. Vague behavioural changes have also been attributed to lolitrem-B, but are unsubstantiated. Cunningham and Hartley (1959) first reported PRGS in horses. They observed that horses, under the same conditions as cattle and sheep, show a 'propping stance and, on movement, exhibit a reeling, drunken gait', from which they recovered within a few days of changing pasture (Cunningham & Hartley, 1959, pg. 2). Two trials report that horses fed PRG seed cleanings or hay displayed signs of PRGS (Hunt, et al., 1983; Munday, Monkhouse, & Gallagher, 1985). The clinical signs reported parallel those in ruminants with PRGS; clinical signs appeared after 10 – 14 days and involved tremors, hyperaesthesia, a dysmetric ataxia and weaving when standing still. However, clinical signs are also reported that do not appear in the literature on PRGS in ruminants including tenesmus (Munday, et al., 1985), posterior paralysis (Cunningham & Hartley,

1959) and hyperproteinaemia (Hunt, et al., 1983). These differences require substantiation.

In the trial reported by Munday et al (1985) horses ingested feed containing 5.3 ppm lolitrem-B – more than twice the toxic threshold for sheep and cattle (Blythe, et al., 2007; Di Menna, et al., 1992; Fisher, et al., 2004; Galey, et al., 1991; Miyazaki, et al., 2004; Tor-Agbidye, et al., 2001). The high levels of lolitrem-B used by Munday et al (1985) may have resulted in a somewhat atypical clinical picture, as horses would rarely ingest such quantities of lolitrem-B over a relatively short period of time. This study also provides no evidence for or against the notion that horses are particularly sensitive to PRGS (Prestidge, 1993; Smith & Towers, 2002).

The detection of other soil fungi that produce tremorgenic compounds confounds the interpretation of results by Hunt et al (1983). The pellets made from PRG straw that were used in the trial were found to contain *Penicillium cyclopium*. This soil fungus can produce tremorgens: penitrem A, verrucologen and fumitremorgin B; which can result in similar clinical signs to lolitrem-B intoxication including tremor and ataxia (Gallagher, Keogh, Latch, & Reid, 1977; Mayhew, 2007). Hunt et al (1983) did not investigate the presence of lolitrem-B or endophyte so it cannot be assumed that the observed signs were due to lolitrem-B intoxication.

Therefore, literature on lolitrem-B intoxication in horses inadequately describes the relationship between natural exposure to lolitrem-B and the associated clinical signs displayed. It is uncertain whether lolitrem-B levels that are reported to be toxic for ruminants will cause similar signs in horses due to the high levels used on one study (Munday, et al., 1985) while others did not determine lolitrem-B levels in feed (Cunningham & Hartley, 1959) (Hunt, et al., 1983). Exposure of horses to known amounts of lolitrem-B in a controlled situation is required to determine the clinical signs associated with PRGS in horses.

2.4: The inadequacies of current methods of diagnosing of perennial ryegrass staggers in horses

At present, current literature on PRGS in horses provides merely a speculative framework for diagnosis. Case diagnosis is based on extrapolation of the clinical signs and epidemiologic features from ruminant species, ruling out other differentials, or a response to changing the feed to non-PRG sources. Our inability to diagnose PRGS in horses with any degree of certainty is problematic on multiple levels: for the individual horse owner the expense of re-sowing pastures is rarely justified; from a research perspective it is difficult to justify and design large scale investigations into

prevention or treatment without knowing the prevalence, the estimated cost of the disease or the individual variability; and epidemiological investigations and attempts to realise the cost of lolitrem-B intoxication to the NZ equine industry are futile without an accurate case description.

The clinical signs reported in ruminants and horses are not pathognomonic and result in many differential diagnoses. **Table 2.1** lists other diseases that involve syndromes of ataxia, hyperaesthesia, tremors, and weakness that must be entertained in individual patients. Weakness was included as Cunningham and Hartley (1958) reported posterior paralysis in horses with PRGS. The list is not exhaustive, but highlights that there are many equine diseases that stand as differential diagnoses for PRGS under the current description. Ruling out other disorders can be a costly procedure; the alternative approach involves changing the feed and waiting for a response. This may have serious consequences if another disease is misdiagnosed. Alternatively, if the horse recovers, no definitive diagnosis is attained. Reports of ruminant studies lack detailed descriptions of the precise nature of clinical signs and fail to define medical terms used. Ataxia, for example, is a commonly reported sign of PRGS. Ataxia refers to unpredictable irregularities in movement (Greek origin: *a*- without + *taxis*- ordered, regular) and has different characteristics depending on whether it is associated with general proprioceptive (GP), vestibular or cerebellar dysfunction (Mayhew, 2009). Descriptions of these characteristics or classification of ataxia are often lacking in reports of ruminant studies. The clinical signs associated with lolitrem-B intoxication need to be further described so that the list of differentials **(table 2.1)** can be addressed and PRGS can be distinguished from other, potentially more harmful, diseases.

There are several further limitations in extrapolating information from ruminant studies. Firstly, when diagnosing PRGS in ruminants, substantial weight is placed on epidemiological factors such as morbidity, the time of year and a history of exposure to endophyte-infected PRG. In horses, these epidemiological factors are often complicated or non-existent. Horses are often kept as individuals or in small (usually non-genetically related) herds, in which horses likely have substantially different degrees of susceptibility to lolitrem-B intoxication, making the assessment of morbidity inaccurate or impossible. Secondly, supplementary feeding is common and may contain a variety of pasture species from varied sources; conserved PRG forages can maintain lolitrem-B levels for long periods, despite the tendency for the fungus to die out (Fisher, et al., 2004; Fletcher & Harvey, 1981). Therefore, intoxication can occur independent of season. Thirdly, confidence in determining the intake of lolitrem-B by horses requires analysis of all feeds for lolitrem-B by high-performance liquid

chromatography. An enzyme-linked immunosorbent assay (ELISA) test that detects lolitrem-B has been developed for serum or plasma, urine and faeces, but has not been validated for horses and is not commercially available. Therefore, despite the cumulative costs of feed testing, it is currently the only method available to substantiate suspicions of lolitrem-B intake.

Table 2.1: Listing of diseases and disorders to be considered when making a clinical diagnosis of lolitrem-B intoxication in horses (C. Hahn, 2006; Lewis, 1995; Mayhew, 2007, 2009; Smith & Towers, 2002)

Disease	Etiology	Clinical Signs	Prognosis
Cervical vertebral malformation	Compression of the spinal cord due to either growth-related malformation of the vertebrae or secondary to caudal cervical arthritis.	Spinal ataxia and weakness	Poor without surgery.
Temporohyoid osteoarthropathy	Fusion of the temporohyoid joint predisposes to fractures of this area, which contains the middle and inner ear.	Vestibular ataxia, wide-based stance, short strides, and a reluctance to move. Unilateral cases will be defined from PRGS due to head tilt, but blindfolding may be required to induce head tilt due to visual compensation.	Guarded
Cerebellar abiotrophy	Degeneration of the cerebellum. Predominantly seen in Arabian horses from very young to 6 months of age.	Cerebellar ataxia – hypermetria, intention tremors and reduced menace response with intact vision.	Poor
Equine protozoal myeloencephalitis	A sarcocyst protozoan (*Sarcocystis neurona),* which is transmitted by the American opossum.	Wide array of focal or multifocal neurological signs – frequent stumbling and behaviour problems	Poor
Bacterial meningitis	Bacterial infection of the meninges – *Streptococcus* sp*, salmonella* sp, *Actinobacillus equuli* and various coliforms.	Hyperaesthesia, a stiff neck, tremor, somnolence, seizures, opisthotonus and blindness.	Guarded
West Nile Virus	Flavivirus.	Disease causes an encephalomyelitis, but many cases present with ataxia and not encephalopathy.	Poor
Rabies	Rhabdoviris – *Lyssavirus sp*.	Aggresiveness, hyperaesthesia, paresis/ paralysis, fever, ataxia, seizures.	Fatal
Tetanus	*Clostridia tetani* neurotoxin	Hyperaesthesia, stiff gait, erect ears, opisthotonus, protrusion of the nicotating membrane, awkward stilted hypometric gait, tail head elevation, recumbency with extensor rigidity and opisthotonus.	Poor to good.
Hyperkalaemic periodic paralysis	Autosomal dominant disease of quarter horse and related breeds, due to defect in a Na^+ channel.	Attacks of tremor, staggering, buckling, marked muscle spasms and paraplegia that may culminate in sever tremor and tetany of many muscles with recumbency and sweating.	Guarded to Good
Exertional rhabdomyolysis	Skeletal muscle damage associated with accumulation of excessive glycogen and/or complex polysaccaride.	Stiff gait, tremors, myoglobinuria, raised muscle enzymes.	Guarded to Good
Hypomagnesaemia	Low serum Mg^{2+}	Fasciculations, tremors, nervousness, tachycardia, stiff stilted gait and reluctance to move. May cause recumbency and convulsions.	Guarded to Good
Hypocalcaemia	Low serum Ca^{2+}	Tremor, stiffness, staggering, high-stepping gait, diaphragmatic flutter	Good.
Lead toxicity	Lead is proposed to interfere with neuronal cellular energy metabolism.	Incoordination and tremors and recumbency at terminal stages	Poor
Metaldehyde poisoning (eg. snail bait ingestion)	Metaldehyde crosses blood-brain barrier and causes neural excitement.	Depression, tremors, hyperaesthesia, ataxia, sweating, salivation, opisthotonus and seizures.	Poor
Ionophore toxicity (eg. monensin).	Myoneurotoxin which interfers with ion transfer across cell membranes.	Hypermetric ataxia, weakness, tremors, recumbency and death.	Poor
Macrocyclic lactone toxicity (eg. ivermectin and moxidectin overdose).	Potentiation of inhibitory neurotransmission.	Ataxia and blindness	Good

Nitrogen, urea and ammonia toxicity	Encephalopathy	Uneasiness, muscle twitching, incoordination, blindness, hyperaesthesia, seizures.	Poor
Organophosphate toxicity	Inhibits acetylcholinesterase	Nervousness, apprehension, ataxia, tremor, weakness, convulsions with opisthotonus, coma.	Poor
Mouldy corn poisoning	*Fusarium moniliforme* and *F. proliferatum,* which produce fumonisin mycotoxin that damage blood vessels resulting in leukoencephalomalacia.	Incoordination, depression and sudden death. Less often liver failure.	Poor
Locoweed toxicity	Toxin Swainsonine, isolated from various species of *Swainsona, Astragalus* and *Oxytropis*. Toxin results in a lysosomal storage disease that causes irreversible nerve cell damage.	Incoordination, hypermetria, head bobbing, hyperaesthesia	Poor
Sage sickness	Monoterpene toxin produced by *Artemisia* species.	Abnormal behaviour, hyperaesthesia and incoordination (especially involving the forelimbs).	Good
Phalaris toxicity	Philaris alkaloid toxins interfere with serotonic neurotransmitter release.	Tetany, tremor, dysmetric ataxia exacerbated by exercise or excitement; seizures, cardiac arrhythmia and sudden death.	Poor
Annual Ryegrass staggers	Annual or Wimmera ryegrass (*Lolium rigidum)* is colonised by bacteria *Corynebacterium rathayl* via a nematode (*Anguina agrostis)* and produces tunicamycin-like corynetoxins.	Tremor, tetany, opisthotonus, convulsions	Poor
White Snakeroot	Tremetol	Tremors	Good to guarded
Dallis and paspallum staggers	*Claviceps paspali* produces indole-diterpenoid tremorgens that are structurally related to lolitrem-B, during the flowering stages of the host plant.	Ataxia, tremor and rarely convulsions.	Good
Ergot poisoning	*Claviceps purpurea* in ryegrass and triticale and *C. paspali* in paspalum grasses produce mycotoxic ergots. Poisoning of horses generally a result of consuming mycotoxin-contaminated grains.	Behavioural effects: hyperexcitability, aggression, lethargy, convulsions, incoordination, excess salivation, diarrhea; reproductive effects and dry gangrene of extremities.	Good
Penicillium toxins	Janthitrem A and penitrem A	Ataxia and tremors	Good
Consumption of snake grass *(Equiseturn arvense*) and braken fern (*Pteridium equalinum*)	Thiaminase resulting in thiamine deficiency.	Incoordination, muscle tremors, hindlimb weakness, depression, corneal opacity.	Poor
Aspergillus tremor syndrome	Sorghum beer residue is host to *Aspergillus clavatus*, which produces aspergillus tremorgens that cause degeneration of motor neurons.	Tremor, ataxia, paralysis and death.	Poor
Botulism	*Clostridia botulinum* toxin	Muscle trembling, extensor weakness, recumbency	Poor
Cyanide toxicity associated with ingestion of plants containing cyanogenic glycosides eg. *Sorghum* sp, *Brassica* sp, Hemlock (*Conium maculatum).*	Interference with cellular respiration.	Dyspnea, excitement, lethargy, tremor, convulsions.	Poor

Conclusion

PRGS is a disease that is ingrained in New Zealand pastures. Despite the purported commonness of the disease in horses, diagnosis of PRGS is never definitive. Research on PRGS that has been conducted in horses either used extreme concentrations of lolitrem-B or did not investigate lolitrem-B as the cause. The limitations of extrapolating from ruminant studies, the multiple differentials for ataxia and tremor, the ambiguity and risk involved in waiting for a response to changing feeds, the costs involved in feed testing and the unavailability of ELISA tests for lolitrem-B in horse body fluids all contribute to the challenge of diagnosing PRGS in horses. There is a need to confirm the occurrence of lolitrem-B intoxication in horses and to describe the clinical signs involved.

A further limitation of reported research on PRGS, in both ruminant and equine species, is that most reports precede the determination of the molecular mechanisms of lolitrem-B. The physiology of BK channels may serve as a guide to possible subclinical and clinical effects of lolitrem-B intoxication and may indicate potential diagnostic indices or therapeutic avenues. The next chapter will discuss the structure and function of BK channels and how their roles relate to the clinical signs observed in ruminant studies, and will identify potential pathways for assisting the diagnosis of lolitrem-B intoxication that are yet to be explored.

Chapter 3: The roles of large-conductance Ca^{2+}-activated K^+ channels

Electrochemical *in vitro* studies have demonstrated that lolitrem-B binds to the α-subunit of large-conductance Ca^{2+}-activated K^+ (BK) channels, inhibiting K^+ currents out of the cell in a concentration-dependent manner (Dalziel, Finch, & Dunlop, 2005). The significance of lolitrem-B binding to BK channels *in vivo* was demonstrated in knockout mice where intraperitoneal administration of lolitrem-B caused tremor and ataxia in wild type (WT) mice but no change in mice lacking the α-subunit ($BK_{\alpha}^{-/-}$), even at doses that induced convulsive episodes WT mice (Imlach, et al., 2008). Therefore, lolitrem-B impairs motor function by specifically inhibiting the α-subunit of BK channels. In light of these findings, the aim of this chapter is to link the roles of BK channels with the clinical signs of perennial ryegrass staggers (PRGS). This will be achieved by discussing the results of ruminant trials and by identifying further physiological disturbances that may result from lolitrem-B intoxication according to the roles of BK channels. The structure, biochemical and pharmacological properties of BK channels will be described followed by a review on the disputed roles of BK channels in neural excitability, the mechanisms of tremor and ataxia, and the influence of lolitrem-B on auditory function. The focus will then turn to explaining the non-neurological signs that have been reported in ruminant trials. Finally, the role of BK channels in K^+ excretion will be discussed.

3.1: The structure and functional diversity of large-conductance Ca^{2+}-activated K^+ channels

BK (also termed maxi-K or Slo (Gribkoff, Starrett, & Dworetzky, 2001)) channels are a unique subset of K^+ channels in that they have a large conductance of 200 – 400 pS (Marty, 1981) and display dual activation, involving both membrane depolarisation and Ca^{2+} binding (Pallotta, 1985b). These characteristics differentiate BK channels from the other Ca^{2+}-activated K^+-channels, namely small- (SK) and intermediate- (IK) conductance Ca^{2+}-activated K^+-channels (Ghatta, Nimmagadda, Xu, & O'Rourke, 2006). BK channels are ubiquitous and have multiple roles including the mediation of neural and smooth muscle excitability (Sausbier, et al., 2005; Sausbier, et al., 2004), enabling hormone secretion (Stojilkovic, Zemkova, & Van Goor, 2005) and participating in renal and colonic K^+ excretion (Pluznick & Sansom, 2006; Sausbier, et al., 2006), as well as proposed roles in hearing (Dallos, et al., 1997; Hafidi, Beurg, & Dulon, 2005;

Oliver, et al., 2006; Pyott, et al., 2007; Ruttiger, et al., 2004; Skinner, et al., 2003) and immunity (Ahluwalia, et al., 2004; Essin, et al., 2007; Femling, et al., 2006).

BK channels have a pore forming α-subunit and a regulatory β-subunit that changes the opening properties of the channel pore (Ghatta, et al., 2006). There are four different β-subunit types, each having a unique distribution. In brief: β_1 is predominantly expressed in smooth muscle, β_2 in the ovary, $\boldsymbol{\beta_3}$ in the testis, and β_4 in neurons (Brenner, Jegla, Wickenden, Liu, & Aldrich, 2000; Ghatta, et al., 2006; Imlach, et al., 2008). The β-subunit, along with splice variants and post-translational modifications such as oxidation-reduction, phosphorylation and glycosylation, impart functional diversity to BK channels by changing their sensitivity to intracellular Ca^{2+} ($[Ca^{2+}]_i$), membrane depolarisation and intracellular messengers (Ghatta, et al., 2006; R. Lu, et al., 2006; Reinhart, Chung, Martin, Brautigan, & Levitan, 1991; Weiger, et al., 2000). The structure fine-tunes the properties of the BK channel to the requirements of the cell, allowing a great diversity in the roles of these channels.

In addition to lolitrem-B, several other pharmacological blockers of BK channels are known. These include tetraethylammonium (TEA) (Iwatsuki & Petersen, 1985), two scorpion-derived peptides charybdotoxin (CbTX) and iberiotoxin (IbTX) (Galvez, et al., 1990) and other indole-diterpenoid mycotoxins including paspalitrem A and C, alfatrem, penitrem A, paspalinine, paxilline, verruculogen and paspalicine (Knaus, et al., 1994). Of these compounds lolitrem-B, paxilline and IbTX are specific blockers of BK channels (Dalziel, Finch, & Dunlop, 2005; Ghatta, et al., 2006). The β_4-subunit imparts resistance to CbTX and IbTX (Meera, Wallner, & Toro, 2000). Therefore, IbTX may not reliably block BK-β4 channels.

3.2: Neural roles of large-conductance Ca^{2+}-activated K^+ channels

The clinical signs reported from ruminant PRGS studies (Blythe, et al., 2007; Di Menna, et al., 1992; Fisher, et al., 2004; Galey, et al., 1991; Miyazaki, et al., 2004; Tor-Agbidye, et al., 2001) and observed in WT mice dosed with lolitrem-B (Imlach, et al., 2008) emphasise the occurrence of neuromotor dysfunction during lolitrem-B intoxication. BK channels are widely distributed in the central nervous system (CNS) especially in the cerebral cortex and hippocampus (Knaus, et al., 1996) and have been located in axon terminals, soma and dendrites indicating a wide distribution within the neuron (Gribkoff, et al., 2001). The role of BK channels in neural excitability is unclear, and appears to vary between neurons and between each cellular region.

However, the contribution of BK channels to repolarisation and their role as the sole current that produces the fast afterhyperpolarisation (fAHP) of action potentials (APs) are undisputed. In a single AP spike, the peak depolarisation produced by voltage-gated Na^+ (VGNa) channels (and to a lesser degree voltage-gated Ca^{2+} (VGC) channels) is terminated by depolarisation-induced inactivation of VGNa channels and concurrent activation of outward rectifying K^+ channels, resulting in sudden repolarisation (Guyton & Hall, 2000; Hodgkin & Huxley, 1952). Membrane polarity continues to fall below resting membrane potential (RMP), marking the phase transition from repolarisation to hyperpolarisation (Guyton & Hall, 2000). fAHP lasts for 1-10 ms and is then followed by a more gradual and prolonged hyperpolarisation (medium- & slow-AHP) (Faber & Sah, 2003b). *In vitro* studies revealed that repolarisation was slowed and the fAHP was blocked by the addition of either a fast-activating Ca^{2+}-chelator, TEA, CbTX, IbTX or paxilline, incriminating a role for BK channels (Faber & Sah, 2003b; Lancaster & Nicoll, 1987; Sah & Faber, 2002; Sah & McLachlan, 1992; Storm, 1987a, 1987b). This role aligns with the characteristics of BK channels. That is they activate in response to increased $[Ca^{2+}]_i$ and depolarisation of the membrane potential, resulting in K^+ efflux and a subsequent repolarisation, which is dramatic due to their high conductance (Gribkoff, et al., 2001). Characteristic APs with prolonged repolarisations and a lack of fAHP have been demonstrated by the administration of IbTX or paxilline in CA1 pyramidal cells of the hippocampus (Gu, Vervaeke, & Storm, 2007), lateral amygdala projection neurons (Faber & Sah, 2003a), Purkinje cells (Sausbier, et al., 2004; Womack & Khodakhah, 2002), cluster 1 neurons of the suprachiasmatic nucleus (Cloues & Sather, 2003), vagal motorneurons (Sah & McLachlan, 1992), vestibular neurons (A. B. Nelson, Krispel, Sekirnjak, & du Lac, 2003), neurons of the dorsal root ganglia (Zhang, Gopalakrishnan, & Shieh, 2003) and in vestibulocerebellar unipolar brush cells (Diana, et al., 2007). Purkinje cells from $BK_{\alpha}^{-/-}$ mice show a similar AP phenotype (Sausbier, et al., 2004). Therefore, the contribution of BK channels to spike repolarisation and fAHP seems likely to be a widespread phenomenon.

The role of BK channels in repolarisation and hyperpolarisation, in addition to the general tendency for K^+ currents to attenuate cell excitability, lead to the concept that BK channels are inhibitory (Gribkoff, et al., 2001). However, the overall effect of BK channels on neural excitability is complex due to the intricate and multifaceted network of neurons that make up the CNS. Indeed, inhibition of inhibitory neurons can cause disinhibition (i.e. activation) of neural circuits (Sausbier, et al., 2004). In addition, there are reports of both inhibitory and excitatory roles for BK channels.

The association between BK channels and the CNS depressant effects of ethanol (Davies, et al., 2003) demonstrates the role of BK channels in depressing neural excitability. Ethanol has been shown to increase BK channel activity in neurohypophysial terminals (Dopico, Lemos, & Treistman, 1996), pituitary clonal cells (Jakab, Weiger, & Hermann, 1997), and dorsal root ganglia cells (Gruss, et al., 2001). IbTX reverses the ethanol-induced changes to the AP phenotype, which included prolongation of the AP refractory period and reduced firing rates (Gruss, et al., 2001). Furthermore, nematode (*Caenorhabditis elegans*) mutants that display increased resistance to ethanol exhibit DNA with numerous loss-of-function mutations of the slo-1 gene that encodes BK channels (Davies, et al., 2003), while the behaviour of slo-1 gain-of-function mutants resembles that of ethanol-intoxicated animals (Davies, et al., 2003). These studies indicate that the CNS depressant effects of ethanol are due to activation of BK channels. Another indicator that BK channels depress the CNS is the downregulation of BK channel expression that occurs in the hippocampus and cortex of chronically epileptic rats (Otalora, et al., 2008). These studies indicate that BK channels depress neural excitability.

However, other studies indicate an excitatory role. A gain-of-function mutation of slo-1 was found to underlie the coexisting human epilepsy and paroxysmal movement disorder (Du, et al., 2005). Also, mice lacking the β4-subunit (the β-subunit that slows the gating kinetics of the α-subunit (Brenner, Jegla, et al., 2000)) show gain-of-function of BK channels and display an epileptic phenotype (Brenner, et al., 2005). Furthermore, chemoconvulsant-induced seizures provoked a gain-of-function in BK channels, which manifested *in vitro* as a faster AP decay time and an increase in firing activity that was reduced to normal firing rates by paxilline (Shruti, Clem, & Barth, 2008). BK channel blockade by IbTX inhibited rapid firing of cultured rat cerebral cortical neurons, indicating that BK channels with fast gating kinetics are required for epileptic, bursting behaviour in cortical neurons (Jin, Sugaya, Tsuda, Ohguchi, & Sugaya, 2000). Also, injection of paxilline into the ventricles of mice, presumably blocking BK channels, did not produce seizure activity (Juhng, et al., 1999).

Thus BK channels seemingly depress and excite the CNS, prevent and cause epilepsy and increase and decrease AP firing rates. These conflicting reports preclude any gross generalisation about whether BK channels excite or depress the CNS. However, understanding the proposed mechanisms by which BK channels mediate the RMP, AP firing rates and synaptic transmission, and how modulators of BK channels can modify

the role of BK channels in these neural functions reveals that these reports do not necessarily contradict; rather, they collectively exemplify the functional diversity of BK channels.

3.2.1: Large-conductance Ca^{2+}-activated K^+ channels may contribute to the negative resting membrane potential of excitable cells

As a group, K^+ channels are crucial in determining the RMP of excitable cells (Guyton & Hall, 2000; Salkoff, Butler, Ferreira, Santi, & Wei, 2006). However, *in vitro* studies show that at RMP and stable $[Ca^{2+}]_i$, the probability that BK channels are open is extremely low, predicting that they would have little influence on RMP (Womack & Khodakhah, 2002). But, many modifiers of BK channel activation kinetics are eliminated in excised patches and dissolved intracellular factors are also lost during whole-cell recordings by solution exchange between the cytosol and the pipette (T Hoa, et al., 2007). Therefore, the idea that BK channels require substantial membrane depolarisation to be activated at stable $[Ca^{2+}]_i$ may not always be true for the intact *in vivo* cell. In evidence of this, patch clamping of rat clonal pituitary cells showed that at RMP 40% of these cells displayed K^+ currents that were characteristic of BK channels (Haug & Sand, 1997) and in rat motorcortex neurons BK channels demonstrate enhanced sensitivity to $[Ca^{2+}]_i$ due to phosphorylation (Lee, Rowe, & Ashford, 1995). Therefore, *in vivo*, BK channels may contribute considerably to the permeability of the membrane to K^+ and thus help maintain the negative RMP.

3.2.2: The role of large-conductance Ca^{2+}-activated K^+ channels in determining the rate and pattern of action potential firing in neurons

Encoding of information in the nervous system not only depends on the phenotype of single APs but also on the pattern of AP firing. The influence of BK channels on the pattern of AP firing depends on their location, as BK channels in dendrites reduce burst firing (Benhassine & Berger, 2009; Golding, Jung, Mickus, & Spruston, 1999; Rancz & Hausser, 2006), while those in the soma assist rapid firing (Jin, et al., 2000; Raman & Bean, 1999; Sausbier, et al., 2004). In some neurons, such as CA1 pyramidal neurons in the hippocampus (Golding, et al., 1999), neocortical neurons (Benhassine & Berger, 2009) and Purkinje cells of the cerebellum (Haghdoost-Yazdi, Janahmadi, & Behzadi, 2008; Swensen & Bean, 2003; Womack & Khodakhah, 2004), specific spatial and temporal combinations of synaptic input generate a Ca^{2+} spike in the dendrite, which is propagated to the soma and produces intense depolarisation that in turn evokes a

burst of high frequency APs. BK channels regulate the initiation and duration of the Ca^{2+} spike. BK channel activation increases the threshold for initiation and reduces the amplitude and duration of dendritic Ca^{2+} spikes (Benhassine & Berger, 2009), while blockade of BK channels enhances dendritic excitability, delays repolarisation of Ca^{2+} spikes and subsequently increases the duration of repetitive AP firing (Golding, et al., 1999; Rancz & Hausser, 2006). Therefore, BK channel activation may reduce bursting activity of neurons by lowering the RMP and providing negative feedback in dendrites. In contrast, isolated Purkinje neurons (Raman & Bean, 1999; Sausbier, et al., 2004) and neocortical neurons (Jin, et al., 2000) in the absence of dendritic input show enhanced burst firing at the soma during BK channel activation, characterised by brief rhythmic membrane depolarisations on which several high-amplitude fast spikes are observed. Here, BK channel blockade results in depolarisation-induced inactivation of VGNa channels and culminates in silent membrane activity (Raman & Bean, 1999; Sausbier, et al., 2004). Therefore, activation of BK channels in dendrites reduces bursting activity, while bursting activity originating at the soma requires somatic BK channel activation.

The role of BK channels at the soma exemplifies the paradoxical role of BK channels during high frequency AP trains as the dramatic repolarisation and fAHP influences the activation state of other Na^+ and K^+ channels, allowing rapid initiation of the subsequent spike (Gu, et al., 2007). During depolarisation, VGNa channels become inactivated and require repolarisation (to which BK channel current contributes) to remove the inactivation (Guyton & Hall, 2000). The degree of remnant VGNa channels inactivation determines the absolute refractory period and the threshold required to induce the following AP (Guyton & Hall, 2000). Delayed rectifying K^+ (DRK) channels are voltage-gated K^+ channels that contribute to the repolarisation of the AP. BK channels may accelerate the deactivation of DRK channels by producing the fAHP, limiting DRK channel inhibition of the subsequent depolarisation (Gu, et al., 2007). Accordingly, BK channel blockade and the resulting delay in repolarisation and lack of fAHP cause a reduction in the ensuing gradient of depolarisation and increase the threshold of subsequent APs to more depolarised potentials (Gu, et al., 2007). In hippocampal CA1 pyramidal cells, the effect of BK channels was reduced at frequencies less than 100 Hz (Gu, et al., 2007). This finding is in accordance with the effect of BK channels on VGNa channels and DRK channels as lower AP frequencies would allow a near complete recovery from Na^+ inactivation between spikes and sufficient time for DRK channels to close before the next spike, independent of BK channel function (Gu, et al., 2007). Alternatively, or in conjunction, the BK

channel-induced fAHP might activate hyperpolarisation-activated cation currents, which would then depolarise the cell and contribute to the generation of subsequent APs (Biel, Wahl-Schott, Michalakis, & Zong, 2009; Wahl-Schott & Biel, 2009). Therefore, the influence of BK channels on firing rate depends on its interaction with other ion channels and the importance of these interactions depends on the firing rate of the neuron.

Hodgkin & Huxley (1952) recognised that repolarising K^+ currents were required for the fast firing rates of neurons. The BK channel is an ideal candidate due to its rapid activation kinetics by depolarisation and $[Ca^{2+}]_i$, its large conductance allowing dramatic repolarisation within a short time, and deactivation kinetics that ensure sufficient time to decrease the membrane potential to values that deactivate DRK channels yet are sufficiently fast that BK channels close before initiation of the following spike (Gu, et al., 2007). The enabling of rapid firing is clearly demonstrated in the auditory afferent nerve which can fire at rates up to 400 Hz when evoked by an auditory stimulus (Oliver, et al., 2006); a feat for which BK channels are essential, evidenced by recordings of auditory-evoked firing rates in WT mice averaging 297 ± 8.0 Hz compared with 165 ± 8.4 Hz in $BK_{\alpha}^{-/-}$ mice. Another example of BK channels interacting with other ion channels to determine AP firing patterns is their role in generating and terminating plateau bursting. BK channels that are co-localised with VGC channels rapidly activate in response to the localised increase in $[Ca^{2+}]_i$ during APs. The K^+ efflux truncates the AP spike amplitude and limits the participation of DRK channels, which would otherwise cause continued repolarisation (Stojilkovic, et al., 2005; Van Goor, Li, & Stojilkovic, 2001). Together the BK and DRK channels cause partial repolarisation and subsequent lowering of $[Ca^{2+}]_i$. This leads to rapid deactivation of the BK channels. Because the DRK channel current is insufficient to repolarise the membrane fully, a plateau potential is generated, which is attributable to the balance between the outward current of K^+ via BK and DRK channels and inward Ca^{2+} currents through VGC channels (Van Goor, et al., 2001). The termination of the plateau potential occurs when BK channels that are not co-localised with VGC channels are activated by the global increase in $[Ca^{2+}]_i$ (Tsaneva-Atanasova, Sherman, Van Goor, & Stojilkovic, 2007), exemplifying the different roles of BK channels depending their location in relation to other channels. The association between the role of BK channels in plateau potential generation and the clinical observations in PRGS is discussed in section 3.4. To conclude, BK channels have varied roles in the patterns and rate of AP firing depending on their location along the cell membrane, their interaction with other ion channels and the firing frequency required by the neuron.

3.2.3: The disputed role of large-conductance Ca^{2+}-activated K^+ channels in neurotransmitter release.

The influence of BK channels on synaptic strength is perhaps the greatest dispute about the role of BK channels in neural transmission. Transmission of electrical potentials between sensory cells, neurons and effector organs occurs at synapses. Depolarisation at the presynaptic terminals of sensory cells or axons stimulates the release of vesicles that contain neurotransmitters, by allowing Ca^{2+} to enter via VGC channels (Guyton & Hall, 2000). The localised increase in $[Ca^{2+}]_i$ causes exocytosis of neurotransmitters, which cross the synaptic cleft and bind to receptors on the post-synaptic neuron or effector organ, inducing post-synaptic potentials (Sjaastad, Hove, & Sand, 2003). BK channels are prominently expressed at presynaptic nerve endings where they co-localise with VGC channels (Berkefeld & Fakler, 2008; Berkefeld, et al., 2006; Hu, et al., 2001; Robitaille, Garcia, Kaczorowski, & Charlton, 1993). This strategic positioning indicates a role in synaptic transmission, but evidence conflicts as to whether this role is to terminate or facilitate neurotransmitter release.

The role of BK channels in repolarisation in response to depolarisation and Ca^{2+} influx would indicate that BK channels are an ideal candidates for mediating the negative feedback of neurotransmitter release. BK channel-induced repolarisation in the locale of co-localised VGC channels would close these channels, terminating the release of neurotransmitters (Berkefeld, et al., 2006; Flink & Atchison, 2003; Ghatta, et al., 2006; Robitaille, et al., 1993). An increase in neurotransmitter release in response to IbTX blockade of BK channels has been demonstrated in neuromuscular junctions of the mouse and frog (D. L. Marshall, et al., 1994; Robitaille, et al., 1993), motor nerve terminals (Flink & Atchison, 2003), cholinergic neurons (Liu, Patel, Khawaja, Belvisi, & Rogers, 1999), CA3 pyramidal cells of the hippocampus (Raffaelli, Saviane, Mohajerani, Pedarzani, & Cherubini, 2004) and neurons of the superficial dorsal horn (Furukawa, Takasusuki, Fukushima, & Hori, 2008). Also, increased neurotransmitter release occurs at the neuromuscular junction of $BK_{\alpha}^{-/-}$ nematodes (*C. elegans*) compared to WT nematodes (Wang, Saifee, Nonet, & Salkoff, 2001). These studies relate AP broadening at the soma to an increased duration of neurotransmitter release and an increased rate of excitatory postsynaptic potentials (EPSPs) recorded in the receiving neuron (Raffaelli, et al., 2004; Wang, et al., 2001). The mechanisms of neurotransmitter release are not affected, as IbTX had no effect on AP-independent EPSPs (Raffaelli, et al., 2004). Based on these observations, Raffaelli et al. (2004) proposed that BK channels increase synaptic strength indirectly via regulation of Ca^{2+}

influx. This hypothesis is supported by evidence that at the cerebellar synapses between granule cells and Purkinje cells broadening of the presynaptic AP by 25% results in an near equal increase in Ca^{2+} entry but neurotransmitter release doubles, indicating that synaptic strength is extremely sensitive to increases in $[Ca^{2+}]_i$ (Sabatini & Regehr, 1997). Therefore, Ca^{2+} entry through VGC channels devoid of BK channel control could dramatically increase neurotransmitter release. BK channels not only control $[Ca^{2+}]_i$ by repolarisation-induced closure of VGC channels, but also prevent opening of VGC channels that require strong depolarisation for activation. At motor nerve terminals, BK channels truncate the AP before it reaches fully depolarised values that are required to activate L-type VGC channels and blockade of BK channels activates normally silent L-type VGC channels (Flink & Atchison, 2003). These studies support the hypothesis that BK channels limit neurotransmitter release by their influence on VGC channel activity and may explain the increased spontaneous release of neurotransmitters from synaptosome tissue taken from the cerebral cortex of sheep displaying severe signs of PRGS compared with similar tissue obtained from sheep grazing endophyte-free pasture (Mantle, 1983).

Another study proposed that BK channels, although capable of doing so, do not influence neurotransmitter release under basal conditions, rather their role is to act as an emergency break (Hu, et al., 2001). This was based on the observation that BK channels only influenced synaptic strength of hippocampal CA3–CA1 pyramidal cell synapses when voltage-dependent K^+ channels were blocked. Hu et al. (2001) proposed that under normal conditions voltage-dependent K^+ channels regulate repolarisation and terminate synaptic activity and that BK channels may be recruited under pathological conditions, such as ishaemia or seizure activity, in which an increase in $[Ca^{2+}]_i$ depolarises the cell to the extent that voltage-dependent K^+ channels inactivate, allowing BK channels to take over. In accordance with this hypothesis, one report found that in preparations containing cortical neurons BK channels were only activated in injured neurons (Kang, Huguenard, & Prince, 2000). BK channel openers have been proposed as neuroprotectants during ischaemic stroke because activation of BK channels would reduce the excess release of excitatory amino acids and prevent excitotoxicity and cell death (Hewawasam, et al., 2002).

In contrast to the negative feed back model, blockade of BK channels at the neuromuscular junction of embryonic xenopus (Pattillo, et al., 2001) and at the ribbon synapse of vertebrate rod photoreceptors (J. W. Xu & Slaughter, 2005) reduced neurotransmitter release. It was proposed that BK channel activity in the locale of VGC

channels increases the extracellular K^+ concentration in synaptic cleft, which in turn enhances the electrostatic driving force for Ca^{2+} influx (Pattillo, et al., 2001; J. W. Xu & Slaughter, 2005). In this way, co-localised BK channels and VGC channels form a positive coupled loop that amplifies synaptic transmission. A biphasic action of BK channels was observed at the ribbon synapse of rod photoreceptors. Initially BK currents enhanced VGC channel currents but eventually BK channel current overwhelmed other currents and hyperpolarised the cell, thereby BK channels acted as a safety break (J. W. Xu & Slaughter, 2005).

The role of BK channels in synaptic transmission is ambiguous. As a hyperpolarising channel, its block or genetic removal is expected to increase transmitter release by allowing continued VGC channel activity. This was observed in some systems (Flink & Atchison, 2003; Furukawa, et al., 2008; Liu, et al., 1999; Raffaelli, et al., 2004; Robitaille, et al., 1993; Wang, et al., 2001), other studies indicate BK channels are limited to pathological conditions (Hewawasam, et al., 2002; Hu, et al., 2001; Kang, et al., 2000), while others indicate a facilitative role (Pattillo, et al., 2001; Spencer, Przysiezniak, Acostaurquidi, & Basarsky, 1989; J. W. Xu & Slaughter, 2005). These findings need not contradict; rather, they may represent the plasticity of BK channels. β-subunits, splicing and post-translational modification considerably alter the voltage and $[Ca^{2+}]_i$ required for BK channel activation (Ghatta, et al., 2006; Reinhart, et al., 1991; Weiger, et al., 2000) and therefore would alter the way in which BK channels contribute to the shape of the AP. Computer simulation showed that the precise timing of AP broadening determined whether AP broadening would increase or decrease neurotransmitter release (Pattillo, et al., 2001), with an increase seen if broadening originated at the peak and a decrease if broadening began at 20% repolarisation. BK channel block broadens the AP to varying degrees and at different time points of repolarisation depending on the cell type (Sah & McLachlan, 1992; Storm, 1987b), the developmental age of the synapse (Pattillo, et al., 2001) and the location of the BK channel along the cell membrane (Hu, et al., 2001; Pattillo, et al., 2001). Blockade of BK channels, having fast activation kinetics that normally truncate depolarisation and thus prevent activation of L-type VGC channels (Flink & Atchison, 2003), would presumably broaden and increase the amplitude of the AP peak, thus increase neurotransmitter release. In contrast, the blockade of slower activated BK channels would cause broadening late in the repolarisation phase, which might decrease the driving force for Ca^{2+}, decreasing neurotransmitter release (Pattillo, et al., 2001). Furthermore, BK channels co-localise with various types of VGC channels, which display distinct gating kinetics and generate BK channel currents with distinct profiles

that depend on both VGC channel kinetics and BK channel kinetics (Berkefeld & Fakler, 2008). Therefore, the consequence of BK channel blockade may also depend on the type of VGC channels that populate the synapse. In addition to physiological properties of the synapse, the morphological structure of the synapse may also dictate the effects of BK channels. In rod photoreceptors, BK channel activation facilitates Ca^{2+} entry by increasing the extracellular K^+ concentration (J. W. Xu & Slaughter, 2005). However, this facilitating effect may depend on the invaginating structure of ribbon synapses and may not apply to synapses with a more open geometry where K^+ may readily diffuses away from the synapse. Therefore, modulation of BK channel kinetics and the morphologic structure of the neural synapse may determine whether BK channels reduce or augment neurotransmitter release.

3.2.4: The role of large-conductance Ca^{2+}-activated K^+ channels in neural excitability depends on the entire biophysical context of the individual channel

In conclusion, the influence of BK channels on neural excitability appears to depend on the entire biophysical context in which the channel operates, enabling BK channels to play different roles in different types of neurons, in different parts of the same neuron and in different circumstances. β-subunits, alternate splicing and post-translational modifiers can alter the kinetics of BK channels (Brenner, et al., 2005; Lee, et al., 1995; T Hoa, et al., 2007) as can co-localised VGC channels (Berkefeld & Fakler, 2008). In addition, the location of BK channels along the cell membrane (Benhassine & Berger, 2009; Golding, et al., 1999; Jin, et al., 2000; Raman & Bean, 1999; Rancz & Hausser, 2006; Sausbier, et al., 2004), their interactions with other ion channels (Gu, et al., 2007), the morphology of their location (J. W. Xu & Slaughter, 2005) and the required frequency of AP firing of the neuron (Gu, et al., 2007) can all influence the function of BK channels. BK channels should not be considered as having an inhibitory or an excitatory role per se, rather, their role is to shape the AP, regulate the firing rate and contribute to synaptic strength according to the requirements of the individual neuron. Therefore, BK channel blockade by lolitrem-B will not strictly produce functional neural excitement or neural suppression, but rather could be expected to disrupt the neural encoding of messages. Miscoded messages may therefore have caused the neuromotor dysfunction observed in $BK_{\alpha}^{-/-}$ mice, in WT mice dosed with lolitrem-B and in PRGS (Imlach, et al., 2008; Sausbier, et al., 2004). However, studies have failed to decipher which neurons are involved in these miscoded messages. The following section will discuss the pathophysiology of tremor and ataxia in light of the neural roles

of BK channels and identifies the need for a more detailed clinical description of lolitrem-B.

3.3: Proposed mechanisms for tremor and ataxia displayed in lolitrem-B intoxication

Tremor and ataxia are the common neurological signs identified in ruminant trials on PRGS (Blythe, et al., 2007; Di Menna, et al., 1992; Fisher, et al., 2004; Galey, et al., 1991; Miyazaki, et al., 2004; Tor-Agbidye, et al., 2001), in mice bioassays (Gallagher & Hawkes, 1986) and in knockout mice experiments (Imlach, et al., 2008). The exact mechanisms by which lolitrem-B blockade of BK channels causes ataxia and tremor are unknown. Tremor can result from dysfunction of the muscle cell membrane, neuromuscular spindle and associated myotatic reflex arc, spinal cord, brainstem, thalamus, cerebellum or cerebrum (Guyton & Hall, 2000; Mayhew, 2009), while ataxia can be due to dysfunction of the general proprioceptive (GP) pathways, the vestibular system or the cerebellum (Mayhew, 2009). The diffuse nature of BK channels and their various roles throughout the nervous system confounds the determination of the exact mechanisms and provides little indication as to the nature of tremor and ataxia that could be expected in lolitrem-B intoxication. A more detailed clinical description of the nature of tremor and ataxia displayed in PRGS is required.

3.3.1: Tremor

Muscle tremor is defined as an involuntary movement, characterised by regular or irregular oscillations of one or several body segments (Engin, et al., 2007). Normally, muscle contraction and relaxation are balanced by the neuromuscular spindle and the myotatic reflex arc, which receive modulatory input from the CNS including the spinal cord, brainstem, thalamus, basal ganglia, cerebellum and cerebrum (Guyton & Hall, 2000; Mayhew, 2009). Many tremor disorders occur without structural unsoundness (Shaikh, et al., 2007) and are more often related to changes in neural excitability, such as neural disinhibition, alterations in normal synaptic transmission, receptor abnormalities, or dysfunction of the muscle cell membrane (de Lahunta, Glass, & Kent, 2006). Cerebellar dysfunction results in 'intention tremor' (Guyton & Hall, 2000), a term that has been used to describe tremor associated with the $BK_{\alpha}^{-/-}$ phenotype (Sausbier, et al., 2004). Weakness can also manifest as tremor of anti-gravity muscles (Mayhew, 2009). The body of literature discussed previously in this chapter that identified the roles of BK channels in inhibitory neurons, synaptic transmission and maintaining the RMP, in addition to their wide expression in nerves and muscle cells (Knaus, et al., 1996; Pallotta, 1985a), and reports of intention tremor in $BK_{\alpha}^{-/-}$ (Sausbier, et al., 2004)

and weakness in horses with PRGS (Cunningham & Hartley, 1959) means that literature on BK channels and PRGS provides little indication of the cause of tremor.

3.3.2: Cerebellar ataxia

There are multiple, complex, integrating pathways involved in the production of smooth, precisely timed, regular movement, which can be divided into three classes depending on the involvement of GP, vestibular or cerebellar systems. An abnormal locomotion and deficiency in motor coordination observed in $BK_{\alpha}^{-/-}$ has been suggested to result from cerebellar dysfunction (Sausbier, et al., 2004), specifically, disinhibition of the deep cerebellar nuclei due to Purkinje cell dysfunction. Tonal signals from the deep cerebellar nuclei finely adjust motor activity by influencing brainstem upper motor neurons (UMNs) (de Lahunta & Glass, 2009). The cerebellum receives input from visual and vestibular systems, tendons, joints and muscles, and the cerebral cortex. It constantly compares the actual movement with the intended or programmed movement and makes appropriate corrective adjustments – not only subsequent to inaccuracies but before they occur, and thus produces smooth, precisely timed and positioned movements (Guyton & Hall, 2000). These corrective adjustments rely on output from the cerebellar cortex, which is solely the role of Purkinje cells (de Lahunta & Glass, 2009; Edgerton & Reinhart, 2003; Sausbier, et al., 2004; Womack & Khodakhah, 2002). Lack of this inhibition results in disinhibition of UMNs, resulting in a dysmetric gait (de Lahunta & Glass, 2009).

In situ hybridisation experiments of mRNA and BK channel protein indicate that the expression of BK channels in the cerebellum is predominantly (but not exclusively) in Purkinje cells, including its dendrites, soma and axons (Sausbier, et al., 2004). At the cellular level, Purkinje cells obtained from $BK_{\alpha}^{-/-}$ mice express a dramatic reduction in spontaneous activity due to depolarisation-induced inactivation of the AP mechanism (Raman & Bean, 1999; Sausbier, et al., 2004), as discussed previously in this chapter **(section 3.2.2)**. Application of IbTX demonstrates that BK channels decrease the repolarisation time and enhance the amplitude of the fAHP (Haghdoost-Yazdi, et al., 2008), enabling Purkinje cells to fire at rates of up to 200 Hz (Womack & Khodakhah, 2004). Regulation of firing frequency of Purkinje cells is important for motor coordination (Womack & Khodakhah, 2004); therefore, changes in the rate of Purkinje cell output may result in ataxia. Spinocerebellar ataxia in humans has been associated with dysfunction of P/Q-type VGC channels located in Purkinje cells (Zhuchenko, et al., 1997), whose net effect is to activate BK channels and cause repolarisation (Edgerton & Reinhart, 2003; Raman & Bean, 1999), providing further evidence that BK channel dysfunction in Purkinje cells results in ataxia. In addition, BK channels are thought to

be important modulators of dendritic Ca^{2+} spikes in Purkinje cells and therefore, not only are they important for the intrinsic firing pattern in Purkinje cells but also modulate the influence of synaptic inputs (Edgerton & Reinhart, 2003; Womack & Khodakhah, 2004). These studies advocate that BK channel dysfunction in Purkinje cells may result in cerebellar ataxia.

Episodes described in ruminant trials that involve recumbency, opisthotonus and interludes of limb thrashing, while maintaining consciousness (Blythe, et al., 2007; Cunningham & Hartley, 1959; Galey, et al., 1991; Mackintosh, et al., 1982; Mayhew, 2007; Tor-Agbidye, et al., 2001) depict the occurrence of so-called cerebellar fits. The term cerebellar fits is used in human medicine to describe tonic seizures where the head is drawn back, the legs are fully extended with the feet arching backwards and consciousness is maintained (McCrory, Bladin, & Berkovic, 1999). In quadrepeds, cerebellar fits resemble transient episodes of decerebellate rigidity with opisthotonus and rigid extension of the forelimbs, a posture that is induced by experimental surgical ablation of the entire cerebellum or resection restricted to the rostral lobe of the cerebellum (Holliday, 1980). In cerebellar lesions, the hindlimbs are either tonically extended, extended but flexed at the hips or display alternating clonic and tonic movements (de Lahunta & Glass; Holliday, 1980). The variable degree of hindlimb tonus reflects the ventral extent of the experimental cerebellar lesion and when the lesion extends sufficiently ventral in the rostral lobe to affect the area that somatotopically relates to the rear legs (lingual and lobulus centralis) the hindlimbs and hip joints are extended and rigid as in the forelimbs (Holliday, 1980). Transection of the brainstem between the rostral and caudal colliculi of the midbrain results in a similar phenomenon termed decerebrate rigidity (de Lahunta & Glass). The differentiating feature of these phenomena is that decerebrate rigidity often involves changes in sensorium such as coma, whereas cerebellar lesions that cause decerebellate rigidity do not influence the state of consciousness (de Lahunta & Glass). Episodes that are reported in association with PRGS most closely resemble decerebellate rigidity, as consciousness is maintained during episodes.

The extensor hypertonus displayed in both decerebrate and decerebellate rigidity can be explained as release of the lower motor neurons (LMNs) that maintain tone in extensor muscles, from the inhibitory influence of the caudally projecting UMN tracts, which largely depend on final regulation by the rostral lobe of the cerebellum (de Lahunta & Glass; Holliday, 1980). Therefore, cerebellar fits may result from paroxysmal disruption of output from the cerebellum (particularly from the rostral lobe) and a subsequent release of LMNs from UMN inhibitory control. However, despite the

term ‘cerebellar’ used to describe these episodes, in humans a variety of pathological causes that do not involve the cerebellum also produce cerebellar fits such as a rapid increase in intracranial pressure, encephalitis of the brainstem and sudden cerebral hypoxia (McCrory, et al., 1999). Furthermore, other non-cerebellar causes of paroxysmal, severe, neural disinhibition cause cerebellar fits in quadrupeds such as dysfunction of the glycine receptor on extensor LMN cell membranes (de Lahunta, et al., 2006). Therefore, the inclusion of cerebellar fits in the clinical description of PRGS in ruminants does not require that lolitrem-B causes cerebellar dysfunction.

3.3.3: Vestibular ataxia

It has also been suggested that BK channels play important roles in the vestibular system. The vestibular system is a special proprioceptive pathway that enables animals to maintain orientation of the eyes, trunk and limbs in relation to the positioning of the head and with respects to gravity (Mayhew, 2009). Afferent signals from the vestibular apparatus are integrated by the vestibular nuclei, reticular formation and the cerebellum to produce appropriate motor signals to postural and eye muscles and thus maintain equilibrium and visual fixation (de Lahunta & Glass, 2009; Guyton & Hall, 2000). BK channels participate in efferent-induced synaptic inhibition of type II vestibular hair cell (VHCII) (Kong, et al., 2005; Kong, et al., 2007) and inhibitory-induced excitation of vestibular nucleus neurons (A. B. Nelson, et al., 2003), modulate the excitability of cells in the flocculonodular lobe of the cerebellum (Diana, et al., 2007; Holliday, 1980), and may indirectly influence the composition of endolymph. Therefore, lolitrem-B may disrupt the encoding of vestibular signals.

The mechanoreceptors of the vestibular apparatus are located in 3 semicircular canals and in the maculae of the utricle and saccule. Mechanoreceptors of the utricle and saccule record the static position of the head with respects to gravity, while those in the semicircular ducts detect rotational movement and, via cranial nerve VIII, communicate to the brain the rate and direction of head movement in each of the three planes of space (Guyton & Hall, 2000). There are two types of mechanoreceptors in the vestibular organ; VHCII make direct contact with both afferent and efferent terminals, whereas type I vestibular hair cells only contact afferent nerves (Purcell & Perachio, 1997). As in outer hair cells (OHCs) of the auditory system (Dallos, et al., 1997; Sridhar, Brown, & Sewell, 1997), efferent neurons that synapse with VHCII release the neurotransmitter acetylcholine (Ach), which activates nicotinic receptors and subsequently induces a fast and slow hyperpolarisation (Kong, et al., 2005). Whole-cell patch clamping revealed that Ach stimulation of VHCII produces a sustained non-inactivating K^+ current that was reversibly inhibited by TEA, CbTX and IbTX, but

not by apamin (a SK channel blocker), indicating that BK channel current is responsible for the slow effect of Ach (Kong, et al., 2005; Kong, et al., 2007). Hyperpolarisation of VHCII results in synaptic inhibition at the hair cell afferent nerve synapse (Kong, et al., 2007) and thus adjusts the sensitivity of the vestibular apparatus (Boyle, Rabbitt, & Highstein, 2009). A comparison between novice and highly trained rowers revealed that the greater experience in movement control and rowing-specific balance of the trained rowers was associated with a lower frequency of nystagmus after both groups were subjected to a rotating chair test (Nigg, MacIntosh, & Mester, 2000). This indicates a learned ability to dampen vestibular signals, which may involve the inhibitory effects of efferent nerve stimulation. Lolitrem-B may result in inappropriately increased sensitivity of the vestibular apparatus due to lack of response to efferent stimulation.

Another form of motor learning that is dependant on BK channel function is displayed by neurons of the vestibular nucleus. These neurons show an increase in spontaneous firing rate after inhibition is removed; a phenomenon termed firing rate potentiation (A. B. Nelson, et al., 2003). An inhibitory-induced reduction in BK channel current, either by reducing $[Ca^{2+}]_i$ or by BK channel phosphorylation, underlies the subsequent long-term changes in intrinsic excitability that increase AP firing rate (A. B. Nelson, et al., 2003). IbTX has varying effects on vestibular nucleus neuron depending on whether the neuron has been influenced by firing rate potentiation; BK channel blockade has no significant influences on neuron excitability after firing rate potentiation had occurred, but dramatically increases spontaneous rates prior to potentiation (A. B. Nelson, et al., 2003). This indicates that lolitrem-B intoxication might prevent this phenomenon by causing the neuron to fire at an increased rate independent of inhibitory-induced changes in $[Ca^{2+}]_i$ or changes in BK channel phosphorylation. Therefore, blockade of BK channels may interfere with the learned modulation of vestibular stimulation, resulting in inappropriate vestibular output.

The flocculonodular region of the cerebellum is an important component of the vestibular system and lesions in this area of the cerebellum produce signs of vestibular ataxia (Holliday, 1980). Purkinje cells from the flocculonodular lobe terminate directly on and inhibit neurons of the vestibular nuclei (de Lahunta & Glass, 2009). Therefore, dysfunction of BK channels in Purkinje cells may cause disinhibition of the vestibular nuclei and disrupt vestibular function. In addition, BK channels have a role in determining the excitability of interneurons of the flocculonodular lobe, which receive direct input from vestibular afferents and have been proposed to be key in determining how the cerebellum interprets signals from the vestibular apparatus (Diana, et al.,

2007; Holliday, 1980). Therefore, blockade of BK channels of the interneurons and Purkinje cells in the flocculonodular lobe might contribute to vestibular dysfunction.

BK channels may also influence vestibular signaling by altering the composition of endolymph, the fluid in which vestibular hair cells are bathed. Endolymph is a K^+ rich fluid that has a positive potential with respects to blood or perilymph (Huy, 2002; Marcus, Wu, Wangemann, & Kofuji, 2002). Endolymph is secreted by dark cells in the ampulla of the semicircular canals and in the wall of the utricle (Ciuman, 2009). Purinergic, adrenergic and muscarinic receptors, and mineralocorticosteroid, vasopressin and atrial natriuretic peptide hormones (Ciuman, 2009) carefully regulate the secretion of K^+ by dark cells. K^+, along with the positive polarity of the endolymph, drives transduction currents that generate receptor potentials in vestibular hair cells (Marcus, et al., 2002). Elevation or reduction of K^+ levels in endolymph decreases or increases the ampullar nerve discharge activity, highlighting the importance of adequate regulation of K^+ secretion. BK channels are expressed on dark cells, but their density is low and therefore BK channels are unlikely to have a vital role in K^+ secretion into the endolymph (Ciuman, 2009). Therefore, it is unlikely that blockade of BK channels on dark cells will influence vestibular function by changing the composition of the endolymph. Later in this chapter the influence of BK channels on aldosterone levels is discussed **(section 3.6)**. Alterations in the level of this hormone induced by changes in BK channel activity may in turn influence dark cell K^+ secretion. In addition, neurotransmitter release and thus activation of purinergic, adrenergic and muscarinic receptors may be increased or decreased by blockade of BK channels. Therefore, BK channels may influence the composition of endolymph indirectly.

3.3.4: General proprioceptive ataxia

General proprioceptive ataxia results from dysfunction of the afferent proprioceptive pathways, which originate in muscles, tendons and joints and project to the cerebellum, thalamus and cerebral cortex (de Lahunta & Glass, 2009; Mayhew, 2009). Two basic pathways constitute the GP system: the subconscious proprioceptive pathways that involve the segmental reflex arc and pathways to the cerebellum, and the conscious proprioceptive pathway that involves transmission of proprioceptive information to the cerebrum (de Lahunta & Glass, 2009). Little is known about the role of BK channels in peripheral nerves. However, BK channels have also been located in dorsal root ganglia (Li, et al., 2007), where the cell bodies of afferent proprioceptive neurons are located (de Lahunta & Glass, 2009). IbTX increased EPSPs in the superficial dorsal horn indicating a role for BK channels in neurotransmitter release from peripheral sensory afferent neurons and thus in somatosensory processing of pain, temperature

and touch systems (Furukawa, et al., 2008). However, axons of primary afferent neurons of the subconscious and conscious GP pathways pass through the dorsal horn without synapsing (Bradley, 1991; de Lahunta & Glass, 2009). Therefore, the influence of BK channels on neurotransmitter release in the superficial dorsal horn does not involve GP pathways. However, it does demonstrate that BK channels have a role in determining neurotransmitter release within peripheral sensory pathways and therefore may also influence GP transmission and processing.

3.3.5: Conclusion

According to what is known about the role of BK channels in the nervous system there is no persuasive indicator of the nature of tremor and ataxia to be expected in PRGS. Tremor may result from changes in excitability in the neural circuits involved in muscle control, cerebellar dysfunction and/or weakness. While the role of BK channels in cerebellar, vestibular and peripheral sensory pathways suggests that ataxia reported in association with PRGS could conform to all three systems. There is currently more evidence of BK channel involvement in cerebellar and vestibular systems than GP pathways. As molecular biochemistry provides no definitive indicators as to the nature of tremor and ataxia expected, a more detailed clinical description of tremor and ataxia is required to allow classification of these disorders of motor control.

3.4: Effects of Iolitrem B on auditory function

BK channels are proposed to play an important role in encoding auditory signals. The exact role is uncertain. Research has focused on their role in inner hair cells (IHCs) of the cochlear and evidence is building that BK channels are important for the temporal precision of auditory signals. The mammalian cochlear consists of a coiled tube that is longitudinally divided into three compartments: scala vestibuli, scala media and scala tympani. The organ of Corti is situated in the scala media, boarded from the other compartments by the basilar membrane (BM) and the tectorial membrane (Guyton & Hall, 2000). Sound waves cause transverse vibrations of the tonotopographic BM, which resonates when the wave reaches the portion of the membrane that has a natural resonant frequency equal to the sound wave (Guyton & Hall, 2000). The organ of Corti transforms sound-evoked, transverse, BM vibrations into electrical signals in sensory hair cells, which synapse onto the auditory afferent nerves (Guyton & Hall, 2000). There are two types of sensory receptor cells in the mammalian organ of Corti: IHCs and OHCs. Both types of cells have elongated, actin-based villi named stereocilia, which emerge from their apical surface and project into the overlying,

stationary, tectorial membrane. The bodies of the hair cells are contained in the reticular lamina, which is connected to the BM in such a way that transverse movement of the BM causes a shearing motion between the sterocilia and the tectorial membrane. Distortion of sterocilia in one direction mechanically opens transduction channels allowing entry of K^+, resulting in depolarisation, while distortion in the opposite direction closes the channels resulting in hyperpolarisation of the hair cell (Guyton & Hall, 2000). The receptor potential generated by IHCs facilitates the release of neurotransmitter at their synapse, altering the firing rate of associated auditory nerves, and in this way encoding information about the sound (Glowatzki, Grant, & Fuchs, 2008). The receptor potential produced by OHCs stimulates motor activity, which is believed to contribute to the cochlear amplifier system (Frolenkov, 2006). The different roles of IHCs and OHCs are echoed in their innervation; 90-95% of afferent nerves synapse to IHCs supporting their sensory role, while OHCs receive efferent inputs from the medial dorsal olive (Purcell & Perachio, 1997), allowing central control over cochlear amplification.

In situ hybridisation and immunostaining techniques have revealed the strong expression of BK channels in IHCs and mild presence in OHCs, the soma of auditory afferent nerves, and in the stria vascularis (the vascular lining of the scala media) (Hafidi, et al., 2005; Langer, Grunder, & Rusch, 2003; Pyott, Glowatzki, Trimmer, & Aldrich, 2004; Ruttiger, et al., 2004; Skinner, et al., 2003). The expression of BK channels in IHCs parallels the development hearing in mice, suggesting that acquisition of BK channel current is important in the proper development of the auditory pathway (Hafidi, et al., 2005; Pyott, et al., 2004). Infusion of the cochlear with IbTX dramatically reduces the compound action potential (CAP) of the auditory nerve (Beurg, et al., 2005; Skinner, et al., 2003), further indicating the importance of BK channels in the organ of Corti.

3.4.1: The role of large-conductance Ca^{2+}-activated K^+ channels in inner hair cells and their influence on the temporal precision of auditory signals.

The cochlea is faced with the challenge of encoding a large dynamic range of sound frequencies at differing intensities with high temporal precision. This requires fast, precise neurotransmission (Oliver, et al., 2006) – a feat in which BK channels may partake. *In vitro* IHCs display a fast-activating outward K^+ current that is absent in IHCs from $BK_{\alpha}^{-/-}$ mice and in IHCs from WT mice that are bathed in IbTX (Oliver, et al., 2006; Pyott, et al., 2004; Skinner, et al., 2003). When absent, a smaller residual current that displays slow activation kinetics remains (Oliver, et al., 2006), indicating

that BK channels are solely responsible for the fast portion of the K^+ current and that there are no compensatory upregulations of other outward rectifiers when BK channels are blocked (Pyott, et al., 2004). In the absence of BK channels, the lack of the fast K^+ current causes the timing of the IHC receptor potential to be slowed, and thus lengthens the time constant (Oliver, et al., 2006; Skinner, et al., 2003). Furthermore, Oliver et al. (2006) found that IHCs from $BK_{\alpha}^{-/-}$ mice had a slightly depolarised RMP compared with WT IHCs. These findings indicate that BK channels play a substantial role in IHC function.

The importance of BK channel fast K^+ currents in IHCs has been attributed to modulation of neurotransmitter release (Hafidi, et al., 2005; Skinner, et al., 2003). Skinner et al. (2003) hypothesised that BK channels co-localise with VGC channels and regulate neurotransmitter release at presynaptic active zones of mammalian IHCs. However, Pyott et al. (2004) refuted this idea by demonstrating that BK channels localised in the apical region of IHCs and were not associated with the synaptic zone. Hafidi et al. (2005) also described a prominent distribution of BK channels in the apical region, but also reported discrete immunolabelling for BK channels in the synaptic region. In addition to immunohistochemical techniques, fluorescently labeled IbTX was used to confirm the dual localisation of BK channels within the IHCs (Hafidi, et al., 2005). It is possible that BK channels contribute to modulation of neurotransmitter release. However, the non-apical predominance of BK channel distribution indicates that their role is not limited to synaptic control.

Oliver et al. (2006) proposed a further role where BK channels contribute to the temporal precision of auditory transmission by determining the time constant of IHC receptor potentials, which in turn induces synaptic activity and contributes to the AP firing rate of the auditory afferent nerve. The decreased sound evoked discharge rate in auditory nerves from $BK_{\alpha}^{-/-}$ mice compared with WT mice indicates that BK channels are important in maintaining the temporal precision of auditory signals (Oliver, et al., 2006). Oliver et al. (2006) further demonstrated the role of BK channels in temporal precision; in $BK_{\alpha}^{-/-}$ mice, the degree of synchronisation between the auditory stimulus phase and the spike timing of the auditory nerve was reduced and the majority of fibres showed increased variance in the timing of the first peak in AP trains evoked by an auditory stimulus, compared with WT mice. These results are consistent with the *in vitro* increase of the time constant in IHCs (Oliver, et al., 2006; Skinner, et al., 2003) and the notion that activation of a large-conducting K^+ channels rapidly increases the conductance of IHCs and provides fast dynamics (Kros & Crawford, 1990).

BK channel involvement in determining the IHC time constant and thus the precise timing of APs in auditory afferent nerves provides an explanation for the electrophysiological effects of BK channel knockout or blockade. Brainstem auditory evoked potentials (BAEPs) from $BK_{\alpha}^{-/-}$ mice show a delay in the first peak latency (Pyott, et al., 2007), which is consistent with delayed transmission across the IHC–afferent dendrite synapse. And, the reduced waveform of the CAP after infusing the cochlea with IbTX (Beurg, et al., 2005; Skinner, et al., 2003) is consistent with alterations in the timing of auditory afferent nerve APs. The amplitude of a CAP depends on the precise coordination of the largest possible number of individual afferent APs (Skinner, et al., 2003). Incongruity of AP timing between different auditory afferent nerve fibres is averaged out resulting in dampening of the CAP.

BK channels have also been located in the soma of auditory afferent nerves (Hafidi, et al., 2005; Ruttiger, et al., 2004; Skinner, et al., 2003). As mentioned previously in this chapter, BK channels are believed to play an important role in fAHP (Faber & Sah, 2003b; Lancaster & Nicoll, 1987; Sah & Faber, 2002; Sah & McLachlan, 1992; Storm, 1987a, 1987b) and influence the potential for rapid firing by interacting with other ion channels (Gu, et al., 2007; Guyton & Hall, 2000). Oliver et al. (2006) reported that auditory afferent nerves from $BK_{\alpha}^{-/-}$ mice show an increased absolute refractory period than those from WT mice, which may further limit the rate of auditory-evoked AP firing.

Accurate timing of stimulus onset is critical for the localisation of sounds (Oliver, et al., 2006). Mammals are capable of locating sounds within a precision of a few degrees, but this requires the auditory system to accurately extract and compare the time that the sound arrives at each ear with precision in the order of microseconds (A. R. Palmer, 2004). The fast kinetics of BK channels assist in the temporal precision of auditory signals, indicating that lolitrem-B intoxication may interfere with the ability to determine the horizontal location of sound.

The influence of BK channels on the RMP of IHCs may also influence auditory transmission. Auditory afferent nerves discharge spontaneously in the absence of sound stimulation due to spontaneous release of neurotransmitter from IHCs (Oliver, et al., 2006). Intracochlear perfusion with IbTX caused the spontaneous spike rate of auditory afferent nerves to increase (Beurg, et al., 2005; Hafidi, et al., 2005; Pyott, et al., 2004; Skinner, et al., 2003) and the frequency distribution of the spontaneous rates of auditory afferent nerves from $BK_{\alpha}^{-/-}$ mice showed a lower number of low-rate fibres

than WT mice (Oliver, et al., 2006). In the absence of BK channels, the membrane potential of IHCs displayed a depolarising shift (Oliver, et al., 2006), which would require lower fluctuations in membrane potential to activate synaptic release machinery (Beurg, et al., 2005). The higher spontaneous discharge rate and lower auditory-evoked discharge rate mutes the distinction between these firing patterns and may further confuse auditory signals.

3.4.2: The protective role of large-conductance Ca^{2+}-activated K^+ channels in outer hair cells

Frictional forces against BM motion require that mechanical energy is injected into the organ of Corti on a cycle-by-cycle basis in order to maintain the threshold sensitivity and frequency selectivity for sounds (Dallos, et al., 1997; Frolenkov, 2006). OHCs are believed to generate these mechanical forces by active mechanical feedback generated by sterocilia-induced receptor potentials (Frolenkov, 2006) and by the electromotile response to efferent stimulation (Borko, Batta, & Sziklai, 2005; Dallos, et al., 1997; Dallos, et al., 2008). The molecular mechanism of the latter is based on voltage-dependent conformational changes of the motor protein prestin, which is densely embedded in the lateral membrane of the OHCs (Borko, et al., 2005; Dallos, et al., 1997; Dallos, et al., 2008).

BK channels have been located in OHCs. As with IHCs, there is discrepancy in their cellular localisation with some reporting close apposition to efferent synapses (Hafidi, et al., 2005; Ruttiger, et al., 2004), while others report that BK channels are spatially segregated from the synapse (Blanchet, Erostegui, Sugasawa, & Dulon, 1996). Regardless of cellular location, BK channels may participate in the efferent response (Sridhar, et al., 1997). Efferent nerves release Ach, which binds to nicotinic receptors at the basal pole of the OHC (Sridhar, et al., 1997). The Ach-dependent effects are biphasic; the fast effect causes an influx of Ca^{2+} into the cell via the nicotinic receptors, which activates co-localised SK channels resulting in K^+ efflux, OHC hyperpolarisation, decreased amplification and subsequent decreased stimulation of the IHC (Blanchet, et al., 1996; Sridhar, et al., 1997). The slower effect is due to Ca^{2+}-induced Ca^{2+} release from internal stores and results in phosphorylation and lengthening of prestin, which decreases axial stiffness and induces the electromotile amplification of BM vibrations (Batta, Panyi, Szucs, & Sziklai, 2004; Dallos, et al., 1997). Increased motility of OHC during the electromotile response subsequently induces a shortening response of the cytoskeleton, which increases OHC stiffness and dampens the BM vibrations (Borko, et

al., 2005). Cell shortening is thought to protect the organ of Corti against acoustic overstimulation (Batta, et al., 2004). Coinciding with cell shortening is the presence of BK channel current, activated by the internal Ca^{2+} release (Sridhar, et al., 1997). The BK current induces hyperpolarisation and is thought to contribute to protecting the OHC from acoustic overstimulation (Sridhar, et al., 1997). Ruttiger et al. (2004) reported age-related hearing loss in $BK_{\alpha}^{-/-}$ mice associated with OHC degeneration. However, the strain (C57BL/6) used to inbreed the mice in this study expressed the age-related hearing loss gene (Davis, et al., 2001; Erway, Shiau, Davis, & Krieg, 1996; Johnson, Erway, Cook, Willott, & Zheng, 1997). A similar study, which used a different strain of mice, reported no age-related hearing loss in $BK_{\alpha}^{-/-}$ mice (Pyott, et al., 2007). This indicates that lack of BK channels does not cause age-related hearing loss per se, but may accelerate hearing loss in a strain of mice already predisposed, supporting the proposed role of BK channels as an 'emergency break'.

Dysfunction of cochlear amplification by the OHCs manifests as decreased precision in the selectivity of character frequency (the frequency at which resonance occurs) and as reduced sensitivity to sound energy, requiring higher sound intensities to compensate for the lack of internal amplification (Dallos, et al., 1997). As efferent-induced activation of BK channels does not occur until after the electromotile response has occurred, it is unlikely that lolitrem-B will disrupt cochlear amplification. This view is supported by the lack of difference in the threshold at character frequency recorded from individual auditory nerves (Oliver, et al., 2006) and the threshold of BAEP recordings (Pyott, et al., 2007) from $BK_{\alpha}^{-/-}$ and WT mice. Furthermore, cochlear microphonics are unchanged by IbTX infusion of the cochlear (Skinner, et al., 2003) and distortion product otoacoustic emission are no different between $BK_{\alpha}^{-/-}$ and WT mice (Pyott, et al., 2007). These studies support normal functioning of the cochlear amplifier despite loss of BK channel function. Therefore, although BK channels have been located in OHCs and are involved in the slow effect of the efferent nerve response, their dysfunction does not appear to have significant effects on cochlear amplification. They may however, be important in protecting OHCs from acoustic overstimulation.

3.4.3: Large-conductance Ca^{2+}-activated K^{+} channels do not influence endolymph properties

BK channels have been localised in the stria vascularis (Hafidi, et al., 2005; Ruttiger, et al., 2004; Skinner, et al., 2003), showing strong labeling on the marginal cells (Hafidi, et al., 2005; Takeuchi, Marcus, & Wangemann, 1992). The stria vascularis is a highly

vascularised stratified epithelium on the lateral wall of the scala media and produces endolymph, a fluid characterised by a high K^+ concentration, a low Na^+ concentration and a positive potential (Huy, 2002; Marcus, et al., 2002). The high K^+ concentration in the endolymph allows K^+ to be the major charge carrier for the cochlear mechanoelectrical transduction (Couloigner, Sterkers, & Ferrary, 2006), as in the vestibular labyrinth. Stria abnormalities have been associated with several forms of hearing loss (Huy, 2002). Ruttiger et al. (2004) investigated the phenotypic nature of the stria vascularis in $BK_{\alpha}^{-/-}$ mice, but did not detect any change up to 14 weeks of age compared with WT mice (Ruttiger, et al., 2004). Also, cochlear microphonics, which are sensitive to change in the endocochlear potential, remained unchanged despite infusion of the cochlear with IbTX (Skinner, et al., 2003). These findings indicate that BK channels do not play an essential role in maintaining the K^+ concentration of endolymph or the endocochlear potential. Other K^+ secreting channels have been identified (Takeuchi, et al., 1992), which may compensate for lack of BK channel function.

3.4.4: The proposed influence of lolitrem-B intoxication on auditory transmission

Based on the current knowledge of the roles of BK channels in auditory function it is plausible that lolitrem-B intoxication will result in reduced temporal precision and increased susceptibility to noise-induced hearing loss. The threshold of hearing sensitivity is unlikely to be impaired, as BK channels do not appear to influence cochlear amplification (Oliver, et al., 2006; Pyott, et al., 2007; Skinner, et al., 2003). Endolymph properties are also unlikely to be changed by lolitrem-B intoxication (Ruttiger, et al., 2004; Skinner, et al., 2003).

3.5: The non-neurological clinical signs of perennial ryegrass staggers reported in ruminant trials, explained according to the roles of large-conductance Ca^{2+}-activated K^+ channels

Despite the widespread distribution of BK channels throughout the body, neurological signs predominate in PRGS. *In vitro* studies showed by patch clamping that the affinity of lolitrem-B to BK channels was twofold greater in BK-β_4 channels than BK-β_1 channels, providing a partial explanation for this predominance of neurological disturbances (Imlach, et al., 2008). However, reports of reduced serum prolactin levels, reduced liveweight gain, hypertension, altered gastrointestinal motility and

changes in faecal consistency (Fletcher & Barrell, 1984; McLeay, et al., 1999) indicate that lolitrem-B affects BK channels in other organ systems.

3.5.1: The role of large-conductance Ca^{2+}-activated K^+ channels in maintaining plateau potentials in lactotrophs and somatotrophs may explain the depressed serum prolactin levels and liveweight gain in hoggets grazing perennial ryegrass.

Hoggets grazing perennial ryegrass (PRG) with high levels of endophyte showed depressed serum prolactin levels and reduced liveweight gain (Fletcher & Barrell, 1984). Prolactin is a hormone that is secreted from cells, called lactotrophs, while growth hormone is released from somatotrophs, both in the anterior pituitary gland. These cells are excitable and spontaneously release hormone by firing plateau-bursting APs that increase global $[Ca^{2+}]_i$ and subsequently trigger hormone release (Stojilkovic, et al., 2005; Van Goor, et al., 2001). Activation of BK channels normally limits AP duration and associated Ca^{2+} entry by facilitating membrane repolarisation. However in lactotrophs and somatotrophs, BK channels have a paradoxical role in generating the plateau potential. That is BK channels truncate the initial AP amplitude, which limits the activation of DRK channels and thereby reduces the repolarising current, enabling the generation of the plateau potential, as discussed in section 3.2.2 (Stojilkovic, et al., 2005; Tsaneva-Atanasova, et al., 2007; Van Goor, et al., 2001). Blockade of BK channels by paxilline or IbTX prevents plateau potentials forming in lactotrophs and somatotrophs and results in higher amplitude single spikes because failure to truncate the AP results in many DRK channels being activated (Stojilkovic, et al., 2005). Single APs are insufficient to increase global Ca^{2+}; therefore, hormone secretion is decreased. This explains the decreased prolactin in sheep grazing PRG. Reduced levels of growth hormone may relate to the reduction in liveweight gain.

3.5.2: Ill-thrift, reduced liveweight gain and a change in faecal consistency may result from immune dysfunction

Ill-thrift is a vaguely defined condition with a variety of causes, one of which is immune dysfunction. Reduced liveweight gain and faecal scouring are commonly associated with ill-thrift. The role of BK channels in innate immunity is controversial. Neutrophils kill microorganisms by ingesting them into phagocytic vacuoles and bombarding them with reactive oxygen species and enzymes (Guyton & Hall, 2000). Generation of reactive oxygen species involves movement of negative charges out of the cell into the

phagocytic vacuole and requires charge equilibration. Two equalising currents have been proposed: an H^+ current through voltage-gated proton channels (DeCoursey, Morgan, & Cherny, 2003) and a K^+ current (Reeves, et al., 2002). Ahluwalia et al. (2004) concluded that K^+ channels, rather than proton channels, provide the compensatory current and that BK channels were responsible for the K^+ efflux and are therefore essential for innate immunity. These conclusions were based on the demonstration of K^+ efflux in neutrophils, the blockade of this K^+ current by IbTX and paxilline, the localisation of BK channels in neutrophils, the lack of proton current, and the lack of microbial killing when BK channels were blocked. However, these findings are dramatically different from the prevailing dogma about neutrophil killing and the frequent demonstration of proton currents (Beswick, Brannen, & Hurles, 1986; DeCoursey, et al., 2003; Henderson, Chappell, & Jones, 1987, 1988; Rada, Geiszt, Hably, & Ligeti, 2005). Essin et al. (2007) and Femling et al. (2006) refuted Ahulwalia's findings by performing comparable studies but achieved contrasting results; antibody against BK channels did not detect immunoreactive protein in neutrophils, no BK channel current was observed and IbTX did not decrease any component of outward current nor decrease microbial killing. Although controversial, the bulk of literature is of the opinion that BK channels are not essential for neutrophil function.

Despite the apparent independence of neutrophils from BK channels function, lolitrem-B intoxication may have implications on immunity by impairing monocyte migration and their subsequent function as macrophages. Activation of endothelial BK channels is important for monocyte adhesion to endothelial cells and therefore may be important for monocyte migration from the blood into tissues (Erdogan, et al., 2007), and differentiated macrophages have been reported to express BK channels (Aiyar, 1999; DeCoursey, Kim, Silver, & Quandt, 1996). Furthermore, other fungal mycotoxins can influence immune function (Dew, et al., 1990). Therefore, effects of lolitrem-B on immunity may be associated with the observed ill-thrift, reduced liveweight gain and increased perianal faecal contamination.

3.5.3: Lolitrem-B modifies gastrointestinal motility, which may contribute to the reduced liveweight gain and increased perianal faecal contamination and provides evidence that lolitrem-B influences BK-β_1 channels

The previous section suggested that lolitrem-B may influence the immune status and thus cause ill-thrift, reduced liveweight gain and increased perianal faecal contamination, alternatively, these subclinical signs could result from modification of

gastrointestinal motility. Electromyographic recordings of the rumen, reticulum, abomasal antrum and duodenum of sheep after intravenous administration of lolitrem-B or paxilline show that these tremorgens alter gastrointestinal activity (McLeay, et al., 1999; Smith, et al., 1997). This supports earlier work, which showed that paxilline enhanced the myoelectric activity of the guinea pig ileum and the rat duodenum *in vitro* (DeFarias, Carvalho, Lee, Kaczorowski, & SuarezKurtz, 1996), as did IbTX in the guinea pig colon (Suarezkurtz, Garcia, & Kaczorowski, 1991). McLeay et al. (1999) also investigated the effects of atropine, a muscarinic receptor antagonist, on the disturbed motility of the reticulorumen in response to lolitrem-B administration and found that the induced chaotic activity was partially reduced. At the time of these studies, the molecular mechanisms of lolitrem-B had not been determined but paxilline had been shown to block BK channels (Knaus, et al., 1994; Sanchez, Wunderler, Kamassah, & McManus, 1997). McLeay et al. (1999) proposed that lolitrem-B and paxilline might block BK channels on the presynaptic terminals of the vagus and cause increased release of Ach, which binds and activates muscarinic receptor of the gastrointestinal tract. In retrospect, confirmation of lolitrem-B binding to BK channels (Dalziel, Finch, Imlach, Houssain, & Dunlop, 2005) and the role of BK channels in modulating synaptic strength (Flink & Atchison, 2003; Furukawa, et al., 2008; Liu, et al., 1999; Raffaelli, et al., 2004; Robitaille, et al., 1993; Wang, et al., 2001) support this theory. These studies indicate that lolitrem-B intoxication causes alterations in gastrointestinal motility that may subsequently disturb digestion and contribute to faecal scouring and ill-thrift.

BK channels have been shown to be involved in relaxation and repolarisation of smooth muscles in the gastrointestinal tract (Carl, Bayguinov, Shuttleworth, Ward, & Sanders, 1995), urinary bladder (Herrera & Nelson, 2002; Kobayashi, Adachi-Akahane, & Nagao, 2000; Meredith, Thorneloe, Werner, Nelson, & Aldrich, 2004; Petkov, et al., 2001), uterus (Doheny, Lynch, Smith, & Morrison, 2005), penile and clitoral erectile tissue (Gragasin, et al., 2004; Werner, Zvara, Meredith, Aldrich, & Nelson, 2005) and blood vessels (Eichhorn & Dobrev, 2007; Ledoux, Werner, Brayden, & Nelson, 2006; Sausbier, et al., 2005). BK channels of smooth muscles have β_1-subunits (Ghatta, et al., 2006). These studies and those reported in the previous paragraph demonstrate that lolitrem-B binds to BK channels of smooth muscle and thus BK channels that have β1-sununits, despite the increased affinity conferred by the β_4-subunit (Imlach, et al., 2008). Knockout mice lacking the gene that encodes the β_1-subunit (BK-$\beta_1^{-/-}$) show phenotypic characteristics of BK channel dysfunction in smooth muscles including hypertension (Brenner, Jegla, et al., 2000; Brenner, Perez, et al., 2000) and changes in

faecal consistency (Hagen, Bayguinov, & Sanders, 2003). $BK_{\alpha}^{-/-}$ mice in addition to hypertension (Sausbier, et al., 2005) show urinary incontinence (Meredith, et al., 2004) and penile erectile dysfunction (Werner, et al., 2005). Thus it might be expected that these changes will occur with lolitrem-B intoxication.

3.6: The role of large-conductance Ca^{2+}-activated K^+ channels in renal K^+ secretion.

The previous section demonstrates that lolitrem-B binds to BK-β_1 channels in smooth muscle cells, in spite of there being a predominance of neurological signs in PRGS and a higher affinity of lolitrem-B to BK-β_4 channels (Imlach, et al., 2008). The β_1-subunit is also located in the kidney (Grimm, Foutz, Brenner, & Sansom, 2007; Pluznick, Wei, Grimm, & Sansom, 2005) and is associated with BK channels that are responsible for the increase in K^+ excretion during increased fluid flow rates in the nephron tubules (Taniguchi & Imai, 1998). The following section discusses the role of BK channels in K^+ excretion and how this may be disrupted by lolitrem-B intoxication.

3.6.1: Localisation of large-conductance Ca^{2+}-activated K^+ channels in the kidney

BK channels are widely expressed in renal tissue; they have been identified in the glomerular mesangial cells, proximal convoluted tubule, medullary and cortical thick ascending limb of Henle's loop, distal convoluted tubule, connecting tubule (CNT), cortical collecting duct (CCD) and the outer medullary collecting duct (Frindt & Palmer, 2004; Grunnet, Hay-Schmidt, & Klaerke, 2005; Kudlacek, Pluznick, Ma, Padanilam, & Sansom, 2003; Morita, Hanaoka, Morales, Montrose-Rafizadeh, & Guggino, 1997). The wide distribution of BK channels throughout the nephron contrasts with sparse expression of the β_1-subunit, being confined to mesangial cells (Kudlacek, et al., 2003) and the apical membrane of principal cells (PCs) in the CNT (Grimm, et al., 2007; Pluznick, et al., 2005), and in rabbits, the initial CCD (Pluznick, et al., 2005). As structure and function are closely linked in the nephron, the localisation of BK channels provides insight into their physiological role. In particular, the location of BK-β_1 channels in the apical membrane of the PCs indicates a role in K^+ excretion.

3.6.2: The role of large-conductance Ca^{2+}-activated K^+ channels associated with the β_1-subunit in flow-mediated K^+ secretion

K^+ is predominantly an intracellular ion and has tightly controlled homeostatic mechanisms. Renal K^+ excretion normally equates to 90% of K^+ dietary intake, and the remaining intake is accounted for by colonic excretion (Giebisch, 1998; Gurkan, Estilo, Wei, & Satlin, 2007). The primary site of renal K^+ excretion is the CNT followed by the CCD (Pluznick & Sansom, 2006). Both sections contain two types of cells: PCs which reabsorb Na^+ and secrete K^+, and intercalated cells which are involved in acid-base balance (Gurkan, et al., 2007). In PCs, the basolateral Na^+-K^+-ATPase pump promotes passive diffusion of Na^+ from the tubule lumen into PCs via luminal amiloride-sensitive epithelial Na^+ (ENa) channels (Giebisch, 1998). The Na^+ influx creates a transepithelial potential difference, while the Na^+-K^+-ATPase pump generates a K^+ concentration gradient, both of which drive K^+ secretion via apical channels (Giebisch, 1998; Grimm, et al., 2007; Loffing & Korbmacher, 2009; Pluznick, et al., 2005). Two different types of apical K^+ channels have been isolated in PCs: renal outer medullary K^+ (ROMK) channels (Kohda, et al., 1998; Mennitt, Wade, Ecelbarger, Palmer, & Frindt, 1997; J. Z. Xu, et al., 1997) and BK-β_1 channels (Frindt & Palmer, 2004; Grimm, et al., 2007; Pluznick, et al., 2005). ROMK channels are Ca^{2+}-activated, but unlike BK channels they have a low conductance and are weakly dependent on depolarisation (Frindt & Palmer, 2004). The latter provides reason for their substantially higher probability of being open at RMP compared with BK channels (Frindt & Palmer, 2004; Ghatta, et al., 2006). The contrasting characteristics of these two channels imply that they have different, but complementary roles in K^+ secretion; ROMK channels allow the baseline trickle of K^+, whereas BK channels, with their large single-channel conduction, are recruited when there is increased flow through the nephron or in hyperkalaemic states (Pluznick & Sansom, 2006). Increased fluid flow through the distal tubule has been shown to increase K^+ secretion (Taniguchi & Imai, 1998). This phenomenon is termed flow-mediated K^+ secretion (FMKS) and relies on BK-β_1 channels, as revealed in knockout mice experiments. BK-$\alpha^{-/-}$ and BK-$\beta_1^{-/-}$ mice showed no response to increased flow rates (Pluznick, Wei, Carmines, & Sansom, 2003); whereas mice lacking ROMK channels showed an enhanced K^+ excretion in response to increased flow rates (M. Lu, et al., 2002) that was only reduced by BK channel blockade with IbTX (Bailey, et al., 2006). These studies confirm that FMKS occurs solely by BK channels. Therefore, there is a division of labour, with each type of apical K^+ channel contributing according to its specific properties.

3.6.3: Potential compensation by homeostatic mechanisms

Homeostatic mechanisms work to balance dietary intake of K^+ with renal and colonic excretion. Studies have shown that renal K^+ excretion is increased during hyperkalaemia by two mechanisms: an upregulation of ROMK channels in the CCD (Frindt & Palmer, 2004) and an increase in ENa channel activity in PCs of the CNT in response to the hormone aldosterone (Frindt & Palmer, 2004; Najjar, et al., 2005). Aldosterone induces transcription of genes encoding ENa channels and Na-K-ATPase pumps and subsequently alters membrane trafficking of these induced proteins and other ENa channels stored in intracellular compartments, resulting in a redistribution of ENa channels to the apical membrane (Snyder, 2005). Aldosterone also activates Sgk1 kinase, a regulatory protein that accelerates apical insertion rate and increases the probability of opening of ENa channels (Loffing & Korbmacher, 2009). The increase in intracellular Na^+ that results from increased ENa channel activity depolarises the membrane and subsequently activates BK-β_1 channels (Frindt & Palmer, 2004; Garciafilho, Malnic, & Giebisch, 1980; Pluznick & Sansom, 2006). Therefore, K^+ homeostasis may be maintained despite BK channel blockade if channels are not saturated or if ROMK channel upregulation is sufficient. Hormonal changes may indicate the activation of compensatory mechanisms, as $BK_{\alpha}^{-/-}$ mice that were fed a high K^+ diet maintained normal plasma K^+ levels, but plasma aldosterone was dramatically increased (Pluznick & Sansom, 2006). Therefore, lolitrem-B may either impair renal K^+ secretion during increased renal tubule fluid flow rates or produce compensatory changes in aldosterone.

As mentioned previously, K^+ excretion is divided between renal and colonic pathways (Giebisch, 1998; Gurkan, et al., 2007). In end-stage renal disease, colonic K^+ excretion is greatly enhanced and contributes substantially to K^+ homeostasis (Mathialahan, Maclennan, Sandle, Verbeke, & Sandle, 2005). BK channels are the only luminal K^+ secreting channels in the distal colon (Sausbier, et al., 2006) and the expression and function of BK channels are increased by aldosterone (Sorensen, et al., 2008). Therefore, blockade of colonic BK channels may further disrupt K^+ homeostasis and limit the ability to adapt to reduced renal K^+ excretion.

3.6.4: Lolitrem-B may interfere with flow-mediated K^+ secretion

Neurological signs dominate the clinical presentation of PRGS; however, localisation of BK channels in other organ systems would suggest that lolitrem-B intoxication involves

more than motor impairment. Even though the β_4-subunit has been shown to increase the affinity of BK channels to lolitrem-B (Imlach, et al., 2008), clinical effects have also been associated with BK-β_1 channels located in smooth muscle cells (McLeay, et al., 1999; Smith, et al., 1997). The locality of BK-β_1 channels in the PCs of the CNT, and their apparent necessity in FMKS, indicate that BK-β_1 channels allow for rapid adjustments in renal K^+ excretion. It is plausible that if lolitrem-B intoxication occurred concurrently with either an acute expansion in blood volume, diuretic therapy or an increase in dietary K^+ intake, that BK channel dysfunction may result in K^+ output failing to balance K^+ input, and a subsequent hyperkalaemia. The rapid dynamics of K^+ homeostatic mechanisms may compensate for BK-β_1 channel dysfunction. However, if clinical or subclinical parameters associated with either a decrease in K^+ secretion or an up-regulation of homeostatic mechanisms could be detected, these could assist the diagnosis of PRGS. Further investigation is required to define these parameters and their clinical utility.

Conclusion

Chapter 2 recognised that diagnosis of lolitrem-B intoxication in horses is currently limited to speculation due to multiple differential diagnoses, the cost involved in pasture testing, the unavailability of enzyme-linked immunosorbent assay (ELISA) tests of body fluids, the lack of well documented cases in this species and the limitations associated with extrapolation from ruminant trials. The recent demonstration that lolitrem-B binds to BK channels revealed multiple gaps in the current knowledge on the effects of lolitrem-B in the nervous system, such as the mechanisms of tremor and ataxia and the effects on auditory transmission. In addition, the ubiquitous distribution of BK channels in organ systems other than the nervous system exposes and/or confirms potential effects of lolitrem-B on renal and colonic K^+ excretion, gut motility, blood pressure, immune function and hormone secretion. By applying knowledge of the roles of BK channels other clinical effects may be recognised, which will add detail to the present clinical description and thus strengthen diagnostic capabilities.

Chapter 4: Research Design

The previous chapters identified that the description of lolitrem-B intoxication in horses lacks detail and that application of knowledge about large-conductance Ca^{2+}-activated K^+ (BK) channels could assist in the development of a clinical description. The aim of this chapter is to present the hypotheses and objectives, and to discuss and justify the research design. Research instrumentation will be explained and reasons for excluding tests presented.

4.1: Hypotheses

The current description of perennial ryegrass staggers (PRGS), based on ruminant studies, includes tremor, ataxia, limb stiffness, hyperaesthesia, cerebellar fits and subclinical reductions in milk production, liveweight gain and reproductive performance, and increased perianal faecal contamination, which occur in association with ingestion of feed that contains at least 2 ppm lolitrem-B. BK channels have multiple roles including various influences on neural excitability and smooth muscle tone, controlling the release of neurotransmitters and hormones, contributing to renal and colonic K^+ secretion and enabling temporal precision of acoustic information. BK channels also have potential roles in protecting against noise-induced hearing loss and managing macrophage function. Based on this information, two hypotheses are proposed. Firstly, that horses ingesting lolitrem-B, at levels and for an exposure duration reported as toxic for ruminants, will display signs of BK channel dysfunction that are detectable by clinically applicable tests. Secondly, that plasma and urine levels of lolitrem-B will correlate with the severity of clinical signs.

4.2: Objectives

The objective in defining the relationship between lolitrem-B ingestion and clinical signs was to develop a description of PRGS in horses that will be useful for veterinary clinicians and assist the design of future studies. In order to be applicable to veterinary clinicians, the tests included in this investigation needed to be available in veterinary clinics and not involve high costs.

As there are only two reports of controlled experiments investigating lolitrem-B intoxication in horses, little is known about the disease in this species including the individual variation in susceptibility to lolitrem-B and the toxic dose of lolitrem-B.

Considering these variables, this investigation was designed as a pilot study that would screen for the clinical effects of lolitrem-B in horses and provide a foundation for future investigations.

4.3: Brief overview of the method

Seven healthy New Zealand bred horses, five geldings and two mares aged between approximately 6 and 16 years, were obtained from North Island markets and housed in uncovered, dirt yards in Fielding, Manawatu. Horses were divided into two groups; group 1 was yarded from May 10th until May 29th, group 2 from June 6th until June 26th. Tetanus antitoxin and tetanus toxoid were administered on arrival. The horses were fed non-perennial ryegrass (PRG) feed (meadow and lucerne chaff) for a week prior to exposing them to lolitrem-B, during which time data was collected so that each horse was its own control. After the control period horses were fed endophyte-infected PRG seed and hay, containing an average level of lolitrem-B greater than 2 ppm. The test period was two weeks, based on the reported onset of 7 – 14 days in ruminants (Galey, et al., 1991). The severity of lolitrem-B intoxication displayed by each horse was evaluated daily and scores allocated according to a scoring system adapted from Galey et al. (1991) **(table 4.1)**. Horses were removed from the trial when they scored 3/5, which equates to spontaneous low-intensity tremors and incoordination at rest, and moderate to severe tremors and incoordination with handling or exercise. Daily examinations recorded clinical and neurological signs. Prior to exposure and at the end of the test period a standardised neurological examination, brainstem auditory evoked (BAEP) and magnetic motor evoked (mMEP) potentials, cerebrospinal fluid and blood sampling, and a diuretic challenge were performed. A neurological examination was also performed at the onset of signs of lolitrem-B intoxication. Onset of clinical signs was defined as the initial appearance of a change in behaviour, gait or clinical parameters previously reported in association with lolitrem-B intoxication (refer to descriptions of PRGS in ruminants in chapter 2) that were not present prior to toxin exposure or when horses scored 1/5. The Massey University Animal Ethics Committee approved all procedures used in this investigation (MUAEC Project 09/06).

Score	Clinical Signs
0	No clinical signs
1	No resting tremors or incoordination; Low-intensity tremor and incoordination with handling or exercise
2	No resting tremors or incoordination; Moderate intensity tremors and incoordination with handling or exercise
3*	Spontaneous low-intensity tremors and incoordination at rest; Moderate to severe tremors and incoordination with handling or exercise
4	Pronounced resting tremors and incoordination; Convulsive tremors and severe incoordination with handling
5	Severe spontaneous tremors and incoordination at rest, accompanied by cerebellar fits.

Table 4.1: Scoring system adapted from Galey et al. (1991), used to evaluate quantitatively the severity of clinical signs displayed by horses ingesting lolitrem-B.
* Horses were removed from the study when they scored 3/5 or greater.

4.4: Review of other methods

Previous studies have used a variety of methods to administer lolitrem-B to animals using pasture, hay, straw, seed or purified lolitrem-B. **Table 4.2** compares the form of oral administration used in different studies and the threshold levels for lolitrem-B intoxication. The table demonstrates the variability in toxicity levels between studies, which may be due to differences in susceptibility between species and breeds, grazing behaviour, the duration of exposure, and the analytical methods used. While grazing animals on endophyte-infected PRG is less intensive, less costly and closely simulates natural exposure to lolitrem-B, it provides little control over feed intake and lolitrem-B exposure as the concentration of lolitrem-B varies within the plant and between plants (Ball, et al., 1997; Gallagher, et al., 1982). Another disadvantage is the time required to establish the pasture. The other extreme involves intravenous or intraperitoneal injection of purified extracts of lolitrem-B (Gallagher & Hawkes, 1986; McLeay, et al., 1999). This method allows strict control on the dose of lolitrem-B administered and rules out any variation in absorption. However, this is not a natural method of administration and may produce clinical signs that do not usually occur with oral ingestion, reducing the applicability of results to clinical situations. Feeding endophyte-infected seed provides a less variable method of oral administration, allows greater control of individual animal feed intake and minimises feed wastage. Lolitrem-B levels

in seed remain stable during storage despite a reduction in endophyte after 15 months storage (Fletcher & Harvey, 1981). Hay and straw provide a more natural intake than seed and contain more consistent levels of lolitrem-B than pasture. Ad libitum feeding of hay or straw allows continued grazing throughout the day avoiding abnormal behaviour due to boredom. In the present investigation it was decided to feed a combination of PRG hay and seed according to maintenance requirements. This allowed lolitrem-B intakes to be reasonably controlled, while utilising natural methods of exposure and avoiding boredom. Horses were kept in sprayed yards that contained no vegetation. Group 1 was fed seed once daily and hay twice daily, while group 2 received seed and hay twice daily. PRG hay was sourced from PRG seed crop in Canterbury. The crop contained high infection levels of *N. lolii* and the hay was made after the seed was harvested, in early autumn. Sanson was the PRG seed cultivar that was used as test feed.

Table 4.2 shows that levels of lolitrem-B above 2 ppm were sufficient to produce clinical signs across all studies. It was assumed that 2 ppm lolitrem-B would be sufficient to produce clinical signs in horses. Hay and seed were randomly sampled and tested for lolitrem-B and ergovaline by high performance liquid chromatography at Grasslands Research Center, Ag Research Ltd., Palmerston North. Details on the method of high performance liquid chromatography are described elsewhere (Porter, 1995). Ergovaline levels were analysed in addition to lolitrem-B, as it is another mycotoxin produced by *N. lolii* and therefore is usually present in endophyte infected PRG. Ergovaline is a dopamine, serotonin and adrenergic receptor agonist (Larson, et al., 1995; Schoning, Flieger, & Pertz, 2001) and is reported to cause heat stress, reduced milk production, impaired immune function, lameness and lowered reproductive efficiency (Lean, 2001). Ergovaline is also believed to act synergistically with lolitrem-B and lower the toxic threshold for PRGS (Bluett, et al., 2001). The limitation of administering lolitrem-B by ingestion of naturally infected feed is that it is not possible to separate the effects of lolitrem-B and ergovaline. However, the synergistic effects of ergovaline and lolitrem-B would occur in most natural exposures due to the co-production of these mycotoxins by *N. lolii*.

Table 4.2: Previous clinical studies of PRGS demonstrating the levels of lolitrem-B that were sufficient to produce clinical signs. Shaded boxes indicate studies where lolitrem-B levels were greater than 2 ppm, which was sufficient to produce clinical signs across all studies.

Reference	Species	Feed	Lolitrem-B (ppm)	Morbidity (%)
Munday et al. (1985)	Horse	PRG seed cleanings	5.3	Not reported
Blyth et al. (2007)	Cattle	Chopped PRGS straw	0.0	0
			1.4	0
			2.0	47%
			2.6 - 3.1	100%
Fisher& Barrel (1984)	Cattle	PRG Straw	< 1.6	0
		Pasture	2.0	54%
Di Menna et al. (1992)	Lambs	Pasture	< 1.6	0
		Pasture	2.0 – 2.5	30 – 100%
Miyazaki et al. (2004)	Cattle	Straw: 6.5 kg/day	1.2	100%
		Straw: 3 kg/day	1.2	0
Galey et al. (1991)	Herd A (1989): Cattle	Pasture	0.25 *	'Several' out of herd of 50.
	Herd A (1990): cattle and sheep	Pasture	0.55	Present
	Herd B: sheep	Pasture	0.27 *	50%
	Herd C: sheep	Pasture	3.0	~20%
	Herd C: Cattle	Pasture	3.0	20%

* Pasture samples taken from upper portion of the plant and did not include basal plant material.

4.5: Research instrumentation

4.5.1: Clinical examination

Clinical examinations were performed daily and recorded both subjective features such as behaviour, demeanor, respiratory character, mucus membrane colour, capillary refill time, gastrointestinal sounds, strength of digital pulses, faecal consistency and urination posture and stream characteristics; and objective measures such as heart rate, respiration rate and temperature. **Appendix 1** contains the daily clinical examination form. The purpose of daily clinical examinations was twofold: to detect any clinical changes that may be associated with lolitrem-B intoxication and to monitor the health of the horses to minimise confounding illnesses. Horses were also lunged daily to assess the severity of signs in response to exercise.

4.5.2: Neurological examination

Neurological examination involves a series of stimulations and requested tasks. Observed abnormalities in the accuracy, amplitude, timing, regularity, repeatability and symmetry of the horses' response allows characterisation and localisation of any neurological dysfunction. A standardised neurological examination, including gait analysis, blindfolding and an ophthalmic examination (Mayhew, 2009), was performed prior to exposure, at the onset of signs and at the end of the test period.

Neurological examination was performed in a quiet, naturally lit stall and gait analysis in an outside, sand-arena. Horses were initially examined in their stall from a distance – behaviour, mentation, body symmetry and movement was noted. Horses were then restrained with a halter and cranial nerve function was tested including an assessment of facial symmetry, facial sensation, the menace response, eye position and pupil size, and the pupillary light response. These tests were followed by an ophthalmic examination. The external thoracolaryngeal reflex was performed, followed by palpation of the neck for bone and muscle symmetry and the local cervical and cervicofacial responses examined by stimulation with blunt artery forceps. Sensation tests were continued caudally to the thorax including the cutaneous trunci reflex, which was followed by an assessment of flexion, extension and lateral bending of the thoracolumbosacral vertebral column by firmly stroking the lateral dorsum from the withers to the caudal gluteal region using blunt artery forceps. Finally tail tone, the anal reflex and perineal sensation was examined. Horses were then lead through a series of tasks to assess gait and posture: walking in a straight line, in a serpentine manner, with their head elevated and walking backwards; tight circling in both directions and while simultaneously pulling on both the lead rope and tail to assess strength of

resistance; abrupt release of the tail at cessation of tight circling; hopping on each thoracic limb; and assessing resistance to lateral tension applied to the tail while the horse was stationary and while walking forward. A towel tucked into each side of the halter was used to blindfold horses and then some of the tasks were repeated. Walking, trotting and cantering gaits were also observed on the lunge. Observations from the neurological examinations were recorded in writing and with video. A neurological examination form is included in **Appendix 2**.

4.5.3: Complete blood count and serum biochemistry

Blood was collected from the jugular vein after clinical examination but prior to any other procedure. The jugular furrow was clipped, scrubbed with dilute chlorhexidine and sprayed with methylated spirits prior to collection. A total of 10 mL, 20 mL and 30 mL of blood was collected into 10 mL plain, heparinised and ethylenediaminetetraacetic acid (EDTA) blood tubes, respectively. Samples were stored upright in a chilled container for approximately 8 hours. After which, the plain tube and one of the EDTA blood tubes were delivered to New Zealand Veterinary Pathology Ltd., Palmerston North, for heamatology and serum biochemistry analysis, while the remaining samples were centrifuged (at 5000 rpm for 5 min) and the plasma pipetted into four 2 mL aliquot microvials, which were then stored at -20^0C.

4.5.4: Cerebrospinal fluid collection

Cerebrospinal fluid samples were collected by lumbosacral puncture and, after gross analysis, were stored for future analysis in a separate project. The landmark for lumbosacral puncture was where the transverse line that joined the caudal ventral portion of the tuber coxae and the cranial border of the tuber sacrale intersected the dorsal median plane, which was defined as the midpoint between the right and left tuber sacrale. When palpable, the caudal border of L6 and the cranial edge of S2 were used as the cranial and caudal borders, respectively. The procedure was done with horses standing and lightly sedated. Sedations included acepromazine (0.05 – 0.1 mg/kg iv) or a combination of butorphanol (0.01 mg/kg iv) and detomidine (0.01 mg/kg iv). The site was clipped and surgically prepared with repeated scrubs using chlorhexidine followed by methylated spirits spray. 1 – 2 mL of local anaesthetic (lidocaine 2%) was injected subcutaneously to form a bleb and an additional 2 – 3 mL was injected deeper down the median fascia. A 1 cm skin incision was made at the site prior to penetration with the spinal needle. A 6-inch, 18-gauge, thin-walled, spinal needle with a fitted stylet was used to enter the epidural space. The initial collection of cerebrospinal fluid was discarded until any discolouration had cleared. Clear

cerebrospinal fluid samples were separated into four 2 mL aliquots in screw-capped cryovials and stored in chilled containers for approximately 3 hours prior to storage at -80^{o}C. As analysis will be performed in a future investigation, only gross observations are reported and discussed.

4.5.5: Brainstem auditory evoked potentials

BAEPs record electrical waveforms generated in the auditory pathway through the brainstem to the level of the midbrain following repeated, electrically-induced auditory clicks applied via inserted ear-pieces. Needle electrodes detect the generated waveform. The recording equipment receives and amplifies electrical potentials that occur during the first 10 ms after each click. By averaging the signals it removes random brainwave and muscle potentials, leaving only the short-latency electrical signals that are evoked by each auditory click stimulus. The waveform of these signals contains five characteristic peaks in horses (Mayhew & Washbourne, 1990), which reflect the synchronous activity of auditory cell populations in particular anatomical locations (Biacabe, Chevallier, Avan, & Bonfils, 2001). However, many brainstem auditory structures are simultaneously activated by an acoustical stimulus and the BAEP waveform appearance depends on the summated activity of more than one anatomical site (Biacabe, et al., 2001; Mayhew & Washbourne, 1990).

BAEPs are used in horses to evaluate the integrity of the auditory pathways. Uni- and bi-lateral deafness can be detected by the lack of identifiable peaks. The latency and amplitude of characteristic peaks are indicators of auditory function, allowing partial deficits in auditory transmission to be detected. Reference values for horses and ponies have been determined (A. E. Marshall, 1985; Mayhew & Washbourne, 1992, 1997). The close anatomical proximity of the vestibular and auditory pathways enables BAEP to indirectly test vestibular function in the case of morbid lesions involving both the vestibular and cochlear components of cranial nerve VIII and their brainstem connections (Bedenice, Hoffman, Parrott, & McDonnel, 2001).

In chapter 3, the roles of BK channels in auditory function were discussed. It is plausible that lolitrem-B binding to BK channels of the inner and outer hair cells (IHCs and OHCs), the stria vascularis and the auditory afferent nerve may cause temporal imprecision but is unlikely to reduce the sensitivity and specificity of the auditory system to sound frequencies. As the BAEP waveform relies on the ability of neurons to maintain precise timing and respond synchronously to external stimuli, an increased variance of nerve firing may be reflected by dispersion of the BAEP trace (Oliver, et al., 2006). Latency values may also be delayed as the BAEP of knockout mice lacking the

gene that encodes the α-subunit of BK channels ($BK_{\alpha}^{-/-}$) show a delay in the first peak latency (Pyott, et al., 2007). The BAEP is a non-invasive, safe, inexpensive and easy to perform diagnostic procedure that requires minimal restraint and provides objective information regarding the functional status of the auditory system. The test is ideally suited to research designs where the same horse is used in both control and treatment periods, in that variation within individuals is minimal even over a period of time (Mayhew & Washbourne, 1990).

Horses were placed in a quiet stall and the procedure was carried out with no or minimal sedation – depending on horse cooperation, as for cerebrospinal fluid collection. It is reported that BAEP traces are not affected by sedatives that are normally used in horses (A E Marshall, Byars, Whitlock, & George, 1981; Mayhew & Washbourne, 1990). An ear-piece was inserted in the vertical ear canal and secured with cotton wool and tape wound around the pinna. Traces were recorded using 12 mm, stainless steel, sub-dermal needles; the active electrodes were placed at the base of each ear on the zygomatic process of the temporal bone both ipsilateral and contralateral to the ear receiving the click stimulus; the reference electrode was placed in the frontal region where lines from the eyes to the base of the opposite ear cross (the vertex); and the ground electrode was placed over a bony prominence of the head. These positions have been found to give consistently maximal waveforms that do not change perceptibly with minor changes in positioning (Mayhew & Washbourne, 1997). A 100 μs broadband acoustic click stimulus was applied into the external auditory meatus through the inserted ear-piece and the inserted electrodes recorded the resultant brain electrical activity. The click stimulus was repeated at a frequency of 19 Hz and the response was signal-averaged to obtain a smooth trace on the electodiagnostic computer programme (Medelec Synergy M-2 EMG, LIFEHEALTHCARE, Artarmon, NSW, 2064, Australia). The process was repeated to obtain two traces per horse.

BAEP thresholds were not sought in this study because the main purpose was to screen for BAEP abnormalities related to temporal precision rather than threshold sensitivity. Maximal stimulus intensity (80 – 100 dB, NHL) was selected to compensate for conditions that could induce conductive hearing loss, such as debris in the ear, as a decrease in stimulus intensity causes peak latencies to increase (A. E. Marshall, 1985).

Vertex positive peaks III, V, and I were identified to the best of the authors ability using superimposed replicate tracings to aid in labeling, for both ipsilateral and contralateral traces. Where a peak was not well defined, a midpoint of the positive deflection was estimated for peaks I and III, whereas V was defined as the point immediately prior to

the prominent negative trough in the region of 4 – 7 ms. A comparison was made between the latency for peak V before and after exposure to lolitrem-B by paired t-test. An absence of identifiable BAEP peaks in traces obtained from a horse either before or after exposure to lolitrem-B, excluded the pair of traces from further analysis as latencies could not be determined and compared. The inbuilt delay for the time taken for the auditory stimulus to travel the length of the ear-tip insert tube, and the unknown delay for the remaining air-space within the external auditory meatus was assumed to be consistent so no correction was made to the latency measurements.

4.5.6: Magnetic motor evoked potentials

Magnetic motor cortex stimulation evokes synchronized, descending, excitatory volleys in neural motor pathways and evaluates the functional integrity of the fastest conducting descending motor fibres in the brain and spinal cord (Nollet, Deprez, Van Ham, Verschooten, & Vanderstraeten, 2002). As BK channels are involved in action potential (AP) repolarisation and afterhyperpolarisation (AHP), resting membrane potential (RMP), frequency of AP firing, neurotransmitter release and muscular excitability (Ghatta, et al., 2006; Salkoff, et al., 2006), blocking these channels may change the characteristics of the mMEP trace. A rapidly changing magnetic field, generated by passing an electrical current pulse through a coil of wire, induces electric eddy currents in nearby conductive structures (Nollet, Van Ham, Deprez, & Vanderstraeten, 2003). The induced current flows perpendicularly to the magnetic field and circulates up to a few centimeters away from the coil's external edge (Nollet, Van Ham, Deprez, et al., 2003). Therefore, placing the coil of wire on the median of the horse forehead induces transcranial stimulation of the motor system (Nollet, Van Ham, Deprez, et al., 2003) at the frontal cortex and potentially deeper areas of the brain (Mayhew & Washbourne, 1996). The momentary depolarisation of nerve membranes in the region that is stimulated initiates APs that then propagate by normal methods of nerve conduction to the lower motor neuron (LMN) to induce muscle contraction (Nollet, Van Ham, Deprez, et al., 2003). The electromyographic response is a recording of the electrical activity in the motor units that surround the active electrodes, which are inserted over the muscle belly.

The procedure is not painful as current passes the extracerebral layers with minimal or no activation of the pain receptors (Nollet, Van Ham, Deprez, et al., 2003). Therefore, horses were given sedation only if they were agitated by the procedure, as for cerebrospinal fluid collection. Acepromazine, butorphanol and detomidine cause no significant difference in the mMEP trace (Mayhew & Washbourne, 1996; Nollet, et al., 2002). An electromagnetic 15 cm outside diameter stimulating coil (Digitimer D190;

Digitimer Ltd., Welwyn Garden City, UK) with a maximum induced field of 1.5 T at the surface of the coil was placed over the vertex of the forehead. This position produces the shortest latency in horses (Nollet, Van Ham, Dewulf, Vanderstraeten, & Deprez, 2003). Unlike humans, the direction of current flow in the coil has no influence on the mMEP trace in horses (Nollet, Van Ham, Dewulf, et al., 2003), so the orientation of the coil was not controlled. The stimulation output was initially set to about 20% to accommodate the horse and was then increased to obtain a clear recording. Electromyographic recordings were obtained from electrode needles. Active electrodes were inserted subcutaneously over the middle of the muscle belly – the extensor carpi radialis for forelimb recordings and the tibialis cranialis for hindlimb recordings. The reference electrode was placed subcutaneously on the lateral side over the radial tuberosity of the forelimb and the lateral malleolus of the hindlimb. In the forelimb, the ground electrode was place subcutaneously on the elbow, in the hindlimb it was placed on the groin. Responses produced a trace in the electrodiagnostic computer programme (Medelec Synergy M-2 EMG, LIFEHEALTHCARE, Artarmon, NSW, 2064, Australia). Both take-off and first-peak latencies were measured from the trigger point to the initial (negative or positive) deflection and to the highest point on the first positive peak, respectively. The latencies of traces recorded before and after exposure to lolitrem-B were compared using the paired t-test. As with BAEP recordings, if either the pre- or post-exposure traces from a horse were unable to be interpreted, the pair of traces was excluded from paired t-test analysis.

4.5.7: Frusemide challenge

Chapter 3 discussed the role of renal BK-β_1 channels in flow-mediated K^+ secretion (FMKS) and suggests that dysfunction of renal BK-β_1 channels would result in either reduced K^+ secretion during acute expansion in blood volume, diuretic therapy or an increase in dietary K^+ intake, or changes in aldosterone levels. The frusemide challenge was designed to challenge the renal BK-β_1 channels with an increase in urine flow and tubular pressure and adapted the method used to investigate the relationship between renin and aldosterone in horses (Guthrie, Cecil, Darden, & Kotchen, 1982). Frusemide, a loop diuretic, was administered and the ability of the nephron to increase excretion of K^+ was measured by calculating the fractional excretion of K^+ (FEK^+) sequentially. Hypothetically, lolitrem-B blockade of renal BK channels would diminish the FMKS response. Fractional excretion of Na^+ ($FENa^+$) was also determined. BK channel dysfunction may be compensated for by the actions of aldosterone; therefore, plasma aldosterone was also measured sequentially.

Horses were sedated with acepromazine (1mg/kg iv), as this sedative is not reported to have a diuretic effect (Plumb, 2005). Samples collected included duplicates of heparinised and EDTA jugular blood samples, and urine collected via bladder catheterisation; these were collected prior to intravenous frusemide administration of 1 mg/kg (Salix, Intervet ® 50 mg/mL frusemide) and at approximately 15, 30, 60 and 120 minutes post frusemide administration. These times were selected based on the pharmacokinetics of frusemide, which has peak activity at 15 – 30 minutes post intravenous administration and induces diuresis for a duration of approximately 2 hours (Tobin, Roberts, Swerczek, & Crisman, 1978). The precise time of sample collection relative to frusemide administration was recorded. After urine samples were collected the bladder was allowed to drain until apparently empty. On completion of the challenge, 10 L of water was administered via nasogastric intubation to replace the diuretic-induced fluid deficit. The volume of 10 L was chosen based on the report that urine production averages 14.3 L in 8 hours after IM injection as opposed to 2.6 L for control horses and that the diuretic effect is more profound when administered via intramuscular injection compared with intravenous administration (Hinchcliff & Muir, 1991). Trimethoprimsulfa (30mg/kg, po, BID) was administered for three days for prophylaxis against urinary tract infection.

Heparinised plasma was centrifuged (at 5000 rpm for 5 min) immediately after collection and separated plasma was stored in a chilled container, along with the urine samples, prior to delivery to New Zealand Veterinary Pathology Ltd., Palmerston North, for electrolyte determination. EDTA tubes were stored upright in a chilled container, centrifuged and the plasma separated and stored at -20^{o}C prior to aldosterone analysis at the Christchurch Cardioendocrine Research Group. Due to limited resources, one of the replicate samples was chosen at random for analysis of FE and aldosterone. Fractional excretions were calculated for Na^{+} and K^{+} using the following equation:

$$FE_x = \frac{[X]_{urine}}{[X]_{plasma}} \times \frac{[Creatinine]_{plasma}}{[Creatinine]_{urine}}$$

In New Zealand, no aldosterone assay is available for horses, but radioimmunoassays for sheep and human plasma are available. The sheep assay was chosen because it is a straightforward aldosterone assay, whereas the human assay has been specifically modified to account for other cross-reacting steroids present in human plasma. Additionally, plasma dilutions were performed to assess the fit of horse samples to the standard curve for both the human and the sheep assays. The horse samples had a closer fit to the sheep assay than the human assay.

Dietary levels of K^+ can prime or downregulate homeostatic controls of K^+ excretion (Grimm, Irsik, Settles, Holtzclaw, & Sansom, 2009; Pluznick & Sansom, 2006; Rieg, et al., 2007; Sausbier, et al., 2005); therefore, K^+ levels in the feed were analysed. Duplicate samples composed of 10 random grab samples from the morning feed were collected and stored in airtight chilled containers prior to analysis. The level of K^+ was determined by inductively coupled plasma optical emission spectrometry (Animal Nutrition, Institute of food, nutrition and human health, Massey University, Palmerston North).

4.5.8: Plasma and urine lolitrem-B levels

Frozen aliquots of heparinised plasma and urine, collected prior to frusemide administration, were analysed at Ag Research Ltd., Ruakura, for lolitrem-B by enzyme-linked immunosorbent assay (ELISA). Urine and faecal lolitrem-B levels have been determined using ELISA in ruminants (J. Sprosen, Ag Research Ltd., Ruakura, pers. comm. May 2009) but have not been used previously in horses. Horse plasma has been analysed but not in a research context. The object of ELISA determination of lolitrem-B levels in body fluids was to compare the change in levels that occurred with lolitrem-B exposure and to examine the correlation of values to the severity of clinical signs displayed by individual horses.

4.6: Tests that were excluded

BK channels are involved in many organ systems. The experiment was designed to encompass these within the limits of clinical expediency. This excluded tests that use equipment that is unavailable to clinics, require general anaesthesia, or are highly invasive. Kinematic analysis of body movements has been used as an objective measure of ataxia (Ishihara, Bertone, & Rajala-Schultz, 2005; Keegan, et al., 2004). However, treadmills or force plates are used to collect the kinematic data and this equipment is not available to the majority of veterinary practitioners, research establishments and hospitals. Activation of BK channels in vascular smooth muscle and endothelium have been shown to oppose vasoconstriction (Eichhorn & Dobrev, 2007). This role of BK channels is supported by the common hypertensive phenotype of $BK_{\alpha}^{-/-}$ mice (Sausbier, et al., 2005) with reports of a 10% increase of arterial blood pressure (Sausbier, et al., 2004). However, an accurate assessment of blood pressure in the horse requires direct arterial catheterisation. Therefore, measuring blood pressure was excluded. Other testing that was excluded were electrophysiological tests of gastrointestinal motility and post mortem examination. As discussed in chapter 2 and 3, prolactin has been shown to decrease in sheep that have signs of PRGS

(Fletcher & Barrell, 1984), and cattle and sheep with ergovaline toxicity (Hovermale & Craig, 2001; Waghorn, et al., 1994). However, prolactin levels were not investigated based on the large variation of the hormone between mares and geldings, during stages of the estrus cycle and following daily temperature changes and differing daylight hours (Thompson, Depew, Ortiz, Sticker, & Rahmanian, 1994); the research method used would not be appropriate to gain significant results as contemporary controls would be required. In addition, the influence of ergovaline on prolactin levels could not have been separated from the effects of lolitrem-B.

4.7: Statistical analysis

Computer software, Minitab 15, was used to construct graphs and to perform statistical tests. Data collected prior to and after exposure to lolitrem-B was compared by the paired t-test, with data from each horse composing pairs of data. The mean change in data values for each temporal group (Group 1: May 10 – 29; Group 2: June 6 – 26) was compared using the unpaired 2-sample t-test. One-way ANOVA was used to compare the difference between severity groups according to the PRGS severity grade assigned to each horse on the last day of lolitrem-B exposure. The null hypothesis of the paired t-test stated that there is no difference between data collected prior to exposure and at the end of the test period. The null hypothesis for one-way ANOVA and the unpaired t-test was that the mean between any of the groups are equal and the alternative hypothesis was that there are one or more groups that have a mean that differs from the other groups. It was recognised that the small sample size of seven horses limited the statistical power; however, results were intended to indicate areas that are worthy of further investigation rather than to prove differences.

Conclusion

To test the hypothesis that horses display clinically detectable signs of BK channel dysfunction when ingesting lolitrem-B at levels and for a duration period reported as toxic to ruminants, neurological and clinical examinations, blood and cerebrospinal fluid sampling, electrophysiological tests, and a frusemide challenge test were performed on horses prior to and two weeks after ingesting controlled amounts of seed and hay containing on average more than 2 ppm lolitrem-B. The correlation between plasma and urine levels of lolitrem-B and the severity of clinical signs was assessed by analysing plasma and urine for lolitrem-B using ELISA techniques and these values were compared to the final grade of severity of PRGS assigned to individual horses.

Chapter 5: Results

The trial was designed to test the hypothesis that horses ingesting lolitrem-B at levels greater than 2 ppm will display clinically detectable signs of large-conductance Ca^{2+}-activated K^{+} (BK) channel dysfunction and that lolitrem-B levels in plasma and urine would correlate to the severity of these signs. During the treatment period horses displayed clinical signs that have previously been reported in association with perennial ryegrass staggers (PRGS) and appeared to fully recover within a week of completion of the trial period.

During the testing period horses ingested a mean of 36 ± 10 and 46 ± 13 μg/kgBW of lolitrem-B each day in group 1 and group 2 respectively. These dose rates equate to a mean of 2.1 ± 0.6 and 2.2 ± 0.6 ppm lolitrem-B, respectively, which compare with toxicity levels reported in ruminant studies **(Appendix 3).** Feed ingested by group 1 contained 2.0 ± 0.4 ppm ergovaline, while group 2, who were feed seed twice a day, ingested levels at 3.6 ± 0.7 ppm due to the high content of ergovaline in the seed (**Appendix 3)**.

Low levels of lolitrem-B were detected in the meadow chaff that was consumed during the control period (mean lolitrem-B content of 0.7 ± 0.3 ppm, n=3). One study reports PRGS in cattle and sheep grazing pasture with levels as low as 0.25 and 0.27 ppm, respectively (Galey, et al., 1991). However, in this study lolitrem-B analysis was performed on the upper part of the plant and did not include basal plant material and therefore, may have underestimated the amount of toxin ingested. In addition, no clinical signs that have been associated with PRGS were observed during the control period. Therefore, data can be reliably compared between the control period and the period of lolitrem-B exposure.

5.1: Clinical description

During the treatment period horses displayed signs of neuromuscular and neurosensory dysfunction. The onset of clinical signs ranged from 4 – 9 days after initiation of exposure. The initial signs that marked the onset included rapid (~5 Hz) muscle fasciculation, truncal sway, an increase in stance width and slowed movement. Muscle fasciculation is defined as involuntary muscle contractions that do not affect movement of a body part or segment. These appeared as fine movements of the hairs across the shoulder and quivering of the mane and tail head. Fasciculations increased in amplitude to a tremor involving the proximal forelimb musculature after exercise and feeding. At rest, horses showed a low amplitude sway of the trunk, either in a lateral or

a craniocaudal direction. The lateral sway was most evident by observing the changing angle at the fetlock as it flexed and extended with the changing distribution of the horses' weight. During gait analysis horses adopted a wide-based stance **(fig. 5.1)** and movement was slowed**.**

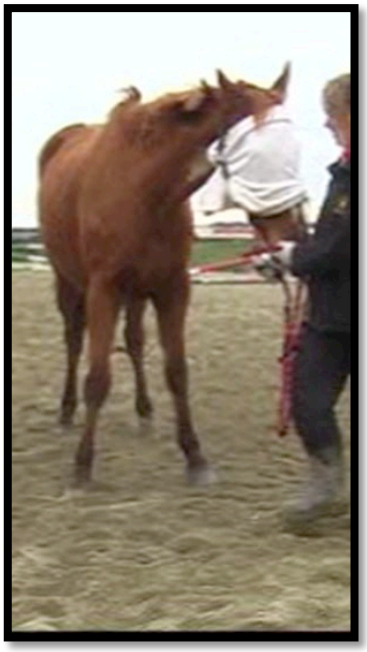


Fig. 5.1: Horse #3, day 12 exposure, displays a wide-based stance that was exaggerated with blindfolding.

Despite the controlled ingestion of about equal doses of lolitrem-B there was significant variation in the severity of signs displayed at the conclusion of the exposure period. Horse #1 and #2 were graded 3/5 on day 9 exposure and were subsequently removed from the study. The remaining horses were exposed for 12 – 14 days; three horses scored 2/5 on the final day of exposure while the remaining two scored 1/5. The severity of signs did not correlate with breed, age, sex or size **(table 5.1)**.

All horses displayed tremor; however, aspects of the tremor characteristics varied between horses and within the individual depending on the circumstances. The forelimb musculature was the predominant anatomic location, with initial fascicular movements of the shoulder and pectoral muscles **(DVD 1)** progressing to involve entire muscle groups with clonic spasms causing rapid flexion and extension of the forelimb (~3 Hz, as measured from real-time video), which in slow motion appeared as a ripple of flexion ascending the limb **(DVD 2)**. Forelimb spasm was frequently noted during eating, after lunging or during complex maneuvers when there was a change in direction or a pause in movement. During these tremors, the carpus buckled during the flexion phase, but the heel bulbs tended to remain on the ground unless the amplitude of the spasm was severe, in which case heels were raised or the foot was lifted and replaced. These forelimb tremors were most noted in the limb bearing least weight with a similar degree of tremor observed in both forelimbs when standing square. Tremor of the left and right thoracic limbs was reciprocated, in that the right limb would flex while the left was extended. However, a 1:1 ratio was not observed, rather an irregular sequence. Involuntary muscle movements were also observed at the flank and over the thorax, particularly in horse #5. At the flank they appeared as irregular vermiform movements as though receiving punches from underlying structures at a frequency of 0.5 – 2 Hz. Over the ribs the movement of the overlying skin was more undulating and wave-like with ripples of muscle contractions (i.e. Myokymia) **(DVD 3)**.

Using an ophthalmoscope it was possible to detect a subtle, rapid (~5 Hz) continuous tremor of the eyeball in six of the seven horses.

Table 5.1: Identification of horses, onset of clinical signs of lolitrem-B intoxication and severity grade assigned on the final day of exposure to lolitrem-B.

	Horse #	Age (years)	Breed	Sex	Weight (kg)	Onset (days)	Grade
Group 1	1	16	TB	Gelding	496	5	3/5
	2	6	SB	Gelding	546	5	3/5
	3	10	TB	Mare	507	4	2/5
	4	7	TB	Gelding	484	6	1/5
Group 2	5	17	TB	Mare	480	5	2/5
	6	9	SB	Gelding	405	9	1/5
	7	14	TB	Gelding	503	5	2/5

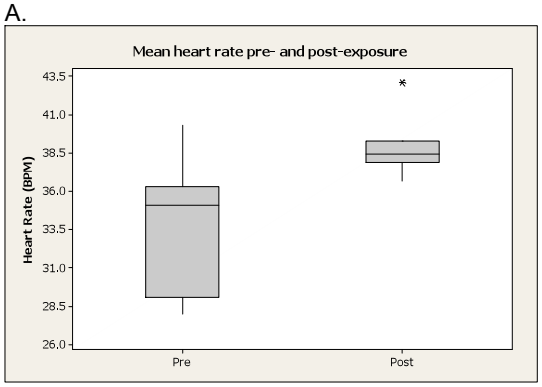
Initial signs of ataxia included a truncal sway at rest and a slowed gait with wide limb placement and an adductive dishing during the protraction phase, particularly when blindfolded or with elevation of the head **(fig. 5.1 and DVD 4).** Ataxia was dramatically exaggerated when horses were blindfolded **(DVD 5).** Occasionally there was hesitancy at the onset of movement, but movement progressed in a smooth manner. When walking in a straight line with minimal pull on the halter the trunk would sway, drift and lean to either side. Movement of the trunk appeared as an exaggeration of the normal sway of the trunk that occurs as weight is transferred for limb protraction. However, there was a delay in the reciprocal sway and the center of gravity remained lateral while limbs were placed. The direction of the truncal sway was followed, and in part compensated for, by foot placements; therefore, although foot placements were irregular they contained a predictable component as they moved according to the change in the center of gravity that was caused by the truncal drift **(DVD 5).** During complex maneuvers, such as tight circling and backing, leaning of the trunk often caused malequilibrium and required rapid, stumbling, irregular movements of the limbs to stop a fall **(DVD 5).** Occasionally during tight circling the outside hindlimb was circumducted, but did not involve hypermetria **(DVD 5)**. Horses that were more severely affected and graded 3/5 showed an increased occurrence of postural deficits during complex maneuvers with a blindfold, such as delayed protraction of the hindlimbs, circumduction of the outside hindlimb when circling and interference with contralateral limbs. In addition their gait became jerky, interrupted and more irregular. However, hypermetria was not characteristic of the ataxia displayed. After an abrupt cessation of rapid movements, horses would stand with abnormal limb positions **(fig.**

5.2), correcting foot placement only after a substantial delay or when a following movement was initiated **(DVD 6).** When led in a serpentine pattern, truncal sway was reduced and horses responded appropriately to the abrupt changes in the direction of halter pull by changing the direction of limb movement mid-protraction **(DVD 7)**. Weakness was not demonstrated by the tail pull, as horses showed strong resistance to tension applied to the tail while stationary and during movement **(DVD 8)**. During hopping on the thoracic limb, horses responded appropriately to the transfer of weight and did not display a tremor or buckling of the weight-bearing limb **(DVD 8),** which would have been taken as indicating weakness. No horses fell or became recumbent during the trial. No change occurred in cranial nerve function or body examination except for the aforementioned eyeball tremor and an increased fright response to the withers slap involved in the thoracolaryngeal test. In the latter, horses would suddenly tense or startle in response to either a slap on the withers or a threatening gesture even though these procedures had been performed numerous times previously (**DVD 9)**. Horses also showed a tendency to startle or hesitate when being led through gateways **(DVD 9)**.

Fig. 5.2: After abrupt cessation of movement, horses showed abnormal awkward limb placement that was not corrected until after a delay or when a subsequent movement was initiated. Left to right: Horse #2, day 5 exposure; Horse #2, day 9 exposure; Horse #3, day 12 exposure; Horse #4, day 12 exposure.

Appendix 4 displays the change in heart rate, temperature and respiration for individual horses and the distribution of mean values. Daily clinical examinations revealed that heart rate increased significantly (p = 0.018, paired t-test of means); however, did not dramatically exceed normal values consistently **(fig. 5.3 A and B)**. No difference in heart rate was observed between horses grouped according to PRGS severity scores (p = 0.938, ANOVA). Temperature and respiration rate showed no significant change (P = 0.51 and 0.348 respectively, paired t-test of means) **(fig. 5.3 C and Appendix 4)**.

A.



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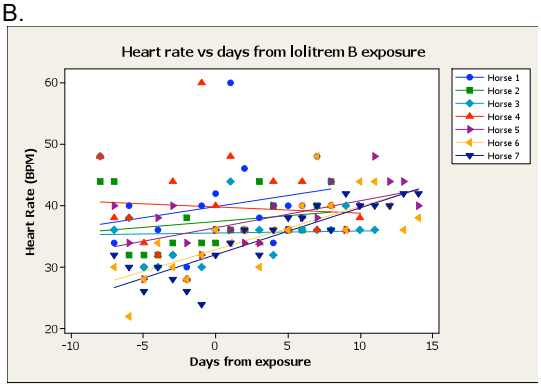


C.

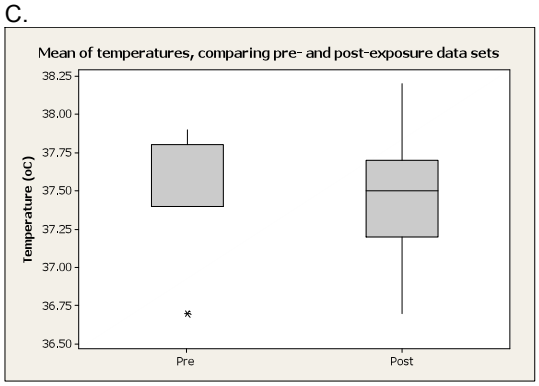


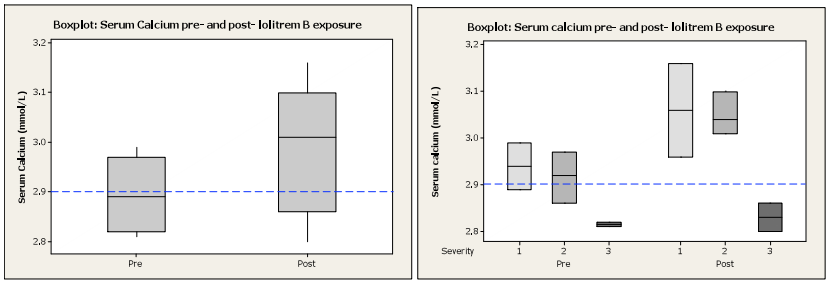
Fig. 5.3: Heart rate increased significantly during the period of lolitrem-B exposure but temperature did not show any substantial change: A. Boxplot showing the distribution of mean heart rate during the pre- and post-exposure periods; B. Scatterplot of heart rate data showing the regression lines that have a positive gradient in all horses except horse #4; C. Boxplot showing the unsubstantial change in distribution of mean temperature between the pre- and post-exposure periods.

Despite ingesting a dry diet, faecal consistency was noted to soften and become more fluid in two of the horses over the period of lolitrem-B exposure. However, no change was detected on gastrointestinal auscultation. Horse #1 and #2 developed swelling and heat in all limbs on day 9 exposure, resulting in loss of definition of flexor tendon and edema in the distal limb. In addition, on day 9 exposure horse #1 developed raised, granulated, fissured lesions around the heel bulbs of all feet reaching proximally to the distal pastern on the planter surface. Lesions oozed large amounts of dark yellow, serous discharge. This horse also showed profuse, dark yellow, serous, nasal discharge from a rhinitis that had been present throughout the trial. Once exposure to lolitrem-B ceased limb swelling reduced, heel lesions began to heal and nasal discharge ceased. Mild rhinitis was intermittently present in all horses over the control and treatment periods.

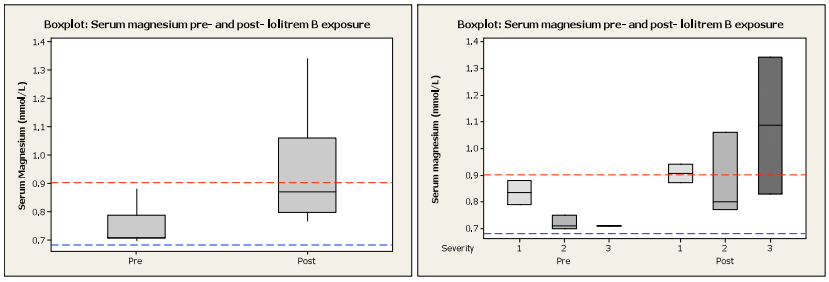
5.2: Complete blood count, serum biochemistry and gross cerebrospinal fluid analysis

In accordance with previous studies (Cunningham & Hartley, 1959), no significant difference was observed in complete blood count values. Serum biochemistry did reveal some statistically significant findings. Raw data values are included in **Appendix 5**. Biochemistry values are independent of frusemide, as samples were taken prior to frusemide administration. Total serum calcium was increased (p = 0.011, paired t-test) and total serum magnesium showed a strong upward trend (p = 0.055, paired t-test) but neither showed a significant difference between severity grades **(fig. 5.4 A and B)**. Total serum calcium remained below

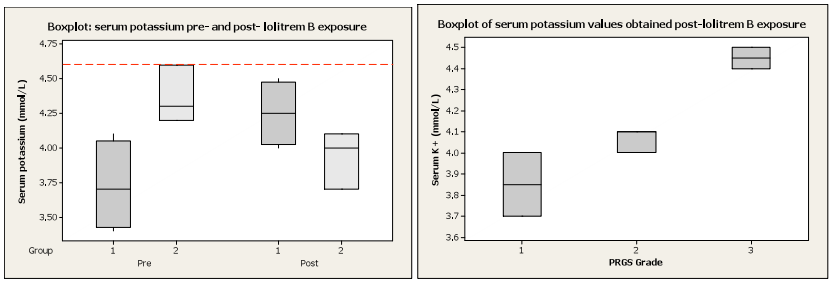
Fig. 5.4: Serum biochemistry results



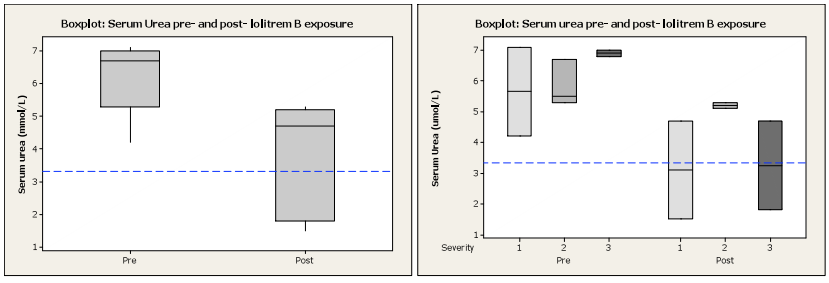
A. There was a significant increase from the pre- to post-exposure values for total serum calcium (p = 0.011, paired t-test), but values remained below the upper reference range (3.3 mmol/L). There was a strong trend for total serum calcium post-exposure values to be lower in horses showing more severe signs with a potential difference in means between the severity groups (p = 0.071, ANOVA). However, this trend was also present before exposure. Severity groups refer to the grade that horses were assigned at the completion of the treatment period according to the scoring system outlined in **table 4.1.**



B. There was a strong trend for total serum magnesium to increase post-exposure (p = 0.055, paired t-test), with some post-exposure values exceeding the upper reference range (0.9 mmol/L: red dotted line). However, there was no significant correlation between the change in serum magnesium and the severity of PRGS observed.



C. No significant difference in serum K^+ between pre- and post-exposure was observed (p = 0.627, paired t-test), but there was a strong temporal group effect (p=0.011, ANOVA) with group 1 increasing, while group 2 decreased. There was also a trend for post-exposure K+ to increase as the severity of clinical signs increased. However, values remained within the reference range (3.0 – 4.6 mmol/L).



D. There was a significant trend for the urea values to decrease post-exposure (p = 0.018, paired t-test), some values falling below the reference range (blue dotted line). Despite a substantial difference between the mean change in urea levels in each severity group (p = 0.094, ANOVA), this change was greatest in groups that scored 1/5 and 3/5 and least in those that scored 2/5. Therefore, the decrease in urea did not correlate with the severity of clinical signs displayed.

the upper limit of the reference range; however, some post-exposure values for total serum magnesium exceeded the upper limit of normality (0.9 mmol/L). There was a strong trend for total serum calcium post-exposure values to be lower in horses graded 3/5, but this trend was also present prior to lolitrem-B exposure. Serum K^+ showed a bidirectional trend, with group 1 increasing, while group 2 decreased ($p = 0.011$, un-paired t-test according to temporal groups). This trend was echoed by serum Na^+ values and serum Cl^- ($p = 0.044$ and $p = 0.029$ respectively, un-paired t-test according to temporal groups). There was also a significant difference between post-exposure K^+ values in each of the PRGS severity score groups ($p = 0.017$, ANOVA according to PRGS score), which increased as the severity grading increased, but remained below the upper limits of normality (**fig. 5.4 C).** AST, GDH and bilirubin showed a significant decrease post-exposure ($p = 0.030$, 0.035 and 0.020 respectively, paired t-test), while GGT increased significantly without exceeding the upper limit of the reference range ($p = 0.021$, paired t-test) **(Appendix 5)**. CK and creatinine did not change significantly, while urea decreased significantly ($p = 0.018$, paired t-test) and although there was a strong difference between PRGS severity score groups ($p = 0.094$, ANOVA according to PRGS score), the values of urea did not show any trend as the severity grade increased (**fig. 5.4 D).** Horses that scored 3/5 showed an increase in fibrinogen post-exposure, with horse #1 exceeding the upper limit of the reference range, and a decrease in serum albumin. But, the difference between the changes in each severity group was not deemed significant by ANOVA ($p = 0.186$ and 0.09, respectively). There was a significant difference between serum globulin values obtained in each severity group ($p = 0.009$, ANOVA according to PRGS score), with horses scoring 3/5 showing the greatest increase **(Appendix 5)**. No statistical change in total protein was observed. Cerebrospinal fluid contained no macroscopic abnormalities **(fig. 5.5).**

Fig. 5.5: Cerebrospinal fluid showed no gross abnormalities, being clear and colourless.

5.3: Electrophysiology

5.3.1: Brainstem auditory evoked potentials

Post-exposure traces with identifiable peaks were obtained from five of the seven horses. Pre- and post-exposure pairs of traces with identifiable peaks were obtained for horses #1, #2 and #7. Either the pre- or post-exposure traces from the remaining horses contained non-identifiable peaks, which excluded interpretation of the change in latency **(Appendix 6).** Traces obtained had a high repeatability within individual horses **(fig. 5.6).** The latency of peak V lengthened post-exposure with the mean of ipsilateral peak V changing from 4.9 ± 0.3 ms to 5.7 ± 0.6 ms, and of contralateral peak V from 5.2 ± 0.3 ms to 5.7 ± 0.6 ms (**fig. 5.7)**; but, the change was not statistically significant.

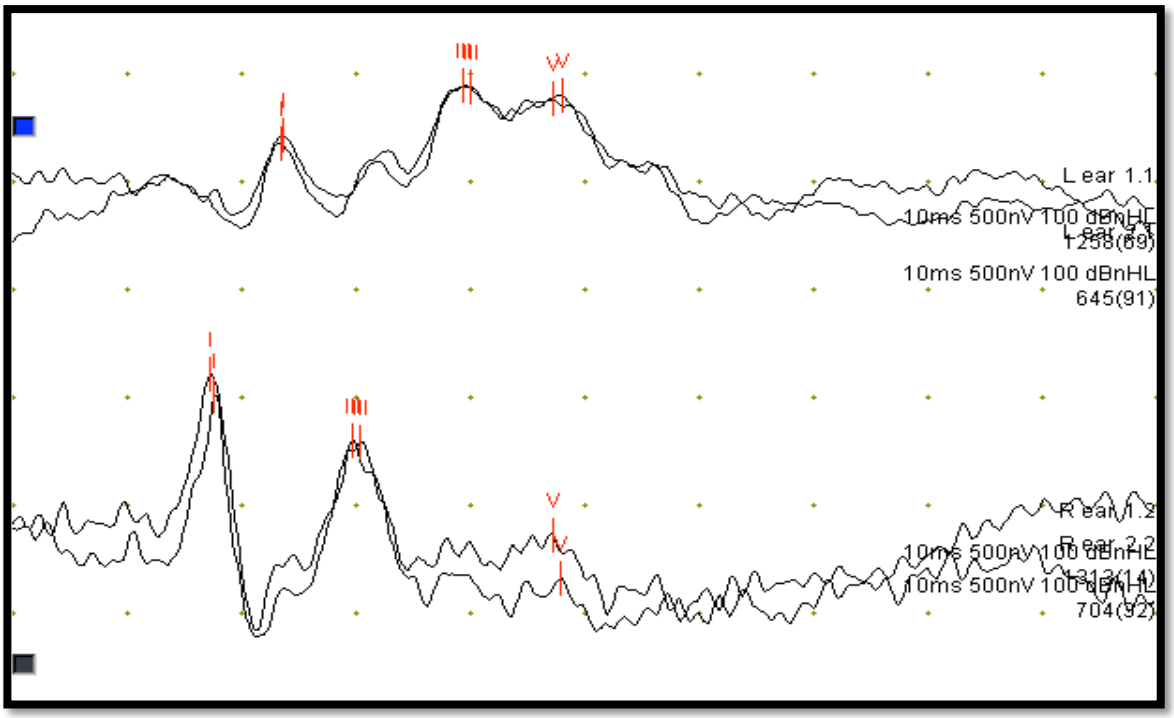


Fig. 5.6: Example BAEP trace (Horse #2, pre-exposure) showing superimposition of repeat recordings and the high degree of repeatability of the BAEP trace. The top trace is the contralateral recording and the bottom trace is the ipsilateral recording.

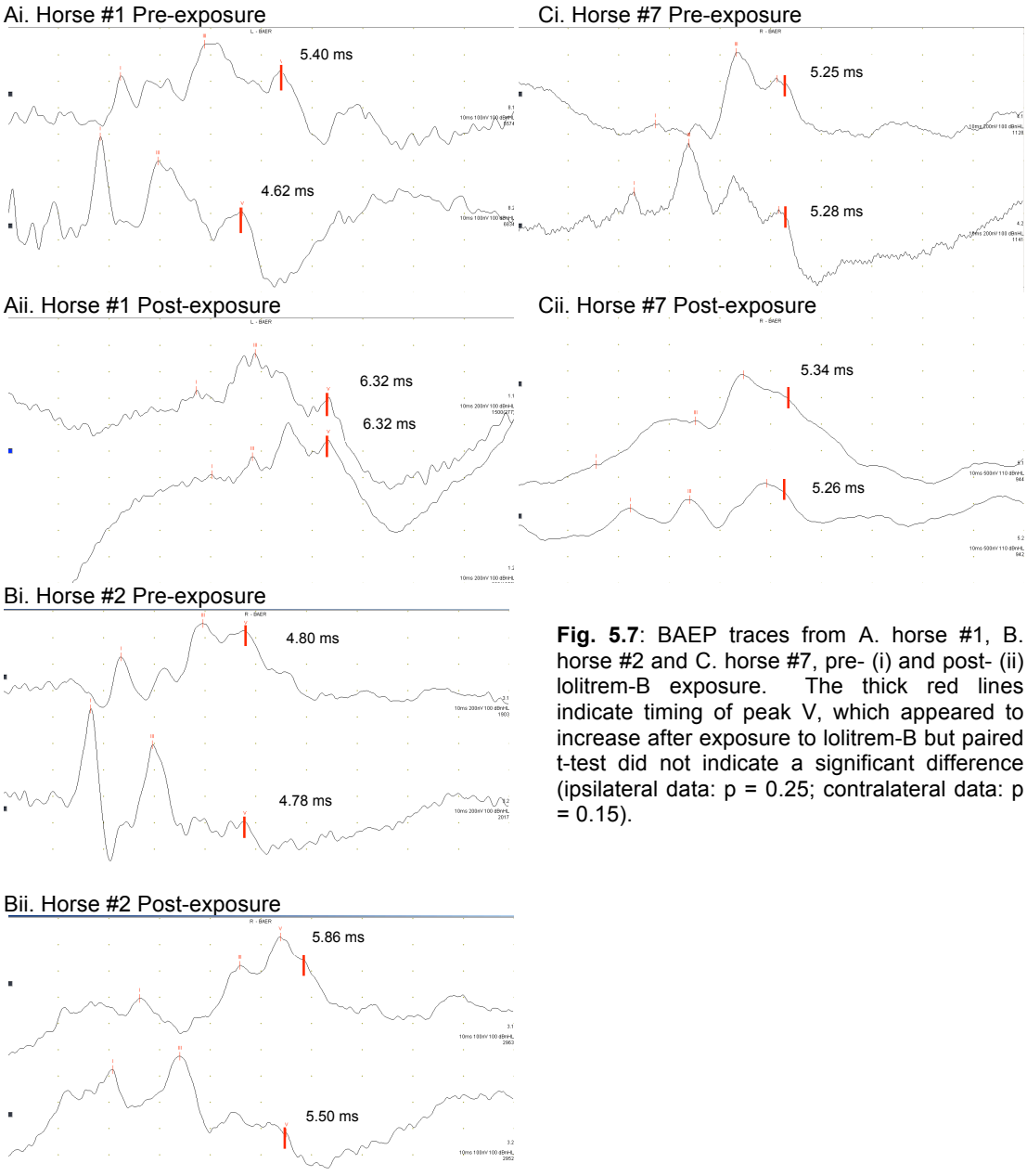


Fig. 5.7: BAEP traces from A. horse #1, B. horse #2 and C. horse #7, pre- (i) and post- (ii) lolitrem-B exposure. The thick red lines indicate timing of peak V, which appeared to increase after exposure to lolitrem-B but paired t-test did not indicate a significant difference (ipsilateral data: $p = 0.25$; contralateral data: $p = 0.15$).

5.3.2: Magnetic motor evoked potentials

Horses #1 – #4 produced traces of magnetic motor evoked potentials (mMEP) recorded from the extensor carpi radialis muscle where the latency to take off and to the first peak could be identified both before and after lolitrem-B exposure, allowing a comparison of the paired traces. The mMEPs for the extensor carpi radialis muscle showed a lengthening in take-off latency and first peak latency ($p = 0.06$ and 0.07, respectively, paired t-test) with the mean latency to take off lengthening from 16.2 ± 6.2 ms (n = 4) to 28.3 ± 9.6 ms (n = 4) and the mean latency to first peak lengthening from 24.5 ± 5.7 ms (n = 4) to 33.8 ± 10.8 ms (n = 4). **Figure 5.8** displays an example trace that was collected from horse #1 pre- and post-exposure. **Appendix 6** displays the remaining pairs of traces available for study.

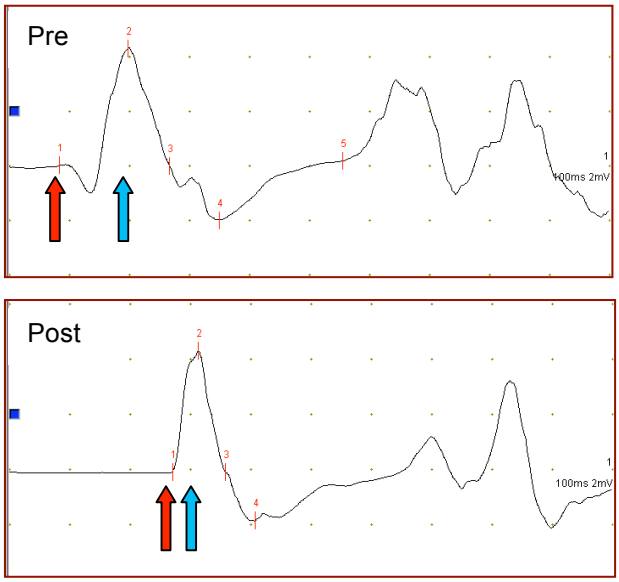


Fig. 5.8: Example mMEP trace pre- and post-lolitrem-B exposure (horse #1). Latency to take off (red arrows) and latency to first peak (blue arrow) are lengthened post exposure.

Traces recorded from the tibialis cranialis muscle and three of the paired extensor carpi radialis traces were excluded from statistical analysis, as one or both of the pairs of traces showed vague or premature (<10ms) take-off or peak latencies **(Appendix 6).**

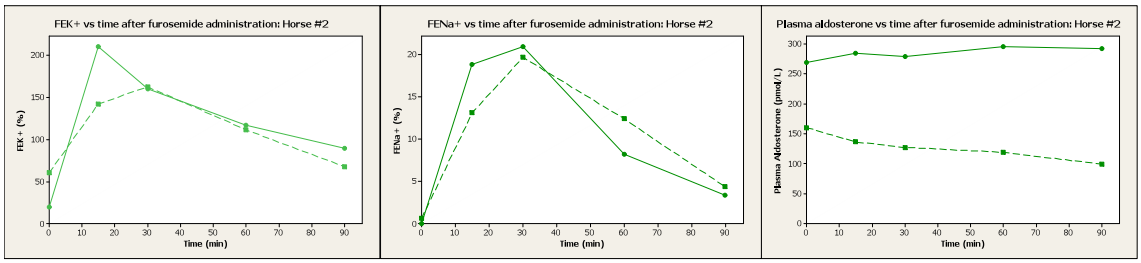
5.4: Frusemide challenge

Fractional excretions were calculated for K^+ and Na^+ (FEK^+ and $FENa^+$). Peak excretion for both ions occurred 15 – 30 minutes after frusemide administration, subsequent to which excretion declined and reached basal levels at around 60 – 100 minutes. During the treatment period, most horses showed a reduced peak fractional excretion value in response to frusemide **(fig. 5.9 A and Appendix 7)**. The graphed results **(fig. 5.9 B)** show that the change in the FEK^+ between 0 and 15 minutes after frusemide administration (ΔFEK^+_{15}) was significantly greater ($p = 0.003$, paired t-test) before horses had been exposed than after two weeks exposure to lolitrem-B. There also appeared to be a strong trend ($p = 0.072$, paired t-test) for an unexpected reduction in the change in $FENa^+$ between 0 and 15 minutes after frusemide administration ($\Delta FENa^+_{15}$) in a manner similar to the frusemide-induced ΔFEK^+_{15}. There was a strong indication ($p = 0.079$, ANOVA) of a difference between the PRGS severity groups in the ΔFEK^+_{15} with horses graded 3/5 showing the greatest reduction **(fig. 5.9 C).** This was not seen for the $\Delta FENa^+_{15}$.

Basal aldosterone values after lolitrem-B exposure were significantly lower than pre-exposure values ($p = 0.027$, paired t-test) **(fig. 5.9 D)**. Aldosterone is reported to peak 30 minutes after frusemide administration (Guthrie, et al., 1982), which was weakly demonstrated in some horses. The peaking trend was most clear in horse #1 pre-, horse #3 pre-, and horse #4 pre- and post-exposure **(Appendix 7 A, C & D)** and although the mean change in serum aldosterone from 0 to 30 minutes after frusemide administration (ΔAld_{30}) was greater prior to lolitrem-B exposure **(fig. 5.9 B)**, the difference between pre- and post-exposure ΔAld_{30} was not significant ($p = 0.165$, paired t-test).

As changes in dietary K^+ intake prime the renal system for K^+ excretion (Grimm, et al., 2009; Pluznick & Sansom, 2006; Rieg, et al., 2007; Sausbier, et al., 2005), differences in K^+ levels in the feed may have influenced the ability of the nephron to respond to frusemide. Therefore, K^+ was analysed in the feed. Control feed contained a total dose of 11.5 ± 0.7 $g_{(K+)}$/kgDM, while treatment feed contained 14.1 ± 2.7 and 13.4 ± 2.5 $g_{(K+)}$/kgDM in group 1 and group 2 respectively **(Appendix 8)**. Neither treatment feeds from group 1 or 2 differ significantly from the control feed ($p = 0.41$ and 0.49 respectively, unpaired t-test).

A.



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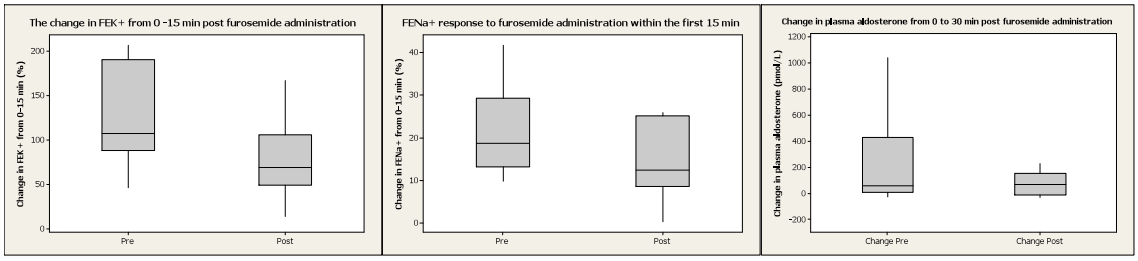

C. **D.**

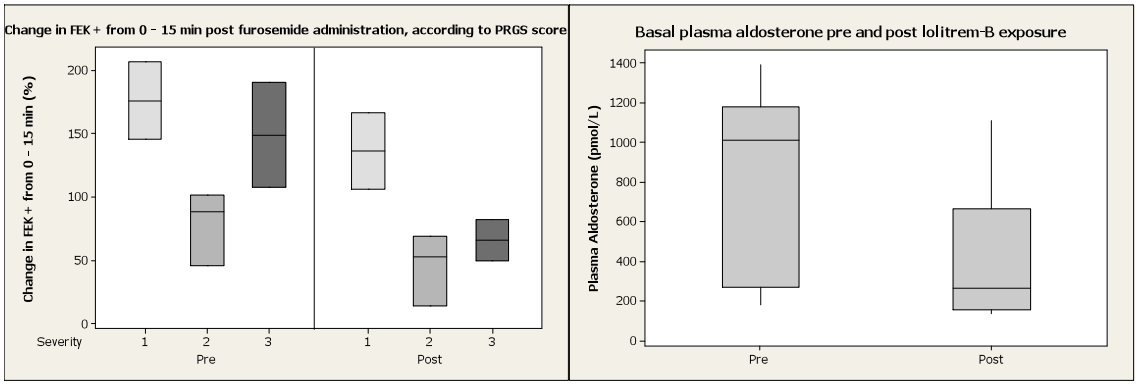

Fig. 5.9: A. Example of frusemide-induced changes in FEK^+, $FENa^+$ and aldosterone following frusemide administration in horse #2. Solid line (—) represents pre-exposure values; interrupted line (- - -) represents post-exposure values; B. boxplots for the ΔFEK^+_{15}, $\Delta FENa^+_{15}$ and ΔAld_{30}; C. boxplot for the ΔFEK^+_{15} for each severity group, which shows that the greatest reduction in ΔFEK^+_{15} occurred in horses graded 3/5 on the final day of the treatment period; D. boxplot demonstrating the decrease in basal plasma aldosterone after exposure to lolitrem-B.

5.5: Lolitrem-B levels in body fluids – urine and plasma

Enzyme-linked immunosorbent assay (ELISA), even using extending detection techniques, was able to detect only minute amounts of lolitrem-B in the urine, for which there was no significant difference between the control and the treatment period. However, there was a significant increase in plasma lolitrem-B from non-detectable during the control period to 0.23 – 0.5 ng/mL after exposure ($p = 0.001$, paired t-test). Interestingly, post exposure levels did not correlate with the severity of signs displayed **(fig. 5.10)** and there was no significant difference between horses in each severity grade ($p = 0.233$, ANOVA).

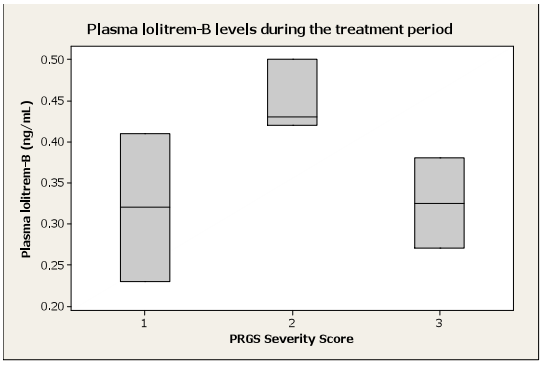

Fig. 5.10: Plasma lolitrem-B values after lolitrem-B exposure. Although there was a significant increase in lolitrem-B values, the level did not correspond to the severity of signs displayed.

Conclusion:

Results support the hypothesis that ingestion of lolitrem-B, at levels and for an exposure duration that is reported as toxic for ruminants, would display signs of BK channel dysfunction that are detectable by clinically applicable tests. Neurological examinations reveal that in horses lolitrem-B intoxication causes tremor, ataxia and an abnormal responsiveness to some stimuli. Result indicate that lolitrem-B may cause an increase in latency of peak V in BAEP traces, an increase in take-off and first peak latency in mMEP traces, a reduction in frusemide-induced ΔFEK^{+}_{15} and $\Delta FENa^{+}_{15}$ and a decrease in basal plasma aldosterone. How these observations relate to the roles of BK channels and their correlation with previous studies of PRGS will be discussed in the following chapter. The second hypothesis was disproved, as although lolitrem-B ingestion produced a significant increase in plasma lolitrem-B values, these values did not correlate with the variations in severity displayed by the intoxicated horses. The limb edema and lesions of the distal limbs that occurred in the two horses that were most severely affected also warrant further discussion.

Chapter 6: Discussion

The aim of this investigation was to describe the clinical effects of lolitrem-B intoxication in horses in relation to the function of large-conductance Ca^{2+}-activated K^{+} (BK) channels and the clinical effects reported in ruminant species, within the limitation of using tests that are applicable to veterinary clinical practice. This was achieved by assessing clinical and neurological parameters, recording electrophysiology traces and applying a frusemide challenge. The following is a discussion of the clinical observations of horses ingesting lolitrem-B, the correlation of these clinical signs with those previously reported in ruminant species in chapter 2 and how they relate to the roles of BK channels that were discussed in chapter 3. Conclusions will be drawn in chapter 7.

6.1: How the clinical signs observed in this investigation compare to those reported in ruminant trials

Horses ingesting feed containing greater than 2 ppm lolitrem-B displayed signs of neuromuscular dysfunction after 4 – 9 days of ingesting treatment feed. Clinical signs were similar to those reported in ruminant studies, including tremor, ataxia and truncal sway (Blythe, et al., 2007; Di Menna, et al., 1992; Fisher, et al., 2004; Galey, et al., 1991; Miyazaki, et al., 2004; Tor-Agbidye, et al., 2001), from which the horses appeared to recover after a week of ingesting non-perennial ryegrass (PRG) feed. This supports the dictum that lolitrem-B intoxication occurs in horses. The onset of 4 – 9 days was shorter than the general 7 – 14 days reported in ruminants (Galey, et al., 1991; Prestidge, 1993); however, early signs were subtle and may not be detected in a herd situation, therefore do not particularly support the preconceived idea that horses are particularly sensitive to lolitrem-B (Prestidge, 1993; Smith & Towers, 2002). As with ruminant studies, exercise exacerbated clinical signs – particularly tremor. Although clinical signs did not increase dramatically with handling or restraint this may represent the horse-human relationship rather than differences in the molecular mechanisms of lolitrem-B intoxication between equids and ruminants. The observed increase in clinical signs during exercise and feeding in horses is consistent with increased demands on the motor system. Therefore, clinical signs of lolitrem-B intoxication in the horse bear close resemblance to the way the disease presents in ruminants.

Ruminant studies on perennial ryegrass staggers (PRGS) report increased perianal faecal contamination (Fletcher, 1993) and electrophysiological changes in gastrointestinal motility (McLeay, et al., 1999). There was no alteration in

gastrointestinal auscultation in study horses that would indicate altered motility, but faecal consistency became more fluid in two of the horses while the others remained soft, despite a diet high in dry-matter. Softening of faeces contrasts with the report that tenesmus occurs in horses with PRGS (Munday, et al., 1985). The opposing change in faecal consistency observed by Munday et al. (1985) and in this investigation may be due to dietary factors rather than lolitrem-B intoxication or alternatively could reflect the different concentrations of lolitrem-B and other mycotoxins administered. The results of this investigation neither support strongly nor refute the effects of lolitrem-B on gastrointestinal function.

This trial signified the individual variability in the degree of clinical signs expressed despite similar intakes of lolitrem-B. Some horses merely showed a slight sway or tremor and no ataxia, even when blindfolded, after 12 – 14 days of exposure; while other horses demonstrated dramatic tremor and obvious ataxia when blindfolded after 8 days of ingesting lolitrem-B. Although varied in severity, all horses showed signs of lolitrem-B intoxication, which presents a much greater morbidity than the commonly reported 10% morbidity in ruminants (Cunningham & Hartley, 1959) but concurs with the findings of other ruminant trials by Blythe et al. (2007) and Di Menna et al. (1992). It should be noted that in studies or case-reports involving pasture grazing, variability in the grazing behaviour of individuals could result in variation in the amount of toxin ingested (Cunningham & Hartley, 1959). Controlling the feed available in this study may have increased the morbidity relative to a pasture situation. The remaining variation, assuming variability due to the amount of toxin ingested is low, is deduced to differences in the handling of the toxin by individual horses, for which there may be a genetic component, as in sheep (Morris, et al., 1998; Morris, et al., 1995). In addition to genetic influences, past exposure may alter the affinity of BK channels to lolitrem-B, the number of active BK channels or the ability to metabolise lolitrem-B, or it may allow animals to adapt to the effects of the drug by neurological compensatory mechanisms (Hernandez & Rathinavelu, 2003). Interestingly, although enzyme-linked immunosorbent assay (ELISA) detected a significant increase in plasma lolitrem-B during the treatment period ($p = 0.001$), the post-exposure levels for each horse did not correlate with the degree of clinical signs displayed; a horse that scored 3/5 had 0.27 ηg/mL detected, while another horse that scored 1/5 had 0.41 ηg/mL detected. Therefore, plasma ELISA may be beneficial in screening for lolitrem-B ingestion but low levels should not be used to rule out PRGS. The discord between clinical signs displayed and the level of lolitrem-B in plasma indicates that the severity of clinical signs may not be associated with the animal's ability to absorb, metabolise and excrete lolitrem-B, as this would be reflected in a correlation between plasma levels and the

severity of disease. Rather, variability in individual expression may relate to receptor-drug interactions and neuro-compensatory mechanisms, resulting in different toxic thresholds for individual animals. However, as this is the first trial involving horses to utilise the ELISA test, further validation of ELISA tests in horse plasma is required before conclusions can be drawn.

ELISA testing of urine detected only trace amounts of lolitrem-B in control and treatment samples. As high-performance liquid chromatography detected low levels of lolitrem-B in the control feed, it is plausible that the trace lolitrem-B detected in the control period is real rather than artifact. Urine ELISA has been used to detect lolitrem-B in sheep and cattle (J. Sprosen, Ag Research Ltd., Ruakura. pers. comm. September 2009), but this was the first time it had been run using horse urine. It is possible that horse urine contains unique factors, such as high Ca^{2+} levels, that interfered with the ELISA test. Alternatively, horses may excrete lolitrem-B predominantly via the hepatobiliary system. This route is supported by the high lipophilicity of lolitrem-B and the detoxicification of paxilline, lolitrem-B's precursor (Weedon & Mantle, 1987), in bile (Rowan, 1993). Sheep trials have found faecal ELISA for lolitrem-B to be more sensitive than urine (L. Fletcher, Ag Research Ltd., Lincoln. pers. comm. May 2009); further investigation is required to determine if this is true in horses.

6.2: Tremor and eye movements

As reported in ruminant trials and case-reports (Blythe, et al., 2007; Cunningham & Hartley, 1959; Galey, et al., 1991; Holmes, et al., 1999; Mackintosh, et al., 1982), lolitrem-B intoxicated horses displayed tremor. The degree of tremor varied dramatically between individuals and within individuals, showing a gradation of severity from fasciculations and vermiform movements involving individual groups of muscle fibres, to the involvement of whole muscle groups producing limb spasms. Tremor was exacerbated with feeding and after exercise and was especially evident in the thoracic limbs. Along with body sway, tremor was the first clinical sign observed and appeared after 4 – 9 days of ingesting lolitrem-B. During the exposure period, horses received 36 – 46 μg/kgBW lolitrem-B each day **(Appendix 3)**. This correlates well with findings by Smith et al. (1997) that intravenous administration of purified lolitrem-B at doses between 45 – 80 μg/kg caused moderate tremors in sheep.

Mentioned previously in chapter 3 are the numerous localities in which dysfunction can result in tremor including the muscle cell membrane, the muscle spindle apparatus, and in neural pathways in the spinal cord, brainstem, thalamus, basal ganglia and

cerebellum (Guyton & Hall, 2000; Mayhew, 2009). Due to the diverse functions of BK channels amongst neurons (Knaus, et al., 1996), it is likely that lolitrem-B intoxication causes tremor at various sites. Describing and classifying the nature of tremor observed in PRGS will be useful diagnostically and may identify similarities with other diseases in which tremor is a prominent sign.

Previous studies have described the tremor displayed with BK channel dysfunction as 'intention tremor' (Sausbier, et al., 2004). This term refers to tremor that occurs with cerebellar dysfunction and is due to a lack of movement precision (Guyton & Hall, 2000; Mayhew, 2009). The neural circuitry of the cerebellum is wired so that input signals divide to directly connect and excite the deep cerebellar nuclei and also branch to a corresponding area of the cerebellar cortex, which is then relayed back to the deep cerebellar nuclei as an inhibitory signal via Purkinje cells (Guyton & Hall, 2000). In this way, motor signals are initially enhanced by the cerebellum and, then when approaching termination, movements are dampened by the inhibitory Purkinje cell signals. Without the dampening influence of the cerebellum, movements overshoot the intended target. Particularly via the visual system, the cerebrum detects the overshoot and corrective motor signals are initiated but again overshoot, resulting in oscillation about the intended target. Although BK channels have been reported to cause dysfunction of Purkinje cells (Sausbier, et al., 2004), as discussed in chapter 3, the muscle tremor observed in horses did not appear to occur in relation to intended movements, indicating that it is not likely they were of cerebellar origin. In addition, tremor caused by lesions restricted to the cerebellum are usually limited to the head and neck region (Mayhew, 2009), whereas the tremor observed in this trial predominantly involved the forelimbs.

Animals that are weak due to diseases such as botulism, rhabdomyolysis and lesions of upper motor neurons (UMNs), as well as those suffering long bone fractures, often tremble (Mayhew, 2009). This trial observed that tremors were most prominent in the limb that was being rested or during movements when weight was transferred off the limb. A weak patient during the hopping response has a tendency to tremble on the thoracic limb bearing weight (Mayhew, 2009); during the hopping response horses did not demonstrate tremor on the weight-bearing limb **(DVD 8)**. These observations indicate that tremor was not likely associated with weakness.

The predominance of forelimb tremor agrees with electromyographic activity over the shoulder being most sensitive to tremor in sheep injected intravenously with purified lolitrem-B (Smith, et al., 1997). The reason for tremor occurring predominantly in the forelimbs is unclear. However, it may relate to the somatotopic organisation of the

motor system (Guyton & Hall, 2000; Holliday, 1980) within which BK channels may have different affinities for lolitrem-B. Alternatively, the greater weight borne by the forelimbs than the hindlimbs may have lead to a predominance in forelimb tremor. However, tremor did not appear to relate to weakness and therefore, the former explanation is more plausible than the latter.

6.2.1: Classifying tremor

de Lahunta et al. (2006) developed a classification system for involuntary, uncontrolled skeletal muscle movements that occur in conscious animals due to muscle or neural pathology (excluding seizure disorders) **(fig. 6.1)**. As alluded to previously, there was a substantial increase in tremor activity associated with exercise and feeding. These circumstances of tremor are characteristic of tetany, myotonia and two subclasses of repetitive myoclonus – action-related and episodic repetitive myoclonus (de Lahunta, et al., 2006). Horses displayed tremors that most closely associate with action-related repetitive myoclonus, but they also showed fasciculations that are characteristic of episodic related repetitive myoclonus. Although horses did not display cerebellar fits, these have been described in other studies of PRGS in ruminants (Cunningham & Hartley, 1959; Mackintosh, et al., 1982) and may represent either tetany or myotonia.

Fig. 6.1: Classification of tremors by de Lahunta et al. (2006)

- Muscle cell membrane dysfunction
  - Myotonia
- Neural dysfunction
  - Tetanus
  - Tetany
  - Myoclonus
    - Sporadic
    - Repetitive
      - Constant
      - Action-related
      - Postural
      - Episodic
      - Resting
  - Movement disorders

Repetitive myoclonus is defined as shock-like, paroxysmal contraction-relaxation cycles of muscle groups that are either constant or occur in relation to particular circumstances (de Lahunta, et al., 2006; Mayhew, 2009). Action-related repetitive myoclonus is a rapid tremor that occurs in relation to increased motor activity and usually involves multiple skeletal muscles (de Lahunta, et al., 2006), describing well the situations in which tremor was exaggerated in this trial. The etiology of this class of tremor involves diffuse neural disorders (de Lahunta, et al., 2006; Mayhew, 2009). Aligned with the role of BK channels in neurotransmitter release (Hu, et al., 2001; Pattillo, et al., 2001; Robitaille, et al., 1993; J. W. Xu & Slaughter, 2005) and their potential to cause neural disinhibition (Sausbier, et al., 2004), this class of tremor includes stiff horse syndrome (a disease due to alterations in neurotransmitter release (Mayhew, 2009)), stiff man syndrome (an autoimmune disease that results in central neuronal disinhibition (Meinck, Ricker, Hulser, & Solimena, 1995; Solimena, Folli,

Aparisi, Pozza, & Decamilli, 1990)), and a multitude of neurotoxicoses including metaldehyde, pyrethrins, lead, organophosphates and other mycotoxins, which (by various mechanisms) alter normal synaptic transmission (de Lahunta, et al., 2006).

Several horses demonstrated fasciculations over the shoulder, trunk and flank. In horse #5 these movements were increased and appeared as irregular undulating vermiform or wave-like movements of the overlying skin **(DVD 3)**. Myokymia is a term used to describe these progressive vermiform movements, which are due to the contraction of small bands of muscle fibres and are classified as episodic repetitive myoclonus (de Lahunta, et al., 2006). In humans, myokymic activity has been related to hyperactivity of peripheral nerve axon and circulating antibodies to voltage-gated K^+ channels have been detected (Mertens & Zschocke, 1965, as cited in de Lahunta, et al., 2006). Blockade of BK channels may also cause hyperactivity of peripheral nerve axons and manifest as myokymia. Flank tremors also resembled abdominal movement that occurs in synchronous diaphragmatic flutter. The mechanisms of this disease involves irritability of the phrenic nerve due to hypocalcaemia or hypochloraemic metabolic alkalosis, which allows cardiac potentials to influence the phrenic nerve causing unilateral or bilateral diaphragmatic contractions (Mayhew, 2009). Dysfunction of BK channels in the phrenic nerve could lower the resting membrane potential (RMP) and thus increase nerve irritability. However, in synchronous diaphragmatic flutter, contraction of the diaphragm is synchronized with the cardiac rhythm and also causes rhythmic respirations and associated nostril flaring. This was not observed; therefore diaphragmatic contractions are unlikely to be the cause of the movements at the flank.

Tetany, which involves intermittent contraction of extensor muscles, is exaggerated by stimulation and movement and can culminate in cerebellar fits (de Lahunta, et al., 2006; Mayhew, 2009). Common to both inherited and acquired forms of tetany is dysfunction of the glycine receptor on extensor LMNs cell membranes, which ultimately results in disinhibition (de Lahunta, et al., 2006). Several reports of cerebellar fits occur in the literature on PRGS (Cunningham & Hartley, 1959; Galey, et al., 1991; Mackintosh, et al., 1982), indicating that lolitrem-B might cause severe disinhibition of extensor LMNs. The mechanisms by which lolitrem-B causes this disinhibition is uncertain, but may involve altered conduction in UMNs or LMNs, or may be due to silencing of Purkinje cells as is reported to occur in $BK_{\alpha}^{-/-}$ mice (Sausbier, et al., 2004). Myotonia is another classification of tremor that can cause paroxysms of tonic and hypometric gait, recumbency and limb rigidity following excitement or sudden exertion. Myotonia results from dysfunction of the muscle cell membrane and by definition refers to continued physical and electrical contraction of muscle well after the voluntary effort

or the reflex, chemical, mechanical, or electrical stimulus has ceased (Mayhew, 2009). BK channels are expressed on the cell membrane of skeletal muscle (Pallotta, 1985a) and therefore lolitrem-B intoxication may disturb the conductance of the muscle cell membrane. Muscle hypertrophy, which often occurs in animals with myotonia, is not reported to occur with PRGS, but this may relate to the transient nature of lolitrem-B intoxication. Electromyographic studies may differentiate between tetany (i.e. a neurogenic disorder) and myotonia (i.e. a myogenic disorder) as the cause of cerebellar fits in animals severely intoxicated with lolitrem-B (Wijnberg, Van der Kolk, Franssen, & Breukink, 2003).

The tremor observed in study horses most closely resembled action-related repetitive myoclonus, but also had features of episodic repetitive myoclonus. Other reports of tremor associated with PRGS would, in addition to the aforementioned classifications, include tetany and/or myoclonus. The pathophysiology common to these classifications suggests that the role of BK channels in neurotransmitter release, peripheral nerve excitability, neural disinhibition and/or muscle cell membrane conduction may be of importance in the production of tremor by lolitrem-B.

6.2.2. Eyeball tremor

Jerky eye movements were reported in sheep injected intravenously with purified lolitrem-B in association with pronounced body tremor (McLeay, et al., 1999). This study did not observe such eye movements; however, using an ophthalmoscope it was possible to detect near-continual, subtle, low amplitude, rapid (~5 Hz) tremor of the eyeball in six of the seven horses. These eye movements may be an exaggeration of normal microsaccadic eye movements. Microsaccades are defined as high frequency conjugate oscillations of the eye, occurring without intervening periods of steady fixation (Engbert & Mergenthaler, 2006; Ramat, Leigh, Zee, & Optican, 2005; Shaikh, et al., 2007). Microsaccades only occur in animals that contain a fovea – the area of the retina that contains slim elongated cones specialised for detailed vision (Collewijn & Kowler, 2008). The horse eye contains an area comparable to the human fovea, named the visual streak (Guo & Sugita, 2000; Murphy, Hall, & Arkins, 2009) and therefore is likely to demonstrate microsaccades. Although microsaccades occur normally, pathological microsaccades also occur in humans and are associated with paroxysms of ‘shimmering’, ‘jiggling’ or ‘wavy’ visual disturbance and are often coupled with dizziness and disequilibrium (Brazis, Masdeu, & Biller, 2007). To validate the hypothesis that the eye movements observed in this study were pathological microsaccades, optic instruments suitable for use in horses that differentiate movements of the eye, the head and the observer’s hand and that measure tremor

frequency and amplitude need to be considered for use. In the meantime, an ophthalmic examination should be performed in horses suspected of suffering from PRGS.

The combination of abnormal eye movement and forelimb tremor that is exaggerated by stress or motor activity resembles human progressive neurological disorders such as essential tremor (Helmchen, et al., 2003) and familial microsaccadic eye oscillations and limb tremor (μSOLT) (Shaikh, et al., 2007). Although it is unlikely that the etiology is the same (i.e. dysfunction of BK channels), it is possible that essential tremor, μSOLT and lolitrem-B intoxication involve functional pathology of the same neural circuitry. It has been hypothesised that increased membrane excitability of the inherently unstable circuits involved in agonist-antagonist muscle control underlies the pathogenesis to essential tremor and μSOLT (Shaikh, et al., 2008). Two features contribute to the instability of the pre-motor circuits: reciprocal inhibition of agonist and antagonist muscles and a phenomenon termed post-inhibitory rebound. The former is a feature at many levels of the motor system and is advantageous in that it ensures that only agonist muscles are activated, while antagonist muscles are inhibited. However, reciprocal inhibition requires cross coupling of neurons, an anatomic configuration that allows circuits to oscillate (Shaikh, et al., 2008). Secondly, neurons of the deep cerebellar nuclei, vestibular nuclei, thalamus, and ocular premotor burst neuron circuits display post-inhibitory rebound firing, where the withdrawal of inhibition causes a neuron to respond by depolarising (Ramat, et al., 2005). If neurons in a reciprocally inhibitory circuit display post-inhibitory rebound then the deactivation of one neuron results in removal of inhibition of another inhibitory neuron, which then responds by rebound depolarisation, inhibiting a subsequent neuron which will also fire when its inhibition is removed (Ramat, et al., 2005). Thus pre-motor, reciprocally inhibitory circuits that display post-inhibitory rebound are inherently unstable and are prone to oscillate, which manifests as tremor of the muscles that the circuit controls. These mechanisms have been thoroughly studied in the saccadic circuit that controls eye movements (Ramat, et al., 2005) and similarities have been drawn between this circuit and other circuits that control limb movement, such as the thalamic network (Shaikh, et al., 2008). The cerebellothalamocortical loop extends from the cerebellum to the thalamus, then cerebrum, back to the thalamus and then to the reticular formation and down the reticulospinal tracts and is important in coordinating the reciprocal contractions of agonist and antagonist muscles in the distal limbs (Guyton & Hall, 2000). Therefore, instability of the thalamic network may cause limb tremor. Both saccadic and thalamic circuits receive external tonic inhibition provided by omnipause neurons and neurons of the globus pallidus internus, respectively (Shaikh, et al., 2008),

without which even small membrane noise can cause the circuits to oscillate (Ramat, et al., 2005). It has been hypothesised that the lack of inhibitory tone from brainstem omnipause neurons is the cause of microsaccadic flutter (Ashe, Hain, Zee, & Schatz, 1991) and computer modeling has shown that reducing the level of hyperpolarisation of neurons in these circuits can cause oscillation within the circuit (Shaikh, et al., 2007). Blockade of BK channels may result in instability of such circuits by influencing the polarisation of neurons within the circuits and/or by influencing the degree of external tonic inhibition. Blockade of BK channels may have a direct effect on the neurons within the saccadic or thalamic circuits by reducing the rate and level of hyperpolarisation (Sah & McLachlan, 1992; Storm, 1987a, 1987b), depolarising the RMP (Haug & Sand, 1997) or changing the activity of other membrane channels such as hyperpolarising-activated cation channels, whose activity is regulated by binding of cAMP and is thus dependant on the concentration of intracellular Ca^{2+} ions ($[Ca^{2+}]_i$) (Wahl-Schott & Biel, 2009). Alternatively or in conjunction, BK channel blockade may alter the level of external tonic inhibition. Firstly, lack of BK channel activity may result in depolarisation-induced inactivation of omnipause and globus pallidus internus neurons, causing disinhibition of the saccadic and thalamic circuits, as was reported to occur in Purkinje cells from knockout mice lacking the α-subunit of BK channels ($BK_{\alpha}^{-/-}$), causing disinhibition at the deep cerebellar nuclei (Raman & Bean, 1999; Sausbier, et al., 2004). Secondly, BK channels may facilitate neurotransmitter release from omnipause and globus pallidus internus neurons by enhancing the electrostatic driving force for Ca^{2+} influx (Pattillo, et al., 2001; J. W. Xu & Slaughter, 2005). Therefore, lolitrem-B intoxication may suppress the release of inhibitory neurotransmitters from these neurons. Of further interest is the finding that ethanol is an effective treatment for essential tremor (Klebe, et al., 2005) and has been found to enhance the probability of BK channels opening (Brodie, Scholz, Weiger, & Dopico, 2007; Dopico, Anantharam, & Treistman, 1998). This supports a role of BK channels in the circuitry involved in essential tremor and suggests a potential link between the tremors observed in this human disease and PRGS.

6.3: Ataxia

Ataxia is defined as a lack of order or inconsistencies in movement resulting in irregular and unpredictable movement and placement of the head, neck, trunk and limbs (Mayhew, 2009). As discussed in the background chapters, there are three qualities of ataxia that are classified according to the predominant pathway involved: general proprioceptive (GP), vestibular or cerebellar. Neither reports from ruminant studies on PRGS nor current information on the role of BK channels indicate persuasively the

nature of ataxia associated with PRGS, identifying the need for a detailed description of the nature of ataxia demonstrated by animals intoxicated with lolitrem-B.

Horses displayed mild ataxia that was exaggerated by blindfolding, in the form of a resting wide-based stance and truncal sway and a cautious wide-based gait involving truncal sway and irregular foot placements that were strong, often multiple and hurried, and were corrective for the lateral movement of the trunk. This gait is typical of symmetrical dysfunction of the vestibular system (including the flocculonodular lobe of the cerebellum) and is termed disequilibrium (Holliday, 1980). During tight circling, postural deficits indicative of GP dysfunction were elicited, including delayed onset of protraction of the hindlimbs and wide placement of the outside pelvic limb. Delayed correction of awkward limb positions after abrupt cessation of movement also indicated a degree of GP dysfunction. Due to the diffuse nature of BK channels, it is possible that GP pathways and the cerebellum are affected in addition to the vestibular system. Therefore, the ataxia observed may be a combined spinovestibulocerebellar disorder that primarily involves vestibular dysfunction during the early stages and as signs progress GP and cerebellar deficits may become evident.

6.3.1: The quality of ataxia did not resemble cerebellar ataxia

Plausible mechanisms for cerebellar ataxia were presented in chapter 3; however, the nature of ataxia observed was not characteristic of cerebellar dysfunction. The cerebellum is the regulator of movement — planning and preempting inaccuracies and imbalance so that movement conforms precisely to the volitional motor signals from the cerebrum and so that appropriate responses maintain subconscious equilibrium. Signals from the cerebellum to the cerebrum plan the next sequential movement in advance, producing a smooth progression of movements (Guyton & Hall, 2000). The cerebellum neither initiates nor terminates movements, rather, it acts as an integrator to coordinate movements (Holliday, 1980). The neural circuitry of the cerebellum, as discussed in association with tremors, is key to the initial enhancement and dampening of motor signals, which meticulously provides the correct timing, force, range and speed required to carry out the intended task (Guyton & Hall, 2000; Holliday, 1980). The cerebellum uses vestibular, visual and GP input to calculate in advance body positions and make anticipatory corrections in order to maintain equilibrium during rapid changes in body position (Guyton & Hall, 2000). Disorders of the cerebellum therefore manifest as variable delays in the onset of movement followed by a highly unpredictable dysmetric gait involving hypermetria and hypometria, overshoot and over-correction, overreaching and forceful foot placement. There is a lurching quality to body movement as the trunk attempts to maintain alignment with the irregular limb

movement. However, blindfolding does not necessarily produce a dramatic deterioration in balance (Bradley, 1991).

In this trial, horses did demonstrate irregular movement; however, limb placements were not entirely unpredictable, rather, they were compensative in that they followed the center of mass according to the direction of truncal drift. So, unlike cerebellar ataxia where the trunk lurches as a result of the disordered limb movement, the disordered movement observed in this trial was initially an excessive sway of the trunk, which necessitated irregular, but corrective, limb movements. The irregular limb movements included abduction, adduction and crossed limb placements, but hypermetria was not featured. Leading the horse in a serpentine path enforces a change in the intended direction of movement midway through the protraction phase and tests the horses' ability to rapidly and smoothly alter the movement of the limb and maintain equilibrium during rapid changes of body position (Mayhew, 2009). Such complex maneuvers involve integration of volitional, postural and reflex systems by the cerebellum (Holliday, 1980). Horses in this study rarely showed deficiencies in the ability to rapidly correct the direction of limb movement mid-motion, even when blindfolded **(DVD 7)**. The lack of signs associated specifically with dysfunction of the cerebellum suggests that although horses demonstrated irregularities in movement, these were not very consistent with cerebellar dysfunction.

At rest, horses displayed both lateral and craniocaudal sway of the trunk that involved a transfer of weight between limbs causing angular fluctuations of the joints, particularly noticeable at the fetlock. All qualities of ataxia involve a truncal sway at rest; however, the mechanisms of sway are different. In cerebellar dysfunction, truncal sway results from a lack of movement precision and in a sense can be considered a postural intention tremor (Mayhew, 2009); the intended position for the body is upright but the motor signals, lacking dampening input from the cerebellum, result in excessive pendular motion. Excess motion is detected by the visual, vestibular and GP sensors and correction is attempted, but again the position is overshot (Guyton & Hall, 2000). This overshoot and overcorrection produces a gross jerky movement of the entire body, termed titubations (de Lahunta & Glass, 2009). Vestibular and GP dysfunction reduce the sensory input to the equilibrium centers, which results in amplification of body movements that occur at rest. Studies of humans with normal neural function have demonstrated that when standing upright the body is not in a state of fixed, motionless stability but rather sways laterally and anteroposteriorly, even during conscious attempts to remain motionless (Day, Steiger, Thompson, & Marsden, 1993). This movement is not of a magnitude that threatens equilibrium and is thought to ready

the body for any perturbation of balance (Nigg, et al., 2000). However, a reduction in sensory input to the equilibrium centers causes excessive sway and can result in the vertical projection of the body's center of mass exceeding the boundaries of limb support (Nigg, et al., 2000). The mild increase in sway that occurs when one sensory pathway is absent has been demonstrated by measuring the movement of the human upright body while attempting to stand still with various stance widths and eyes closed or open (Day, et al., 1993). The speed and amplitude of body sway was increased by closure of eyes, especially when stance width was narrowed. Therefore, a reduction in sensory input to the equilibrium centers results in increased sway of the trunk. Other than the influence of blindfolding, how these different mechanisms of truncal sway manifest clinically has not been defined. However, there may be differences in the frequency, amplitude and regularity of the truncal sway, and each type of sway may occur under different circumstances. Further comparison between sway resulting from cerebellar, vestibular and GP deficits may assist the characterisation of ataxia.

6.3.2. Blindfolding indicates that sensory input to the equilibrium centers is reduced by lolitrem-B intoxication

The profound effect of blindfolding further indicates that cerebellar dysfunction was not the primary cause of the ataxia demonstrated by horses in this trial. The excessive drift of the trunk during movement and the dramatic deterioration of balance and regular movement when blindfolded align with either vestibular or GP deficits. Input from vestibular, visual and GP pathways are integrated by the cerebellum, vestibular nucleus and the reticular nucleus, which generate motor signals to the appropriate agonist and antagonist postural muscles in order to maintain an upright stance (Guyton & Hall, 2000). Each sensory modality is important in maintaining equilibrium. Slight linear or rotational movement of the body instantaneously shifts the visual image on the retina (Nigg, et al., 2000), GP pathways detect changes in pressure, tension and stretch in joints, muscles and tendons (Bradley, 1991), while the vestibular system detects changes in body position with respect to gravity and the rate and direction of head movement (Guyton & Hall, 2000). In this way visual input complements input from the other sensory modalities and visual correction can substantially compensate for vestibular or GP deficits (Nigg, et al., 2000).

Sway at rest was accentuated by blindfolding. This may be considered an adaptation to the Romberg's test, which is used in human medicine to differentiate cerebellar ataxia from GP and vestibular ataxia (Khasnis & Gokula, 2003; Pearce, 2005). In the Romberg's test, the patient is made to stand with their feet close together and arms by their side and any sway or tendency to fall is compared between when eyes are open

and when they are closed (Khasnis & Gokula, 2003). If there is dysfunction of either the GP pathways or the vestibular pathway closing the eyes removes a second sensory input, which leaves insufficient information to maintain balance, resulting in increased sway and a tendency to fall (Khasnis & Gokula, 2003; Pearce, 2005). This is different to cerebellar diseases where patients tend to be unsteady with their eyes open and do not dramatically deteriorate when their eyes are closed, because cerebellar ataxia results from a lack of integrative power that is independent of the amount of sensory input received (Khasnis & Gokula, 2003).

Horses also demonstrated truncal sway during movements, which were exaggerated by blindfolding. When moving, the trunk tended to drift or sway laterally, which was compensated in part by adducted or crossed placement of the limb contralateral to the direction of sway and abducted placement of the ipsilateral limb. In a normal horse, a change in the center of gravity caused by movement of the rider, or tack, can cause gait deficits as the horse attempts to keep limbs under the center of mass (Adams & Stashak, 2002). Likewise, blindfolding a horse with a deficiency in either vestibular or GP input causes excess motion of the center of mass and requires compensatory gait deficits to maintain balance (Nigg, et al., 2000).

Blindfolding established that the ataxia displayed by horses ingesting lolitrem-B is due to a deficient sensory input rather than cerebellar dysfunction. In humans, instability that is accentuated by blindfolding is most commonly due to GP deficits (Khasnis & Gokula, 2003), whereas in quadrupeds blindfolding accentuates ataxia that is due to dysfunction of the vestibular system, cranial cervical spinal cord disease or diffuse spinocerebellar disease. The latter two pathways are involved in subconscious GP. Differentiating between vestibular or GP deficits as the cause for the exacerbation of clinical signs when blindfolded is difficult, especially in that lolitrem-B intoxication produces functional disturbances rather than morbid lesions and therefore may result in atypical qualities of ataxia. The next section will discuss the qualities of ataxia that suggest that lolitrem-B intoxication primarily involves miscoding of vestibular information, rather than of GP input.

6.3.3. Further defining the nature of ataxia observed

As discussed in chapter 3, the vestibular apparatus detects static and dynamic equilibrium and via the integrative workings of the central vestibular, cerebellar and reticular motor systems activates the appropriate postural muscles and modulates the interplay between antagonist and agonist muscle to maintain equilibrium (Guyton & Hall, 2000). Therefore, along with vision and GP pathways, the vestibular system is

important in maintaining static and dynamic equilibrium. Lolitrem-B may interfere with the learned modulation of vestibular signals by binding BK channels on type II vestibular hair cells (VHCII) and/or neurons of the vestibular nucleus, may disrupt the function of the interneurons and Purkinje cells of the flocculonodular lobe, or may indirectly alter the composition of vestibular endolymph. Often, vestibular ataxia is characterised by signs that are due to inappropriately imbalanced input resulting from unilateral lesions that interrupt signals from one side. These signs include an asymmetric posture that favors a particular side and directional nystagmus (Mayhew, 2009). However, lolitrem-B is assumed to be distributed symmetrically, and therefore, would result in symmetrical dysfunction of the vestibular system. Symmetrical vestibular dysfunction in quadrupeds manifests as a truncal sway causing the animal to lean, fall and drift sideways during movement, with a wide-based slowed gait that has irregular but relatively predictable foot placements and a tendency for hypometria (Mayhew, 2009). These clinical signs were demonstrated by horses in this trial and were accentuated by blindfolding. A hypometric gait was particularly noted in horse #1, in that during the swing phase the limb weaved under the body and then was placed in a wide-based position, demonstrating a decreased degree of flexion.

When blindfolded, horses lacked visual input and if we assume that vestibular input was miscoded due to lolitrem-B intoxication the integrative system relied solely on GP input. It is proposed that, when blindfolded, GP input rescued horses from falling. GP pathways provide neural input about the body's position in space, the progress of movement as it occurs, the state of muscle contraction and the surface of the ground (Bradley, 1991). Functioning GP pathways were indicated by the following observations. Firstly, GP input to the integrative centers may have enabled the increased load bearing on a limb and thus the excessive movement of the center of mass to be perceived. However, this is a perception of present consequences rather than pre-emption of future consequences, as joints, muscles and tendons can only detect an increased load once it has occurred (Bradley, 1991). This is unlike the vestibular system, where a prediction of malequilibrium is made, enabling anticipatory adjustments of movement. This difference was observed in that although horses were able to prevent falling, they were unable to prevent imbalance. Secondly, rapid, strong, corrective limb movements restored imbalance. Spastic paresis due to disruption of UMN pathways often prevents rapid movements in GP ataxia, yet rapid and short limb movements are characteristic of vestibular ataxia (de Lahunta & Glass, 2009). Corrective movements require an awareness of the position of the neck, trunk and limbs in space (information provided by GP pathways) in order to place the limb in accordance to the movement of the trunk. Finally, blindfolded horses responded

appropriately to being led in a serpentine manner, which involves strong direction leads via the halter that move the head and neck. Receptors of the GP pathways in the neck muscles must detect the ordered change in direction and via the cervicospinocerebellar tract inform the cerebellum, enabling rapid changes in trunk and limb movement (de Lahunta & Glass, 2009). The regular gait during serpentine movements contrasts the way horses moved when minimal guidance was given from the handler: horses were unable to maintain their direction of movement and the trunk tended to drift, necessitating rapid, compensatory movements of the limbs. This indicates that when blindfolded, horses relied on reference information from body movements rather than the perception of body position with respect to gravity.

A wide-based stance is a biomechanical adaptation that improves stability by broadening the boundaries of limb support and is characteristic of all qualities of ataxia. However, in addition to physical stability, in the presence of a functional GP system a wide-based stance enhances the sensitivity of GP input to perturbations of trunk movement. This is because when the distal limb is positioned wider than the shoulder or hip joints, movement of the distal limb results in proportionally greater movement in the proximal limb than when the same degree of distal limb movement occurs with a base-narrow stance. And thus a wide-based stance amplifies sensory input from the proximal muscles and joints (Day, et al., 1993). Therefore, the effectiveness of wide-based stance will be greater with a functional GP pathway. A clinical comparison of the degree of imbalance that occurs at different stance widths between vestibular and GP ataxia has not been investigated in quadrupeds or humans. However, this is a concept worthy of thought that may provide a differentiating feature between vestibular and GP ataxia.

Vestibular dysfunction appeared to dominate the quality of ataxia observed in lolitrem-B intoxication of horses; however, aspects of GP ataxia were also observed. After abrupt cessation of rapid movements, horses showed a delay in correcting their initial awkward posture and often did not correct until the following movement was initiated. This observation is indicative of GP abnormalities in large animals (Mayhew, 2009) and therefore suggests that a degree of GP dysfunction occurs in lolitrem-B intoxication. In addition, horses that were most severely affected by lolitrem-B intoxication showed an increased occurrence of postural deficits during complex maneuvers when blindfolded. Complex maneuvers, such as head elevation, tight circling, tail pull and backing, increase the demand on the UMN and GP pathways and elicit or exacerbate clinical signs associated with GP ataxia (de Lahunta & Glass, 2009). Postural deficits displayed by horses #1 and #2 include delayed initiation of protraction, especially of the

pelvic limbs during circling; excessive abduction of the outside hindlimb during circling, resulting in a wide swinging motion termed circumduction; and interference between contralateral pelvic limbs. Vestibular dysfunction would not have been expected to cause these clinical signs, as it is the only system involved with movement that when deficient does not cause postural deficits (de Lahunta & Glass, 2009). Other aspects of GP ataxia were not displayed. These would have included a longer thoracic limb stride that hesitates briefly before placement of the hoof, giving an appearance of floating and overreaching of limbs during swing phase, and highly variable and unpredictable foot placement (de Lahunta & Glass, 2009; Mayhew, 2009). The syndrome of GP ataxia also normally includes weakness, because morbid lesions usually disrupt both sensory and motor tracts, which tend to run adjacent throughout the central and peripheral nervous systems (Mayhew, 2009). Other than a delay in the onset of protraction displayed by horses graded 3/5, UMN and LMN paresis were not evident during the treatment period. However, this need not discount GP ataxia as lolitrem-B intoxication may influence the conduction properties of sensory tracts independent of motor pathways, resulting in an unusual presentation of GP ataxia. Therefore, it appears that partial GP deficits occurred as a result of lolitrem-B intoxication. However, ataxia was dominated by qualities of vestibular ataxia initially and features of GP ataxia only became evident when horses were more severely affected.

A combined reduction of both vestibular and GP function is possible due to the wide distribution of BK channels and the possibility that lolitrem-B blockade may not act like an morbid lesion in that nerve conduction may still occur but the encoding of information may be inaccurate. Indeed, horses showed signs of bilateral vestibular dysfunction yet continued to display normal physiological nystagmus. This indicates that information from the peripheral vestibular system was not obliterated (de Lahunta & Glass, 2009). Rather, it appears that information was miscoded, which is consistent with the roles of BK channels in the vestibular system as discussed in chapter 3. Lolitrem-B intoxication is a functional rather than structural disease, therefore the clinical signs need not conform to the typical presentation of ataxia that results from morbid lesions of the nervous system.

6.3.4: Involvement of the cerebellum in severe lolitrem-B intoxication

Nystagmus, hypermetria and cerebellar fits have been reported in association with PRGS (Cunningham & Hartley, 1959; Galey, et al., 1991; Hunt, et al., 1983; Mackintosh, et al., 1982; Mayhew, 2007; McLeay, et al., 1999) and as all three occur with cerebellar dysfunction (Holliday, 1980), the involvement of the cerebellum cannot

be discarded. However, the lack of clinical descriptions included in these reports requires the assumption that terminology has been applied appropriately. In addition, each of these signs can also be explained by mechanisms independent of the cerebellum. Abnormal nystagmus was not observed in horses in this study, but nystagmus has been reported in sheep and deer in association with severe PRGS (Mackintosh, et al., 1982; McLeay, et al., 1999). Physiological nystagmus is controlled via the vestibular ocular reflex, which involves afferent input from the vestibular system, integration by the cerebellum and via the medial longitudinal fasciculus connects to the nuclei of cranial nerves III, IV and VI that control normal eye position, saccades and physiological nystagmus (de Lahunta & Glass, 2009). Abnormal nystagmus indicates an imbalance of sensory information from the peripheral vestibular receptors or dysfunction of the central vestibular and cerebellar components, causing inappropriate input to the motor neurons that control the muscles of the orbit (de Lahunta & Glass, 2009). As vestibular dysfunction caused by lolitrem-B intoxication appears to be symmetrical, the nystagmus observed in ruminant trials may be due to cerebellar dysfunction. Alternatively, previous observers may have mistaken tremor of the eyeball for nystagmus. The fine tremor observed with an ophthalmoscope in this trial may increase in amplitude as the severity of lolitrem-B intoxication advances, resulting in eyeball tremor detectable by the unaided human eye. Another explanation is that severe tremor of the head may have given the appearance of nystagmus. Like nystagmus, hypermetria may have been mistakenly diagnosed for the overreaching during the swing phase that occurs with GP dysfunction (de Lahunta & Glass, 2009; Mayhew, 2009), while cerebellar fits may be caused by changes in neural or muscle cell membrane conduction independent of the cerebellum, as discussed in section 3.3.2 and 6.2.1.

Sausbier et al. (2004) reported that the phenotypic deficiency in motor function and coordination of $BK_{\alpha}^{-/-}$ mice represented cerebellar dysfunction. The deficiencies particularly related to the accelerated rotarod test, which is sensitive to cerebellar and basal ganglia deficits, and also sensorimotor learning in the eye-blink reflex, a test specific to the cerebellum. These findings were correlated to the cellular level, where Purkinje cells from $BK_{\alpha}^{-/-}$ showed a reduction in spontaneous firing activity due to depolarisation-induced inactivation of the action potential (AP) mechanisms (Sausbier, et al., 2004). Although cerebellar signs were not observed in the present study, this does not disprove that lolitrem-B may influence Purkinje cell function and cause cerebellar dysfunction as the severity of PRGS advances. A progression to include cerebellar dysfunction occurs in essential tremor, where in addition to a postural tremor the disease can progress to include an intention tremor, eye movement disorders

characteristic of cerebellar dysfunction and ataxia (Helmchen, et al., 2003; Stolze, Petersen, Raethjen, Wenzelburger, & Deuschl, 2001). Ruminant studies generally report that cerebellar fits are restricted to the most severely affected animals (Cunningham & Hartley, 1959; Galey, et al., 1991; Mackintosh, et al., 1982). However, more mildly affected animals show sway and stiffness in gait, signs that indicate vestibular dysfunction. Therefore with moderate intoxication vestibular ataxia predominates; however, GP and cerebellar dysfunction may feature with severe intoxication. Furthermore, there appears to be a difference in concentration requirements of lolitrem-B in order to generate tremor and ataxia; in mice, ataxia requires higher doses of lolitrem-B than tremor (Imlach, et al., 2008). This indicates that a progression of neuromuscular signs occurs from muscle tremor followed by a predominately vestibular ataxia with some components of GP dysfunction that become increasingly evident as the severity of PRGS increases, and a potential subsequent development of cerebellar dysfunction. The different susceptibility of neural systems to lolitrem-B may depend on altered affinity for lolitrem-B by variance in β-subunit, alternate splicing, associated voltage gated Ca^{2+} (VGC) channels, phosphorylation and other modulators, the availability of compensatory ion channels or neural pathways, the concentration of lolitrem-B distributed to each locality and/or our ability to exacerbate the clinical signs associated with each type of ataxia. Based on these observations a scoring system that reflects the progression of clinical signs in lolitrem-B intoxication is presented in **table 6.1**.

Score	Clinical Signs
0	No clinical signs
1	Slight sway and/or slight tremor when feeding and after exercise No ataxia with or without blindfold
2	Obvious sway and/or tremor when feeding and after exercise Mild to moderate disequilibrium when blindfolded No ataxia when not blindfolded
3	Obvious sway and/or tremor when feeding and after exercise Moderate to severe disequilibrium and postural deficits when blindfolded Mild ataxia when not blindfolded
4	Sever tremors and ataxia
5	Collapse and cerebellar fits

Table 6.1: Scoring system proposed for use in evaluating the severity of PRGS in horses.

6.4: Horses intoxicated with lolitrem-B displayed allodynia and an increased resting heart rate

Previous reports in ruminant and horse trials on PRGS report that animals become hyperaesthetic (Hunt, et al., 1983; Munday, et al., 1985; Rowan, 1993), which by definition refers to an exaggerated response to any external stimulus. During lolitrem-B exposure, horses in this study showed an increased response to a repeated, conditioned slap on the withers and a tendency to startle or hesitate while walking through gateways. The latter behaviour was possibly due to vertigo associated with the miscoding of vestibular signal, but may also reflect an increased response to an external stimulation. However, horses did not show an exaggerated response to sensation tests involving stimulation with blunt artery forceps, firm stroking of the lateral dorsum from the withers to the caudal gluteal region using blunt artery forceps, intravenous injections, threatening gesture of the hand towards the eye in the menace response test, or auditory stimuli such as a dog barking. This suggests that the term allodynia, rather than hyperaesthesia, is more apt in describing the increased response observed in this trial. Allodynia is a subclass of hyperaesthesia that refers here to an abnormal, increased responsiveness to a physiological stimulus (de Lahunta & Glass, 2009). The spectrum of stimuli that result in an exaggerated response is more particular than in hyperaesthesia, where the animal is hypersensitive to any external stimuli. Although the increased response that occurs with allodynia does not necessarily involve pain, allodynia is often associated with a change in the modality of the sensation evoked by mechanoreceptors from touch to pain. A model is suggested where a central presynaptic link between mechanoreceptor and nociceptor afferent neurons results in activation of second order neurons, that are normally stimulated by nociceptor input, in response to the physiological stimulus of touch (Cervero & Laird, 1996). As mentioned previously in section 3.3.4, BK channels are expressed in the dorsal root ganglia (Li, et al., 2007) as well as in the superficial dorsal horn of the spinal cord (Furukawa, et al., 2008), and regulate the firing frequency, AP duration and refractory period, and synaptic transmission of afferent neurons in these regions. Therefore lolitrem-B blockade of BK channels in the dorsal root ganglia and superficial dorsal horn may modify the conduction properties and synapses of mechanoreceptor and nociceptor afferents at the spinal level. The characteristics of BK channels, and thus the influence of lolitrem-B, are determined by the entire biophysical context of individual channels (section 3.2.4), and this may explain why only selective stimuli cause an increased response, rather than all external stimuli.

Horses also showed a significant increase in heart rate during the treatment period ($p = 0.018$), indicating an increased degree of sympathetic tone, which may lower the threshold for the sympathetic stress response and may contribute to the generation of allodynia. Independent of, or in addition to the neural actions of BK channels, lolitrem-B intoxication may influence the degree of sympathetic tone by increasing the release of catecholamines from the adrenal medulla (Gonzalezgarcia, Cena, Keiser, & Rojas, 1993). Chromaffin cells of the adrenal medulla secrete catecholamines in response to the increase in $[Ca^{2+}]_i$, which occurs when chromaffin cells receive neural stimulation (Guyton & Hall, 2000; Marty & Neher, 1985). Immunohistochemistry and Western blot analysis show that BK channels are highly expressed in the adrenal glands (Brunton, et al., 2007) and patch clamping and whole cell recordings of chromaffin cells have detected K^+ currents characteristic of BK channels that are inhibited by tetraethylammonium (TEA) and Iberiotoxin (IbTX) (Marty & Neher, 1985; Neely & Lingle, 1992; Wada, et al., 1995). It has been proposed that BK channels control the gating of VGC channels and thus the release of catecholamines that occurs in response to nicotinic receptor activation in chromaffin cells. Also, blockade of BK channels has been shown to augment catecholamine release (Gonzalezgarcia, et al., 1993; Nagayama, et al., 1998; Wada, et al., 1995). Furthermore, it has been suggested that BK channel blockade may increase catecholamine secretion by interfering with the BK channel-mediated negative feedback control of neurotransmitter release at the presynaptic terminal of the neural–chromaffin cell synapse (Nagayama, et al., 2000). Dopamine, via binding to D_1 and D_2 receptors, also modulates catecholamine release from the adrenal medulla (Pivonello, et al., 2004). Therefore, ergovaline, a D_2 receptor agonist (Larson, et al., 1995), may act synergistically with lolitrem-B causing a sympathomimetic effect.

The increase in heart rate observed in horses during the treatment period supports an increase in sympathetic tone, but contrasts with previous studies. Fisher et al. (2004) reported that the mean heart rate was not different in steers fed PRG straw containing <0.1 ppm lolitrem-B and those receiving 1.55 ppm lolitrem-B for 25 days. Furthermore, mice lacking BK channels show an increase in blood pressure, but do not show an increase in heart rate (Sausbier, et al., 2005). The conflicting results may be due to the lower dose rate used by Fisher et al. (2004) compared to >2.0 ppm lolitrem-B used in this trial. Knockout mice show a degree of homeostatic remodeling, as they show markedly milder signs compared to wild type (WT) mice that are dosed with lolitrem-B (Imlach, et al., 2008); therefore, other channels or neurons involved in the regulation of heart rate may have modified their roles to help compensate for the lack of BK channels.

6.5: Complete blood count and serum biochemistry

The trend for total serum calcium to increase during the treatment period, although significant, neither reflected the severity of clinical signs nor increased above reference ranges. Therefore, the change could relate to different levels of calcium in the feed, rather than a direct influence of lolitrem-B. Post-treatment values for total serum calcium tended to be lowest and below the reference range in horses graded 3/5; however, this trend was also present prior to exposure so is unlikely to be due to lolitrem-B intoxication. Total serum magnesium also showed an increasing trend where some post-exposure values did exceed the maximum reference value, but again the change in values did not relate to the severity of clinical signs. The trend for the levels of both these ions to increase during the treatment period supports the independence of lolitrem-B intoxication from the neurological syndrome that results from hypocalcaemia and hypomagnesaemia (Mayhew, 2009). Magnesium drenching has been suggested as a treatment for PRGS; however, results from a trial in sheep and calves indicate nil efficacy (Allsop & Watters, 1984), which is supported by results from this investigation. Post exposure values of serum K^+ showed a positive correlation to the severity of PRGS. This relationship will be discussed later in section 6.8.

Several biochemical tests showed a statistically significant difference between the control and treatment period, but are unlikely to represent pathology induced by lolitrem-B intoxication. For example, serum AST and GDH were significantly decreased, but only elevations in these enzymes are indicative of cell damage in liver, muscle and other cells (Stockham & Scott, 2002). Serum GGT, an indicator of cholestasis (Stockham & Scott, 2002), significantly increased after lolitrem-B exposure, but did not exceed the upper limit of normality and therefore is unlikely to relate to liver dysfunction. The significant decrease in urea is most likely due to dietary difference between control and treatment feeds. Serum urea levels are determined by the formation of urea in the liver from dietary and endogenous amino acids and also by the amount of urea that is reabsorbed in the renal tubule, which is inversely proportional to renal tubule flow rate (R. W. Nelson & Couto, 2003). Therefore, decreased serum urea can result from decreased protein dietary intake, reduced hepatic ability to convert ammonia to urea, or diuresis. As blood samples for serum biochemistry were taken prior to frusemide administration and additional evidence of hepatic dysfunction is lacking, decreased protein dietary intake appears the most likely cause. Therefore, although statistically significant these findings are not likely to be of importance in the clinical description of lolitrem-B intoxication in horses.

The decrease in AST activity observed in this trial contrasts with a previous study where serum AST was elevated in sheep with PRGS and was thus interpreted to indicate liver dysfunction (Piper, 1989). A later study where CK was also raised suggested that elevation of these enzymes was indicative of enzyme leak from damaged muscle cells rather than the liver (Fletcher, 1993). This is supported by reports of secondary myonecrosis in three sheep that were severely affected by PRGS (Cunningham & Hartley, 1959). Horses in this trial showed no significant change in CK, indicating that muscle damage did not occur, which concurs with the AST activity. The severity of tremor experienced and the occurrence of recumbency may influence the level of these enzymes and explain the discrepancy between studies.

There was a substantial trend for horses graded 3/5 to show an increase in fibrinogen and globulin levels and a decrease in serum albumin. These changes are indicative of an inflammatory process, which may or may not involve infectious agents (R. W. Nelson & Couto, 2003), and might relate to the limb swelling and lesions that will be discussed in section 6.9. Although not statistically significant, there was trend for total protein to increase, which is supported by the previously reported hyperproteinaemia in horses showing signs of PRGS (Hunt, et al., 1983). Further investigation is required to determine if these changes relate to lolitrem-B intoxication or to confounding factors. Haematological values did not indicate the presence of infection in horses graded 3/5 or any clinically detectable influence of lolitrem-B on immunity. Grossly, cerebrospinal fluid appears unchanged. Further investigation of the biochemical and cytological features, as well as the levels of neurotransmitters in cerebrospinal fluid may reveal changes that occur in association with lolitrem-B intoxication.

6.6: Brainstem auditory evoked potentials

The presence of characteristic peaks in the brainstem auditory evoked potentials (BAEPs) recorded after lolitrem-B exposure in five of the seven horses indicates that lolitrem-B intoxication does not cause deafness. However, comparing the latency of peak V in the pre- and post-exposure traces suggests that the latency may be prolonged post-exposure. The mean ipsilateral peak V latency obtained from the three pairs of traces from horses #1, #2 and #7 increased from 4.9 ± 0.3 ms pre-exposure to 5.7 ± 0.6 ms post-exposure ($p = 0.250$), while the mean contralateral peak V latency increased from a pre-exposure value of 5.2 ± 0.3 ms to 5.7 ± 0.6 ms post-exposure ($p = 0.159$). Compared with reference values for thoroughbred horses (Mayhew & Washbourne, 1992), pre-lolitrem-B mean exposure values are within the normal reference range (ipsilateral peak V: 4.90 ± 0.207 ms; contralateral peak V: 5.15 ± 0.147

ms); whereas post-exposure mean values for both ipsilateral and contralateral peak V latencies exceeds the maximum limit, which was calculated as the reference mean plus two standard deviations (ipsilateral maximum = 5.52 ms, contralateral maximum = 5.59 ms). Therefore, although relationships were not significant, there did appear to be a tendency for the post-exposure values for peak V latency to be greater than pre-exposure values and to exceed reference ranges.

6.6.1: A delay in peak V latency indicates reduced temporal precision, but is unlikely to influence the behaviour of horses

A delay in peak V latency represents a delay in conduction anywhere along the auditory path from the IHC–afferent nerve synapse to the midbrain (Mayhew & Washbourne, 1997). The lack of clearly defined peaks III and I precluded a more precise location of the delayed conductance. However, a delay along this path is consistent with BAEPs from $BK_{\alpha}^{-/-}$ mice, which show a delay in the first peak latency that was attributed to delayed transmission across the IHC–afferent nerve synapse (Pyott, et al., 2007). A prolonged latency in BAEPs in association with BK channel blockade can be explained by the lengthened time constant of receptor potentials in IHCs and the reduced sound evoked discharge rate in auditory afferent nerves that occur in the absence of BK channel function and results in reduced temporal precision of the encoded auditory signals (Oliver, et al., 2006). Therefore, lolitrem-B intoxication does not cause deafness per se, but may disrupt the encoding of auditory signals.

As discussed in chapter 3, the accurate and precise timing of the encoded stimulus onset is important for locating sounds on a horizontal plane. Mammals deduce the location of sound by calculating the time lag between acoustic signals entering the two ears (Guyton & Hall, 2000). Therefore, despite the potential diagnostic use of changes to the BAEP trace, a reduced ability to locate sounds might influence horse behaviour. Previously it was thought that localisation acuity depended on the interaural distance (R. S. Heffner & H. E. Heffner, 1983). Despite the tightly conserved positive relationship between interaural distance and the BAEP I – V inter-peak latency in horses and ponies (Mayhew & Washbourne, 1997), the horse was found to have poor localisation acuity relative to other large mammals (H. E. Heffner & Heffner, 1984). The range of hearing for a horse is 1 kHz to 16 kHz (R. S. Heffner & H. E. Heffner, 1983) but they can only localise sounds up to 2 kHz by calculating the interaural time difference (H. E. Heffner & R. S. Heffner, 1983; R. S. Heffner & H. E. Heffner, 1983). H.E. Heffner and Heffner (1984) proposed that horses, having wide panoramic vision, could scan nearly the entire horizon and rely on this sense to locate objects rather than auditory cues. In contrast, mammals with a greater degree of frontal vision require

more precise sound localisation to direct their eyes onto an object (H. E. Heffner & Heffner, 1984). Other prey species that evolved in wide open environments, such as cattle and goats, are also relatively inaccurate in localising sound — these animals also have a large interaural distance and a small degree of binocular vision (R. S. Heffner & Heffner, 1992). It appears that horses do not normally depend on auditory cues alone to locate objects (H. E. Heffner & Heffner, 1984) and therefore it is unlikely that a reduction in the ability to localise sound due to BK channel blockade–induced temporal imprecision would significantly alter behaviour and increase the animals tendency to startle. Therefore, if further studies were to confirm a significant prolongation in peak V latencies, this will be diagnostically useful, but not of major behavioural importance.

6.6.2: Limitations of the research design that may have caused the lengthened latency of all peaks and produced traces that were unable to be interpreted

It is possible that the increased latency of all peaks was due to a block in the ear-piece or poor ear-piece placement, as conduction block effectively reduces the intensity of the click stimulus, which is inversely proportional to the latency of BAEP peaks (A. E. Marshall, 1985; Mayhew & Washbourne, 1990). Further investigation is required, using a larger sample size, to determine whether the increased latency is significant and to define the anatomical correlate of the increased latency, as pairs of interpretable traces were obtained from only three horses.

The production of traces where peaks could not be identified, either before and after lolitrem-B exposure, may have been due to poor or incorrect electrode placement, or interference from ground currents. In addition, frusemide may have interfered with auditory function. The frusemide challenge was performed so that frusemide was administered about 2 hours before BAEPs were recorded, by which time the diuretic effects of frusemide cease (Hinchcliff & Muir, 1991). However, in horses frusemide has a gamma half-life of 160 minutes (Hinchcliff & Muir, 1991) and therefore may still be active in other organ systems. Frusemide acts on the stria vascularis of the cochlear and changes the concentration of K^+ in the endolymph (Hinchcliff & Muir, 1991), which in turn profoundly alters its electrochemical features (Huy, 2002). This trial involved many tests of various organ system functions within a limited time period in order that results of the tests correlated to the duration of exposure and so that exposure duration was not prolonged to the extent that horses became severely ataxic. The disadvantage of this trial design is that tests may interfere with each other i.e. as the frusemide challenge may have interfered with the BAEP results. Therefore, this trial has suggested that lolitrem-B may influence peak V latency; however, further investigation is warranted to determine the anatomic location and significance of any

prolongation of latencies. Furthermore, obtaining BAEP traces where peak III is clearly identifiable would enable the investigation of the wave III – V dispersal of BAEP traces. This is calculated by dividing the height of the III – V complex by its base (Mayhew & Washbourne, 1992) and may support the report that IbTX infusion of the cochlea causes dispersion of the compound action potential (CAP) (Beurg, et al., 2005; Skinner, et al., 2003) and thus strengthen the concept that lolitrem-B influences temporal precision of auditory signals.

6.6.3: Brainstem auditory evoked potentials are unlikely to represent the vestibular dysfunction that resulted in ataxia in intoxicated horses

Although BAEP traces have been used to detect morbid vestibular dysfunction in horses (Bedenice, et al., 2001), BAEPs from this trial cannot be used to decipher the origin of vestibular dysfunction that caused ataxia. BAEPs can be used to indirectly locate morbid lesions causing vestibular dysfunction, as the close anatomic relationship of vestibular and auditory pathways allows singular lesions to influence both systems (Bedenice, et al., 2001; Mayhew, 2003). However, conduction disturbances due to channelopathies are not expected to show such anatomical relationships.

6.7: Magnetic motor evoked potentials

Magnetic motor evoked potentials (mMEP) recorded from the extensor carpi radialis muscle showed a strong trend for the take-off and peak latency to increase after lolitrem-B exposure ($p = 0.06$ and 0.07, respectively). The mean latency to take off increased from 16.2 ± 6.2 ms ($n = 4$) to 28.3 ± 9.6 ms ($n = 4$) and the mean latency to first peak increased from 24.5 ± 5.7 ms ($n = 4$) to 33.8 ± 10.8 ms ($n = 4$). Post-exposure values for the take-off latency exceed the value previously reported for horses of 20.81 ± 1.85 ms (Nollet, et al., 2002). Latency is the interval between the delivery of the magnetic stimulus and the resulting electromyography response (Nollet, et al., 2002). It reflects total motor conduction time from the cortex to the target muscle and is influenced by the size of the neuronal fibre, the abundance of myelin and the number and functional characteristics of synapses that the impulse must cross (Nollet, Van Ham, Deprez, et al., 2003).

6.7.1: The neural pathways that mediate the magnetic motor evoked potentials in horses have not been determined and may involve extrapyramidal tracts

Transcranial magnetic stimulation produces a characteristic electromyographic response that is produced by the transmission of synchronised descending excitatory volleys from the forebrain, for which the latency depends on the conduction velocities in

the fastest descending brainstem and spinal tracts that contain the least number of synapses (Nollet, Van Ham, Deprez, et al., 2003). In man, this pathway is the pyramidal corticospinal tract, which connects monosynaptically to lower motor neurons (LMNs) (Nollet, Van Ham, Deprez, et al., 2003). However, it is not certain which pathway mediates the response in horses. Pyramidal corticospinal pathways are poorly developed in the horse, indicating that these pathways do not directly innervate limb musculature (de Lahunta & Glass, 2009). This leaves cortico-rubrospinal, cortico-reticulospinal, cortico-vestibulospinal or other tracts of the extrapyramidal system (Guyton & Hall, 2000). Indeed, there is some evidence that mMEP conduction occurs through extrapyramidal tracts in rats (Adamson, Zappulla, Fraser, Ryder, & Malis, 1989). Extrapyramidal tracts are multisynaptic corticospinal tracts that originate in the motor cortex and descend via basal nuclei and the brainstem relay regions to LMNs in the spinal cord (de Lahunta & Glass, 2009; Guyton & Hall, 2000). The synaptic delays of such multisynaptic pathways inherently increase the latency of neural conduction. However, if structures deep to the motor cortex are depolarised directly by transcranial magnetic stimulation then some synapses may be bypassed, reducing the latency to values that compare with conduction velocities of pyramidal corticospinal mMEP traces. In the horse, the motor cortex occupies the rostral one-half of the dorsal surface of the cerebral hemisphere (Breazile, Swafford, & Biles, 1966); however, it has been suggested that a much larger area of the brain is stimulated by transcranial magnetic stimulation (Nollet, Van Ham, Dewulf, et al., 2003). The depth of penetration achieved by transcranial magnetic stimulation in the horse is debated. The magnetic field produced by the coil attenuates rapidly with increasing distance from the coil, suggesting that deeper motor structures such as the basal nuclei or thalamus would receive minimal stimulation; but, the large coils used in the horse may achieve a reasonable depth of penetration, as depth of penetration is positively correlated with coil size (Nollet, Van Ham, Deprez, et al., 2003). Therefore, extrapyramidal pathways, such as the thalamo-bulbo-spinal tracts, may be stimulated by transcranial magnetic fields reaching deeper areas of the brain, possibly diencephalic or mesencephalic, subcortical structures, in addition to stimulation of extrapyramidal neurons in the motor cortex. Stimulation of deeper structures would produce the initial mMEP trace as some synapsing is avoided, while later signals would correlate to those originating in the motor cortex. In support of this concept, electrical stimulation of different areas of the exposed motor cortex of anaesthetised horses showed that the area of the motor cortex that represents limb muscles was much less sensitive in horses than primates (Breazile, et al., 1966), which is consistent with the requirement to stimulate deeper subcortical structures for initiation of limb muscle activity. Unlike humans, horses do

not show any difference in response with changes in orientation of the magnetic coil or any great sensitivity to the positioning of the coil (Nollet, Van Ham, Dewulf, et al., 2003), indicating that in the horse the magnetic stimulus may spread throughout a broader and deeper area of the brain than in humans. Therefore, it is possible that the mMEP trace in horses is mediated by neural conduction along extrapyramidal pathways with signals originating in deep brain structures, i.e. a thalamo-bulbo-spinal system, in addition to the motor cortex.

6.7.2: Blockade of large-conductance Ca^{2+}-activated K^+ channels may reduce neural conduction in the extrapyramidal pathway

According to the known roles of BK channels at various locations along the neuron (Gribkoff, et al., 2001) lolitrem-B may have increased the latency of the mMEP trace by blocking the action of BK channels at various sites on the neuron. A single transcortical magnetic stimulation is able to produce multiple descending AP volleys from the cortex (Nollet, et al., 2002). Both spatial and temporal summation of UMN impulses in the dendrites of the LMN is required before the LMN is depolarised sufficiently to generate an AP (Guyton & Hall, 2000; Nollet, et al., 2002). BK channels are important for high frequency firing (Gu, et al., 2007; Jin, et al., 2000) and thus for the temporal summation of postsynaptic potentials. Lolitrem-B blockade of BK channels may reduce the firing rate of the stimulated neurons and may result in reduced temporal summation of UMN impulses at the LMN. Therefore, the fastest firing neurons may require spatial summation of inputs from the slower conducting UMNs to generate an AP in the LMN, resulting in an increase in mMEP latency. In addition, BK channel blockade may result in a reduction in neurotransmitter release at the presynaptic terminal of either neuronal or neuromuscular synapses (Pattillo, et al., 2001; Spencer, et al., 1989), causing a further reduction in presynaptic input and therefore requiring the recruitment of other slower conducting neurons before the LMN is depolarised sufficiently. Alternatively, or in conjunction with the previous theory, BK channel blockade may result in depolarisation-induced inactivation of voltage gated Na^+ (VGNa) channels and silencing of UMNs that display burst firing activity (Jin, et al., 2000; Raman & Bean, 1999; Sausbier, et al., 2004). If these neurons mediate the fastest conducting pathway, their silence would increase mMEP latency as slower conducting neurons would remain to excite the LMN. Therefore, BK channel blockade may lengthen the mMEP latency by reducing the firing rate and the amount of neurotransmitters released by UMNs, and/or by silencing the fastest conducting neurons. The exact role of BK channels in mediating the mMEP response will remain

as speculation until the pathway involved in mMEP generation in horses is determined and the role of BK channels in this pathway defined.

A lengthening in mMEP latency is plausible according to the roles of BK channels, but is usually associated clinically with weakness due to LMN or UMN deficits. The delayed onset of protraction displayed by horses graded 3/5 might indicate UMN paresis. However, latency was lengthened in the absence of clinical signs of UMN paresis in horse #4. Horses in this trial displayed no other signs of weakness such as poor ability to maintain posture, support weight or resist gravity. Therefore, lengthening of the mMEP latency may detect subclinical UMN dysfunction. Alternatively, limitations of the research design may have caused a lengthening of the latency.

6.7.3: Limitations of the research design that may have caused the lengthened latency and produced traces that were unable to be interpreted

Supra-maximal stimulations may not have been reached in all recordings as stimulation output was increased only to a level that interpretable traces were achieved without undue startle response from the subjects, which was usually between 60 – 80% of maximal stimulation output. Therefore, 100% stimulation output was not always reached. As the stimulator output increases, traces show a shorter latency and higher amplitude (Mayhew & Washbourne, 1996). Therefore, lower stimulator outputs may have been used in post-exposure traces than pre-exposure traces. In addition, the standing horse has an uncontrolled degree of muscle tone in postural limb muscles (Nollet, Van Ham, Dewulf, et al., 2003). Slight voluntary contraction of the target muscle shortens the onset and first peak latencies, lowers the threshold and increases the amplitude of the recording by summation of descending voluntary impulses from cortical areas with afferent impulses from muscle spindles and with magnetic-induced stimulation of UMNs (Nollet, Van Ham, Dewulf, et al., 2003). This non-controlled facilitation can influence the threshold of maximal stimulation and therefore the extent of neural stimulation is difficult to control in horses (Nollet, Van Ham, Dewulf, et al., 2003).

Facilitation may also have contributed to the production of traces that could not be interpreted because they had premature (<10 ms), movement-derived deflections or they had no clear baseline **(Appendix 6.2.2)**. The clinical observation that horses showed forelimb tremor was not believed to increase the probability of facilitation as both pre- and post-exposure traces caused pairs of traces to be discarded from the paired t-test analysis of latency. Furthermore, perhaps due to sedation, horses were not showing detectable tremor during the test. The other reason for discarding

recordings was that the traces were of low amplitude, lacked a singular prominent peak and showed wide dispersion, likely due to sub-maximal stimulation.

6.8: The extent of frusemide-induced K^+ and Na^+ excretion and basal levels of aldosterone were reduced after exposure to lolitrem-B.

The frusemide challenge was designed to challenge the renal BK channels with an increase in renal tubule flow rate and tubular pressure. Fractional excretions for K^+ and Na^+ (FEK^+ and $FENa^+$) were calculated. The graphed results **(fig. 5.9 A and B)** show that the change in FEK^+ during the first 15 minutes after frusemide administration (ΔFEK^+_{15}) was significantly greater ($p=0.003$) before the horses had been exposed than after two weeks into lolitrem-B exposure.

6.8.1: Mechanisms of reduced flow-mediated K^+ secretion by lolitrem-B

BK-β_1 channels have been located in the principal cells (PCs) of the cortical collecting duct (CCD) and the connecting tubule (CNT) (Grimm, et al., 2007; Pluznick, et al., 2005) and are involved in flow-mediated K^+ secretion (FMKS) (Pluznick & Sansom, 2006; Pluznick, et al., 2003). The apparent decrease in FMKS after lolitrem-B exposure is in keeping with lolitrem-B blocking these BK channels and is supported by the inability of mice lacking either the α- or the β_1-subunit to elevate K^+ secretion in response to increased renal tubular flow rates (Bailey, et al., 2006; Pluznick, et al., 2003; Pluznick, et al., 2005). Post-exposure values for serum K^+ showed a positive correlation to the severity of PRGS ($p = 0.017$), but did not exceed reference maximums **(fig. 5.4 C)**. This trend further supports the concept that lolitrem-B disrupts K^+ secretion.

Lolitrem-B exposure reduced but did not abolish FMKS. This could be due to BK channels not being saturated and therefore, remaining BK channels that are not blocked by lolitrem-B could produce the persisting FMKS. Alternatively, renal outer medullary K^+ (ROMK) channels, which are predominantly responsible for baseline K^+ secretion (Frindt & Palmer, 2004), may be activated to compensate for the lack of BK channel current. ROMK channels are also activated by Ca^{2+}, but are weakly voltage-dependent and have slower kinetics than BK channels (Frindt & Palmer, 2004). Hydrodynamic bending of apical cilia of PCs has been associated with an elevation of $[Ca^{2+}]_i$ (Malnic, Berliner, & Giebisch, 1989), which would occur despite BK channel dysfunction and may activate ROMK channels; the slower activation kinetics of ROMK channels (Frindt & Palmer, 2004) would account for the more gradual rise in FEK^+ after

exposure than before exposure to lolitrem-B. Although FMKS is thought to occur independent of amiloride-sensitive epithelial Na^+ (ENa) channel activity (Malnic, et al., 1989), FMKS is increased in high K^+ states (Pluznick & Sansom, 2006). As discussed in section 3.6.3, a rise in serum K^+ stimulates aldosterone release, which in turn increases Na^+ reabsorption and thus creates a more negative luminal charge, facilitating the opening of BK channels (Frindt & Palmer, 2004; Garciafilho, et al., 1980; Pluznick & Sansom, 2006). Therefore, although not necessary for FMKS, aldosterone may facilitate this phenomenon. However, in this trial basal plasma aldosterone levels were significantly reduced after lolitrem-B exposure ($p = 0.027$) **(fig. 5.9 D)** and therefore, it is unlikely that aldosterone would have enabled the persisting FMKS during the treatment period.

6.8.2: How lolitrem-B intoxication might influence frusemide-induced changes in fractional excretion of Na^+

There appeared to be a strong, but unexpected, trend ($p=0.072$) for a decrease in the frusemide-induced $FENa^+$ in a manner similar to the effect lolitrem-B blockade of BK channels had on frusemide-induced FEK^+. To answer how lolitrem-B intoxication influences frusemide-induced $FENa^+$, the mechanisms of frusemide-induced $FENa^+$ will be discussed, followed by an explanation of how lolitrem-B may influence these mechanisms.

Frusemide prevents the function of Na^+-K^+-$2Cl^-$ pumps in the thick ascending loop of Henle, resulting in increased delivery of Na^+ to the distal tubule (Hinchcliff & Muir, 1991). ENa channel activity is the rate-limiting step in Na^+ reabsorption (Bhalla & Hallows, 2008). Therefore, if ENa channel activity is insufficient to compensate for the increased Na^+ delivery to the distal tubules then naturesis occurs. ENa channel activity is regulated by the hormones aldosterone, angiotensin II and vasopressin, by extracellular and intracellular proteases, by pH, by luminal and cytosolic ion concentrations, by renal tubular flow rates, and by kinases (Bhalla & Hallows, 2008; Loffing & Korbmacher, 2009). Of these control mechanisms, tubular flow rates and ion concentrations are the most likely candidates to influence the degree of ENa channel activity in the initial period following frusemide administration. ENa channels are mechanosensitive; increased tubular flow rates deflect the extracellular loops of ENa channels and the channel is also distorted by cytoskeleton changes of the PCs that occur with increased tubule diameter (Bhalla & Hallows, 2008). These mechanical changes activate ENa channels independent of $[Ca^{2+}]_i$ or hormonal influence (Bhalla & Hallows, 2008). This increase in activity in response to increased flow rate is balanced

by inhibitory mechanisms, which reduce the channels activity in response to ion concentrations (Bhalla & Hallows, 2008). Increased concentration of intracellular Na^+ causes feedback inhibition of ENa channels, but not immediately (Bhalla & Hallows, 2008). In contrast, self-inhibition can induce rapid changes in ENa channel activity over seconds. This phenomenon involves inhibition of ENa channels in response to extracellular cation concentration (Bhalla & Hallows, 2008). Therefore, the initial dramatic frusemide-induced increase in $FENa^+$ is likely the result of increased Na^+ delivery to the distal tubules causing self-inhibition of ENa channels and overwhelming the combined basal and flow-induced ENa channel activity.

The results of this trial indicate that the frusemide-induced increase in $FENa^+$ during the initial 15 minutes following frusemide administration ($\Delta FENa^+_{15}$) is reduced by lolitrem-B exposure. It is proposed that lolitrem-B influences $\Delta FENa^+_{15}$ either by increasing ENa channel activity and thus Na^+ reabsorption during the initial period following frusemide administration, and/or by interfering with the delivery of frusemide to its site of action. ENa channel activity may be increased either by reduced inhibition or increased activation. In disfavor of the latter, BK channel dysfunction has not been reported to influence the mechanically induced increase in ENa channel activity. Self-inhibition may be reduced by BK channel blockade because of the subsequent effects on luminal ion concentrations. Self-inhibition is not only reliant on luminal Na^+ concentration, but is also influenced by other luminal cations (Bize & Horisberger, 2007). Luminal K^+, although less efficient than Na^+, also inhibits ENa channel activity (Bize & Horisberger, 2007). A reduction in FMKS due to BK channel dysfunction would reduce the concentration of K^+ in the lumen, reducing its contribution to self-inhibition and would thus increase ENa channel activity. Bi-valent cations also influence self-inhibition but with differing effects. Zn^{2+} prevents self-inhibition but Ni^{2+} is additive (Sheng, Perry, & Kleyman, 2004). The effects of Ca^{2+} on self-inhibition have not been determined. Assuming that Ca^{2+} is additive to self-inhibition and that BK channel blockade results in increased Ca^{2+} entry through VGC channels by lack of feedback inhibition (Berkefeld, et al., 2006; Flink & Atchison, 2003; Ghatta, et al., 2006; Robitaille, et al., 1993), lolitrem-B may reduce the luminal concentration of Ca^{2+}, causing further dampening of self-inhibition and thus increase ENa channel activity. BK channels have also been located in the intercalated cells of the CNT (L. G. Palmer & Frindt, 2007), which are involved in acid-base balance (Gurkan, et al., 2007). Therefore, BK channel may indirectly influence ENa channel activity by altering the pH as intracellular alkalinisation enhances ENa channel activity while acidification inhibits ENa channel activity (Bhalla & Hallows, 2008). Alternatively, blockade of BK channels that are located in renal blood vessels, glomerular mesangial cells and in the proximal

convoluted tubules (Frindt & Palmer, 2004; Grunnet, et al., 2005; Kudlacek, et al., 2003; Morita, et al., 1997), may disrupt the delivery of frusemide to it's site of action in the lumen of the loop of Henle (Hinchcliff & Muir, 1991); thereby resulting in a lesser degree of diuresis, naturesis and FMKS.

6.8.3: Lolitrem-B intoxication might result in pathological electrolyte disturbances

The positive correlation between post-exposure values for serum K^+ and the severity of PRGS **(fig. 5.4 C)** suggests that if dose rates of lolitrem-B were increased to cause severe PRGS, exceeding grade 3/5, then pathological disturbance of K^+ may occur. Due to the redundancy of BK channels in basal renal K^+ excretion, severe inhibition of K^+ excretion is unlikely. However, as colonic K^+ excretion relies entirely on BK channel function (Sausbier, et al., 2006), compensatory mechanisms for coexisting renal disease are limited. The lolitrem-B–induced reduction in basal aldosterone may also limit K^+ homeostasis. In addition to the increase in ENa channel activity discussed previously in section 6.8.1, aldosterone also augments the increase in ROMK channel density (L. G. Palmer, Antonian, & Frindt, 1994) and increases BK channel expression and activity in the colon (Sorensen, et al., 2008). Therefore, the synergistic effects of reduced FMKS and plasma aldosterone might result in hyperkalaemia but would probably require coexisting renal disease or a high K^+ diet.

6.8.4: Lolitrem-B induces a reduction in basal plasma aldosterone

Aldosterone was measured in this trial as an increase in plasma aldosterone could prime ENa channel activity, and thus enhance K^+ secretion. $BK_{\alpha}^{-/-}$ and BK-$\beta_1^{-/-}$ mice are reported to have variable serum K^+ levels, but unanimously show an increase in plasma aldosterone compared with WT mice (Grimm, et al., 2009; Pluznick & Sansom, 2006; Rieg, et al., 2007; Sausbier, et al., 2005). In contrast, lolitrem-B intoxication appeared to reduce aldosterone levels. Aldosterone production and secretion occurs in the zona glomerulosa of the adrenal cortex and is controlled by a multitude of regulators including the secretagogues angiotensen II, a high plasma K^+ concentration, ACTH and prolactin (Kau, et al., 1999), and the anti-secretagogue, dopamine (Pivonello, et al., 2004; Wu, et al., 2001). Secretagogues cause an increase in $[Ca^{2+}]_i$ and cAMP, resulting in a change in steroid enzyme activity and aldosterone secretion (Kau, et al., 1999). Lolitrem-B may exert inhibitory effects on aldosterone secretion indirectly by reducing prolactin secretion and influencing the hypothalamic-pituitary-adrenal axis or directly by blocking BK channels located in the cells of the zona glomerulosa.

Prolactin has direct effects on adrenal function and enhances aldosterone production and secretion in a concentration-dependent manner by increasing the activity of aldosterone synthase and 21-hydroxylase and increasing cAMP accumulation (Kau, et al., 1999). Prolactin levels are reduced in hoggets grazing PRG with high levels of endophyte (Fletcher & Barrell, 1984). As discussed in chapter 3, this finding can be explained by the role of BK channels in generating a plateau potential in lactotrophs, which allows sufficient accumulation of $[Ca^{2+}]_i$ to enable exocytosis of prolactin (Stojilkovic, et al., 2005; Van Goor, et al., 2001). Therefore, lolitrem-B may block BK channels located in pituitary lactotrophs resulting in a reduction of serum prolactin and a subsequent reduction in aldosterone production and secretion. BK channel dysfunction may also influence the hypothalamic-pituitary-adrenal axis and reduce ACTH-stimulated aldosterone release. $BK_{\alpha}^{-/-}$ mice showed blunted levels of ACTH in response to restraint with WT mice showing a twofold greater increase in ACTH than $BK_{\alpha}^{-/-}$ mice (Brunton, et al., 2007). Therefore BK channel blockade in the hypothalamus and pituitary gland may indirectly reduce plasma aldosterone levels.

Immunohistochemistry and single channel recordings have shown that BK channels are highly expressed in the zona glomerulosa (Sausbier, et al., 2005) (Vassilev, Kanazirska, Quinn, Tillotson, & Williams, 1992). BK channels, by influencing membrane potential, may be important in controlling Ca^{2+} influx through VGC channels in zona glomerulosa cells and thus aldosterone secretion (Sausbier, et al., 2005; Vassilev, et al., 1992). The direction of influence may vary depending on the biophysical context, as in neural synapses.

None of these explanations account for the conflicting results between our study and those involving knockout mice. One factor that may explain this difference is that in addition to lolitrem-B, test-feed contained high levels of ergovaline while ergovaline was not detected in control feed. Ergovaline is a dopamine agonist of D_2-subtype receptors (Larson, et al., 1995) and therefore may have reduced aldosterone levels directly by an anti-secretagogue effect on zona glomerulosa cells or indirectly by reducing prolactin secretion. Dopamine receptor subtypes D_1, D_2 and D_4 have been localised in the zona glomerulosa and medulla of human adrenal gland by RT-PCR and in situ hybridisation (Pivonello, et al., 2004; Wu, et al., 2001). D_2 antagonists (metaclopramide and raclopride) increase aldosterone secretion (Pivonello, et al., 2004; Wu, et al., 2001), whereas D_2 agonists (bromocriptine and cabergoline) inhibit aldosterone secretion (Pivonello, et al., 2004). The agonistic effects of ergovaline at the D_2 receptor (Larson, et al., 1995) is a plausible mechanism for reduced aldosterone levels during the treatment period. Furthermore, ergovaline suppresses the secretion

of prolactin (Aldrich, Rhodes, Miner, Kerley, & Paterson, 1993; Hovermale & Craig, 2001; Waghorn, et al., 1994), so may act synergistically with lolitrem-B in this indirect modulation of aldosterone secretion.

Intravenous administration of 1 mg/kg frusemide in horses is reported to cause a trend in plasma aldosterone, with levels peaking at 30 minutes post administration followed by a steady reduction to basal levels (Guthrie, et al., 1982). This trend was only weakly demonstrated, both pre- and post-exposure. It is possible that this was due to using an aldosterone assay designed for detecting sheep aldosterone rather than horse aldosterone and other equine steroids may have interfered with the assay. However, these effects would have been a constant source of error in both control and treatment periods.

6.9: Other effects of ergovaline

In this trial, the PRG seed contained high levels of ergovaline in addition to lolitrem-B. As discussed previously in chapter 4, ergovaline causes numerous ill-effects on animal health (Lean, 2001) and can act synergistically with lolitrem-B (Bluett, et al., 2001), and therefore warrants discussion. Toxic thresholds of ergovaline associated with clinical disease in horses are reported as 0.3 to 0.5 ppm (Hovermale & Craig, 2001). The combined intake of ergovaline in seed and hay in this trial was equivalent to ingesting feed containing 2.14 to 2.24 ppm ergovaline — a level that far exceeds toxic thresholds. It should be noted that the toxicity reference values (Hovermale & Craig, 2001) have been developed in North America and are associated with tall fescue ingestion, which contains other non-ergovaline bioactive ergot alkaloids that act synergistically with ergovaline (Gadberry, Denard, Spiers, & Piper, 2003). Therefore, for a given measure of ergovaline, tall fescue based pasture may be more toxic than PRG; however, as values detected in this trial exceed toxic thresholds by fourfold the effect of translating toxicity levels between plant species is probably minor. Ergovaline toxicity in horses has been associated with increased sensitivity to heat stress and reproductive problems (Tor-Agbidye, et al., 2001). Despite the high doses of ergovaline ingested during the test period, no increase in temperature was observed ($p = 0.51$, paired t-test). This may be associated with the dependency of ergovaline on environmental temperature in order to raise body temperature. The absence of change in body temperature in horses in this trial is consistent with other studies conducted in temperate or cool climatic conditions (Fisher, et al., 2004).

Horse #1 developed raised, granulated, fissured lesions around the heel bulbs and palpable heat at the coronary bands of all feet on day #9 lolitrem-B exposure,

correlating with a clinical progression to grade 3/5. There was a profound, dark yellow, serous discharge from these lesions in addition to rhinitis and a dark yellow, serous, nasal discharge. The following day the horse was fed non-PRG, after which lesions began to heal and the serous nasal discharge reduced. In colder climatic regions 'fescue foot' occurs in sheep and cattle ingesting ergovaline and involves ischaemic necrosis and sloughing of the hooves, ears and tail (Tor-Agbidye, et al., 2001), but has not been reported horses. This syndrome is due to the vasoconstrictive effects of ergovaline resulting in gangrene of the extremities (Klotz, et al., 2007; Lean, 2001). Signs include palpable heat, limb swelling, interdigital fissures and visible inflammation of the coronary bands involving scaly raised skin with granulation tissue beneath it (Tor-Agbidye, et al., 2001). This describes well the lesions observed in horse #1. Although horses have been reported to display coolness of the ears and nose after intravenous injection of 15 μg/kgBW ergovaline (Bony, Durix, Leblond, & Jaussaud, 2001), fescue foot has not been reported previously in horses (Cross, Redmond, & Strickland, 1995).

In addition, both horses that were graded 3/5 developed generalised limb edema on the last day of exposure. Edema results from increased capillary pressure, decreased plasma proteins, increased capillary permeability or reduced lymph flow (Guyton & Hall, 2000). Plasma total protein did not show any significant change between pre- and post- lolitrem-B exposure **(Appendix 5)**. Limb edema has been associated with 'fescue foot' in sheep (Tor-Agbidye, et al., 2001) and the venoconstrictive effects of ergovaline (Klotz, et al., 2007) may increase capillary pressure and thereby cause such edema in addition to swelling associated with inflammation from ischaemic necrosis of the limb extremities. Horses that were graded 3/5 tended to adopt a stationary stance and the lack of movement could possibly have resulted in limb edema due to the lack of muscular action available to pump fluid in the lymphatics. Alternatively, limb edema may be consequential to the blockade of BK channels located in lymphatic vessels. Penitrem A, a related mycotoxin to lolitrem-B that also binds to BK channels (Knaus, et al., 1994), slows the frequency and increases the amplitude of smooth muscle contraction of sheep mesenteric lymphatic vessels (McLeay, et al., 1999). Therefore, lolitrem-B may cause spasm of lymphatics in the limbs of horses, reducing their ability to remove interstitial fluid.

These clinical effects of ergovaline may be observed alongside those due to lolitrem-B intoxication as the toxins are both produced by *N. lolii*. However, the concentration of ergovaline in the seed that was fed in this trial was high compared to the normal levels in pasture (Hovermale & Craig, 2001).

Chapter 7: Summary and Conclusion

The aim of this investigation was to describe the clinical effects of lolitrem-B intoxication in horses and relate these signs to those reported in previous studies of perennial ryegrass staggers (PRGS) and to the roles of large-conductance Ca^{2+}-activated K^+ (BK) channels. Tests used in the trial were limited to those that could be used in veterinary clinical practice. To achieve this aim, seven horses were exposed to approximately 2 ppm lolitrem-B in perennial ryegrass (PRG) hay and seed after a period of feeding control feed. This allowed a comparison of data collected before and after lolitrem-B exposure for neurological and clinical examinations, electrophysiological recordings, frusemide challenge testing, cerebrospinal fluid gross analysis, and for plasma and urine lolitrem-B levels. Results supported the first hypothesis that ingestion of lolitrem-B at levels and for an exposure duration that is reported as toxic for ruminants, would display signs of BK channel dysfunction. However, the second hypothesis was not supported; urine showed no significant difference in lolitrem-B levels detected by enzyme-linked immunosorbent assay (ELISA), and although plasma lolitrem-B increased during the treatment period the values did not correspond to the severity of clinical signs displayed by individual horses. The relation of these findings to previous studies of PRGS, the roles of BK channels, and the molecular mechanisms of lolitrem-B were discussed. In addition, the clinical effects that were attributable to ergovaline were reported and discussed. This final chapter summarises and draws conclusions from the previous chapter and identifies the significance of these findings. It will identify how this investigation has advanced our ability to describe the clinical effects of lolitrem-B intoxication in horses and thus diagnose PRGS with greater confidence. Potential diagnostic avenues and their worthiness of further investigation will also be stated.

Lolitrem-B intoxication in horses shows many similarities to the clinical history and presentation in ruminant species, according to trials and case reports of PRGS in ruminants. Differences, such as the slightly earlier onset of signs and higher morbidity likely relate to the closer inspection of individual animals in this trial, while the minimal influence of handling on the severity of signs probably reflects the horse-human relationship rather than a difference in the physiological effects of lolitrem-B between equids and ruminants. Substantial individual variability in the severity of clinical signs occurred despite ingestion of approximately equal quantities of lolitrem-B, but did not correlate with the level of lolitrem-B detected in plasma. These findings indicate a sizeable difference in receptor-drug interactions and/or the availability of neuro-compensatory mechanisms between individual horses, which may be

determined by both genetic and environmental influences. However, further validation of the ELISA test in horses is required to confirm the lack of correlation between plasma lolitrem-B and the severity of PRGS, and thus to discount pharmacokinetic differences.

Horses displayed tremor that varied dramatically between and within individuals from fasciculations and vermiform movements to muscular spasms of the forelimb. The nature of the tremor is inconsistent with that of intention tremors or tremor due to weakness; it is best classified as an action-related repetitive myoclonus but may also relate to episodic repetitive myoclonus. These classifications associate PRGS with other tremor diseases caused by alterations in neurotransmitter release, central neuronal disinhibition and peripheral nerve axon hyperactivity — pathogeneses that are consistent with the role of BK channels. In addition, the combination of eyeball and limb tremor indicates that lolitrem-B intoxication may increase the excitability of the inherently unstable, saccadic and thalamic neural circuits that are hypothetically associated with the human tremor diseases essential tremor and μSOLT. Development of optic instruments that enable clear visualisation of fine eyeball movements in horses will assist in characterising the eyeball tremor.

Horses showed symmetrical vestibular ataxia, demonstrated by a wide-based stance and increased truncal sway at rest and at a walk, which resulted in irregular limb movements that compensated for the lateral drift of the trunk. The effect of blindfolding was profound and should be used as part of the diagnostic workup for PRGS in horses. Irregular limb placements after abrupt cessation of movements that were not immediately corrected indicate that, in addition to vestibular dysfunction, general proprioceptive (GP) tracts are also influenced by lolitrem-B intoxication. However, the delay in correcting imbalance, the rapid, strong, corrective limb movements and the strong responses to the changing directions of lead prompts indicate that GP deficits are not as severe as vestibular dysfunction. Cerebellar ataxia was not featured; however, clinical signs reported in ruminant studies and the role of BK channels in Purkinje cells suggest that cerebellar involvement may be incorporated as the severity of PRGS increases. Following this logic, the signs of PRGS in horses progressed from fasciculations and low amplitude truncal sway to tremors and mild symmetrical vestibular ataxia that is exaggerated by blindfolding, to then progress to include GP and, perhaps, cerebellar ataxia. This indicates a difference in susceptibility of neural systems to lolitrem-B, which may relate to the effects of BK channel modulators on the channel's affinity for lolitrem-B, compensatory mechanisms available to each system, differences in the concentration of lolitrem-B delivered to various neural locations, or

our ability to exaggerate each quality of ataxia. Importantly, diagnosis of lolitrem-B intoxication in mild to moderately ataxic horses requires that symmetrical vestibular ataxia is present. Only severely affected horses will demonstrate cerebellar ataxia.

A state of allodynia was indicated by an increased response to non-noxious, conditioned stimuli and a tendency to startle at gateways, while showing normal responses to other external stimuli. Allodynia may have been caused by changes in afferent neuron conduction and synaptic transmission in the dorsal root ganglia and superficial dorsal horn. A significantly higher resting heart rate during the treatment period than the control period suggests that lolitrem-B intoxication increased sympathetic tone, which may have been a contributing cause of allodynia. BK channel blockade may influence sympathetic tone by several mechanisms involving neural excitability and the release of catecholamines from the adrenal medulla. However, ergovaline, by its influence on the adrenal medulla, may act synergistically with lolitrem-B. Nevertheless, determination of animal responses is subjective and heart rate, although statistically significant, was not raised above the normal reference range dramatically. From a veterinarian's perspective either of these observations could occur merely due to their presence as a stranger. Therefore, these observations lack specificity and hold limited weight in definitively diagnosing PRGS in horses, but add evidence if demonstrated along with action-related repetitive myoclonus and symmetrical vestibular ataxia.

Despite several serum biochemistry tests showing statistically significant trends, the biological significance of these changes is doubtful. Complete blood count and serum biochemistry results neither justify magnesium drenching as a treatment for PRGS nor indicate dysfunction of the liver or immune system. It is uncertain whether lolitrem-B intoxication is responsible for the changes in serum fibrinogen, globulin and albumin that occurred in horses graded 3/5, or whether these reflect an inflammatory process associated with ergovaline-induced ishaemic necrosis of the extremities or other confounding factors.

Brainstem auditory evoked potentials (BAEPs) displayed a trend for the latency of peak V to increase after exposure to lolitrem-B and to exceed the maximal limit of reference ranges, which infers a delay in conduction along the auditory pathway. Delayed conduction supports the conceptual role of BK channels in IHC conduction and temporal precision. However, the high proportion of traces in which peaks could not be identified and the potential interference by frusemide requires that further investigation is carried out in order to validate the significance of these findings. Lengthening of the take-off and first peak latencies of the magnetic motor evoked potentials (mMEPs)

were also interpreted as neural conduction being delayed. The role of BK channels in determining firing activity in neurons, influencing synaptic strength and preventing depolarisation-induced inactivation can theoretically justify the delay observed, but requires assumptions about the pathways involved in generating the mMEP and about the roles of BK channels in these pathways. As clinical UMN weakness was only apparent in horses that were graded 3/5 and none of the horses showed LMN weakness, the lengthening of mMEP latency may be interpreted to indicate subclinical UMN deficits or may have occurred due to limitations of the study design such as the subjective nature of neural stimulation in the presence of non-controlled facilitation in the standing horse. Further investigation is required to ascertain the statistical and clinical significance of the increased latency and the reproducibility of these results.

The reduction in frusemide-induced fractional excretion of K^+ (FEK^+) and Na^+ ($FENa^+$) during the treatment period indicates that lolitrem-B blocks BK-β1 receptors in the renal collecting tubule (CNT) and thereby influences electrolyte handling; specifically, the ability to secrete electrolytes during high renal tubular flow rates. The effect on K^+ excretion is most probably a direct result of BK channel blockade with the persisting K^+ secretion occurring via BK channels that were not blocked by lolitrem-B due to unsaturation, or by compensatory activation of renal outer medullary K^+ (ROMK) channels. Whereas the reduction in Na^+ excretion is likely an indirect effect via the change in cation concentrations in the luminal fluid resulting in reduced self-inhibition of amiloride-sensitive epithelial Na^+ (ENa) channels. Alternatively, blockade of BK channels in other regions of the nephron may alter the concentration of frusemide at its site of action resulting in reduced diuresis, naturesis and FMKS. As neither K^+ nor Na^+ flow mediated responses were completely blocked it is unlikely that lolitrem-B intoxication will result in pathological changes in electrolyte concentrations. Even so, while serum K^+ values did not exceed reference values, there was a positive correlation with the severity of clinical signs. The reduction in plasma aldosterone levels during the treatment period may contribute to this correlation. Blockade of BK channels in the zona glomerulosa, or indirect effects of the lolitrem-B on ACTH and prolactin secretion may explain the reduction in aldosterone. Ergovaline may also have contributed to the reduction in aldosterone. The reduced availability of K^+ secreting channels in the CNT and potentially the colon, along with dampened homeostatic mechanisms due to lowered aldosterone, may raise serum K^+ despite the presence of remnant K^+ secreting channels. However, electrolyte disturbances may require co-existing renal disease or high K^+ dietary intakes. Therefore, these findings may not be of pathological importance but detectable changes in electrolyte handling may be diagnostically useful in future.

In addition to the dopaminergic effects of ergovaline on the adrenal gland and prolactin secretion potentially reducing aldosterone secretion, ergovaline may also have caused ischemic necrosis of the distal limbs in horse #1. Fescue foot in horses has not been reported previously, but the climatic conditions and reported descriptions of lesions in cattle and sheep correlated well with those in horse #1. Therefore gangrene of the extremities due to ergovaline toxicosis may occur in horses. The venoconstrictive effects of ergovaline may also have caused the limb edema observed in horses #1 and #2. Alternatively, limb swelling may be due to lack of movement or lolitrem-B–induced spasm of the lymphatic vessels. The influence of ergovaline on the clinical presentation of PRGS in horses is likely to occur in natural situations as they are both produced by *N. lolii*. However, perhaps not to the same extent as ergovaline content of pasture and hay is generally lower than the levels contained in the seed that was fed.

This investigation of the clinical effects of lolitrem-B intoxication has defined more clearly the disease in equids and has provided a clearer appreciation of the nature of the clinical signs and the individual variability. The influence of lolitrem-B on electrolyte handling has been established. Further research aimed at validating the ELISA test for lolitrem-B in horse plasma, quantifying eyeball movement, substantiating the effect of lolitrem-B on nerve conduction velocities in BAEP and mMEP traces, and defining further the changes in electrolyte handling that occur with lolitrem-B intoxication are worth pursuing and may be diagnostically useful in the future.

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Appendices

Appendix 1: Clinical examination

Horse ID		Date	
		Date first exposure	

Feed type	Amount feed (kg)	
	8AM	4PM

Time						
Attn						
Demeanour						
Appetite						
Volume water drunk						
RR (BrPM)						
Resp character						
HR (BPM)						
Temp (^{o}C)						
MMC						
CRT						
GIT	LU	RU				
	LL	RL				
DP	LF	RF				
	LH	RH				
Faecal amount						
Faecal consistency						
Urination						
PRGS Grade (Galey 1991)						

Descriptions				
Attention	BAR	Bright, Alert, Responsive	QAR	Quiet, Alert, responsive
	NR	Not responsive	HS	Hypersensitive
Demeanour	Dull, Depressed (DPD), normal (N), Nervous (Nv),			
Respiratory character	Normal (N), Increased Effort, Dyspnic, Nasal Flare			
MMC	pp – pale pink, pk – pink, cyn – cyanotic, hpa - hyperaemic			
CRT	X<2>X			
GIT auscultation	-absent, + decreased, ++ normal, +++ increased			
DP digital pulse	-absent, + decreased, ++ normal, +++ increased			
Faecal consistency	0	Dry, hard		
	1	Normal, pelleted, dry		
	2	Soft pelleted		
	3	Paste		
	4	Semi-liquid		
	5	Watery, liquid		

Time	Comments

Appendix 2: Neurological Evaluation Form

Animal ID:	**Date**	
Examiner:	**Date Iolitrem-B exposure started**	

NEUROLOGICAL EXAMINATION

VIDEO: Y N

HEAD

Behaviour, seizures

Head Posture
○ Tilt ○ Turn ○ Symmetry

Mental Attitude - Sensorium
○ Alert and responsive ○ Depressed ○ Lethargic
○ Obtunded ○ Semicoma, ○ Stupor ○ Coma
○ Acting vague ○ Disoriented ○ Hyperactive
○ Propulsive ○ Aggressive

Head, Neck & Trunk Movement & Coordination
○ Lowered position of neck, ○ Trembling, ○ Truncal sway○ Extensor tone ○ Tail position ……
……
……

N = normal, AN = abnormal, NR = no response
NR+ = no response but positive head withdrawal, NR- = no response and no head withdrawal
0 = absent; 1+ = hyporeflexic; 2+ = normal; 3+ = hypereflexic; 4+ = hypereflexic & clonus

Left Ophthalmic Exam	N	AN		Description				
Vision; II	N	AN						
Menace; II – VII, Cerebellum	N	AN	NR+/ NR-	-	+	++	+++	
Pupils, PLR; II – III, Symp.	N	AN		Dilation at rest		NR		
Dazzle Response	N	AN		NR				
Horners; Symp.	N	AN						
Strabismus; III, IV, VI, VIII	N	AN		Lateral	Dorsomedial	Ventral	Medial	
Nystagmus	N	AN		Horizontal	Vertical	Rotational	Postitional	Tremor

Right Ophthalmic Exam	N	AN						
Vision; II	N	AN						
Menace; II – VII, Cerebellum	N	AN	NR+/ NR-	-	+	++	+++	
Pupils, PLR; II – III, Symp.	N	AN		Dilation at rest		NR		
Dazzle Response	N	AN		NR				
Horners; Symp.	N	AN						
Strabismus; III, IV, VI, VIII	N	AN		Lateral	Dorsomedial	Ventral	Medial	
Nystagmus	N	AN		Horizontal	Vertical	Rotational	Positional	Tremor

Face			**Location/ Descriptor**	**-**	**+**	**++**	**+++**
Sensation; V(s), cerebrum	N	AN		-	+	++	+++
Muscle mass/ jaw tone; V(m)	N	AN		-	+	++	+++
Ear, eye, nose, lip reflex;V-VII	N	AN		-	+	++	+++
Expression; VII, cerebrum	N	AN		-	+	++	+++
Sweating; Symp.	N	AN		-	+	++	+++
Facial Symmetry	N	AN					

Tongue/ Pharynx/ Larynx					
Tongue mass & use XII, cerebrum	N	AN			
Mastication	N	AN	Dysphagia	Sialosis	Muscle Asym.
Voice; IX, X	N	AN			
Swallow; IX, X, cerebrum	N	AN			
Thoracolaryngeal reflex	N	AN			

Body			
Muscle Symmetry			
Cutaneous sensation			
Bending of vertebral column			
Anal reflex			
Tail tone			

Comments:

Gait & Posture Analysis			
Spontaneous involuntary movements			
Tremor	**Location**	***F*(Tremor)**	**Action related?**
	Eyes		
	Ears		
	Face		
	Neck		
	Trunk		
	Flank		
	Forelimb		
	Hindlimb		
	Tail head		
Knuckling/ joint buckling	Joint:	*F*(occurrence):	
Truncal Sway	Direction:	*F*(occurrence):	
Hypertonia			

Tasks – not blindfolded	**N/ AN**	**Descriptor**
Unrestrained in paddock		
Standing at rest		
Initiation of movement – delayed?		
Walk in straight line		
Trot in straight line		
Walk in serpentine		
Walk with head elevated		
Backing		
Lunging long rein – L/R		
Tight circling – L/R		
Walking up slope		
Tail pull at rest		
Tail pull at walk		
Thoracic Hopping		
Tail and halter pull – L/R		
Tasks – blindfolded	**N/ AN**	**Descriptor**
Standing at rest		
Initiation of movement – delayed?		
Walk in straight line		
Walk in serpentine		
Walk with head elevated		
Backing		
Tight circling – L/R		

Overall Assessment

Severity of PRGS	**0**	**1**	**2**	**3**	**4**	**5**
Pattern of deficit observed	**LMN paresis**					
	UMN paresis					
	General proprioceptive ataxia					
	Vestibular ataxia					
	Cerebellar ataxia					

GRADING SYSTEM FOR THE SEVERITY OF PRGS

(Adapted from Galey et al (1991)

0	No clinical signs
1	No resting tremors or incoordination; Low-intensity tremor and incoordination with handling or exercise
2	No resting tremors or incoordination; Moderate intensity tremors and incoordination with handling or exercise
3	Spontaneous low-intensity tremors and incoordination at rest; Moderate to severe tremors and incoordination with handling or exercise
4	Pronounced resting tremors and incoordination; Convulsive tremors and severe incoordination with handling
5	Severe spontaneous tremors and incoordination at rest, accompanied by convulsive episodes

Appendix 3: Levels of lolitrem-B and ergovaline ingested by horses

Horses were fed known amounts of perennial ryegrass hay and seed according to maintenance requirements. In order to calculate the amount of lolitrem-B and ergovaline ingested the toxin levels were combined and weighted according to the amount of each that was fed.

3.1 Calculations of maintenance requirements for group 1 & 2

Maintenance = 1.8 % of BW (horse in light work)

Horse weights were estimated according to the following equation and results are presented in **table 1.**

$$\text{Horse weight estimate} = \frac{(\text{heart girth (cm)})^2 \times \text{length}^* \text{ (cm)}}{11880}$$

* length was measured as the head of the humerus to the tuber ischii {Davies, 2009 #1188}

Table 1: Estimates of horse weights according to heart girth and length.

Horse	Heart girth (cm)	Length (cm)	Estimated horse weight (kg)	Maintenance requirements (kgDM/day)
1	192	160	496	8.92
2	192	176	546	9.82
3	194	160	507	9.13
4	184	170	484	8.71
5	190	158	480	8.64
6	178	152	405	7.29
7	192	162	503	9.05

The mean maintenance requirement for group 1 = 9.15 kgDM/day and group 2 = 8.33 kgDM/day.

3.2 Dose rates of lolitrem-B

Table2: Lolitrem-B levels in feed, determined by HPLC

Feed	Meadow chaff (control feed)	PRG hay	PRG seed – group 1	PRG seed – group 2
Mean (ppm*)	0.7	1.73	5.16	4.15
SD	0.3	0.67	1.17	1.17
Number samples	3	3	6	15

* ppm = mg/kgDM

It was assumed that hay and seed contained 90% DM and that 10% of hay was wasted. Seed was fed to the horses in individual feed containers to ensure none was wasted. Group 2 were fed seed twice a day, whereas group 1 were fed seed only in the morning, and therefore the levels of lolitrem-B ingested by each group have been calculated separately. For both groups the equivalent net content of lolitrem-B in feed was greater than 2 ppm.

Table 3: Calculations of lolitrem-B dose rates in ppm and μg/kgBW/day.

	Group 1		Group 2	
	Hay	Seed	Hay	Seed
Amount fed (kgDM/horse/day)	8.1	0.9	8.1	1.8
Lolitrem-B content (mg/kgDM)	1.73 ± 0.67	5.16 ± 1.17	1.73 ± 0.67	4.15 ± 1.17
Lolitrem-B fed (mg)	14.01 ± 5.43	4.64 ± 1.05	14.01 ± 5.43	7.47 ± 2.11
Total lolitrem-B fed (mg/horse/day)	18.65 ± 5.53		21.48 ± 5.83	
Total DMI (kgDM)	9		9.9	
Equivalent net lolitrem-B content (ppm)	2.07 ± 0.61		2.17 ± 0.59	
Mean horse weight (kgBW)	508.25		462.67	
Dose of lolitrem-B (μg/kgBW/day)	36 ± 10		46 ± 13	

3.3: Dose rates of ergovaline

Seed contained high levels of ergovaline as shown in **table 4.**

Table 4: Ergovaline levels in feed determined by HPLC

Feed	Meadow chaff	PRG Hay	PRG seed Group 1	PRG seed Group 2
Mean (ppm)	ND	0.53	14.97	17.92
SD		0.32	2.34	3.73
Number of samples	3	3	6	15

Horses in Group 2 ingested a higher net equivalent of ergovaline fed content due to the greater quantities of seed feed **(table 5)**.

Table 5: Dose rate of ergovaline in ppm.

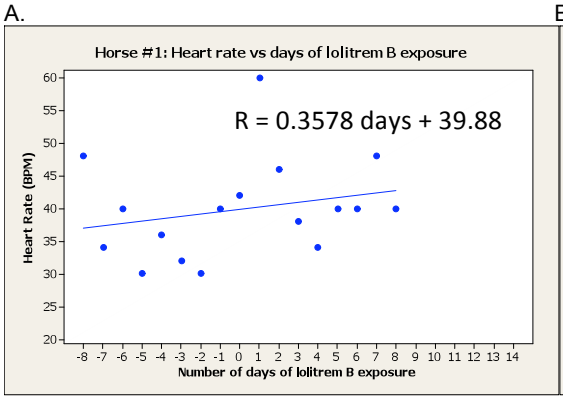
	Group 1		Group 2	
	Hay	Seed	Hay	Seed
Amount fed (kgDM/horse/day)	8.1	0.9	8.1	1.8
Ergovaline content (mg/kgDM)	0.53 ± 0.32	14.97 ± 2.34	0.53 ± 0.32	17.92 ± 3.73
ergovaline fed (mg)	4.3 ± 2.59	13.473 ± 2.11	4.3 ± 2.59	32.256 ± 6.71
Total ergovaline fed (mg)	17.773 ± 3.34		36.556 ± 7.19	
Total DMI (kgDM)	9.0		9.9	
Equivalent net ergovaline content (ppm)	1.97 ± 0.37		3.59 ± 0.73	

Appendix 4: Graphical analysis of objective clinical parameters

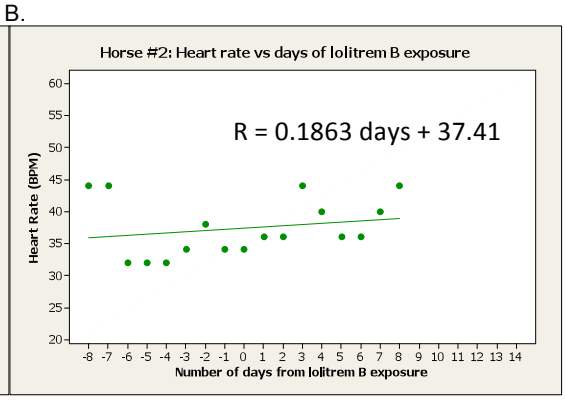
4.1: Heart rate

Graphs of daily heart rate demonstrate an increasing trend in all but horse #4 **(D).** During the treatment period heart the mean heart rate was significantly higher than during the control period (p = 0.018, paired t-test), as shown in the boxplot **(H).**

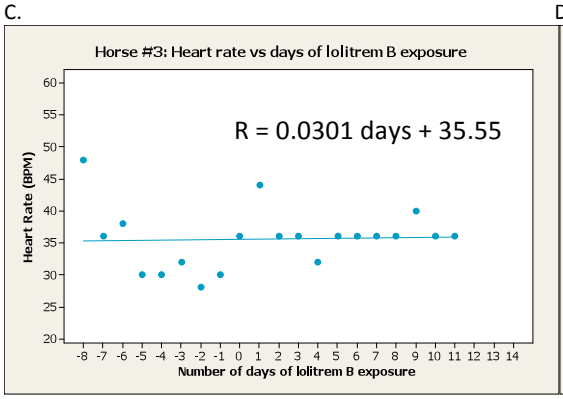
A.



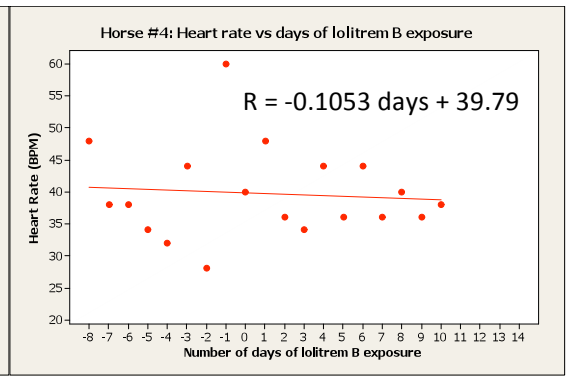
B.



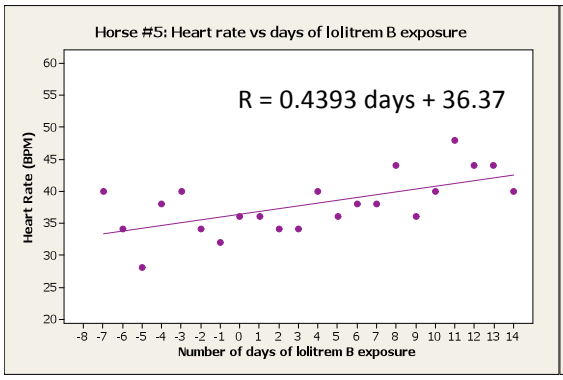
C.



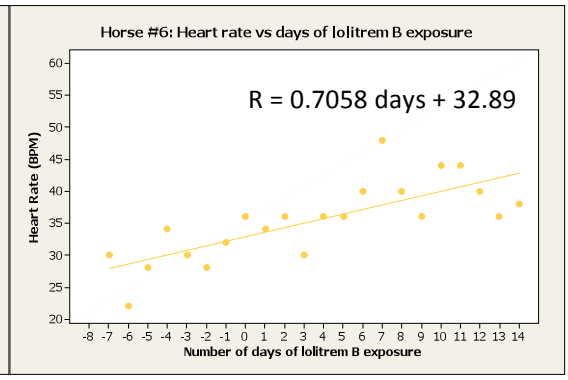
D.



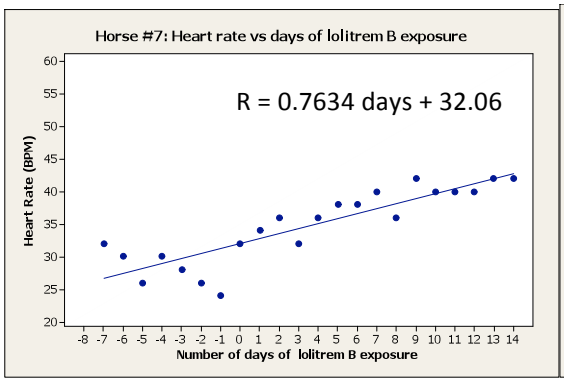
E.



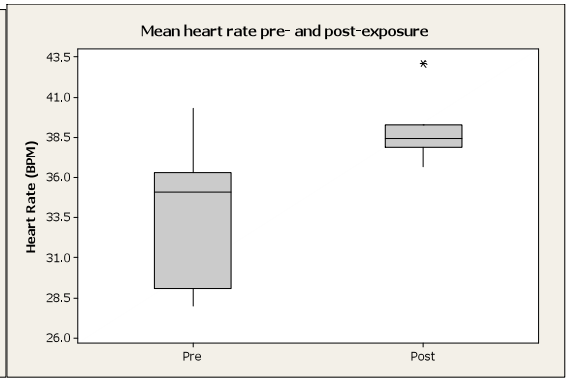
F.



G.



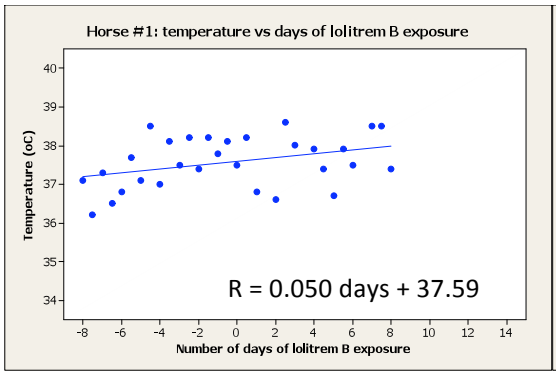
H.



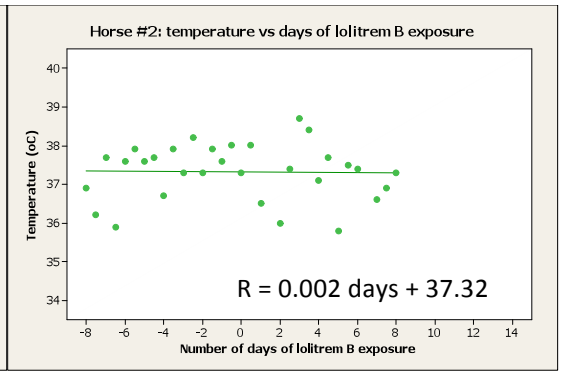
4.2: Temperature

Individual plots of temperature did not show any consistent trends and the mean temperature during the control period and treatment period were not significantly different ($p = 0.51$, paired t-test).

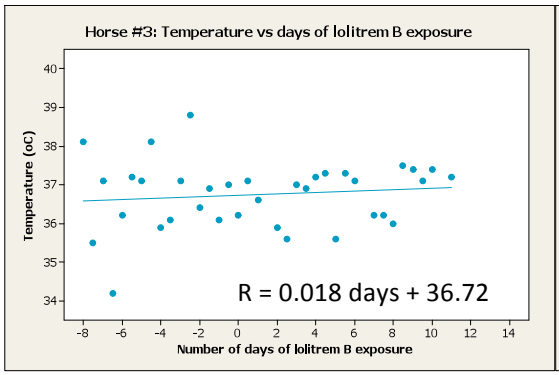
A.



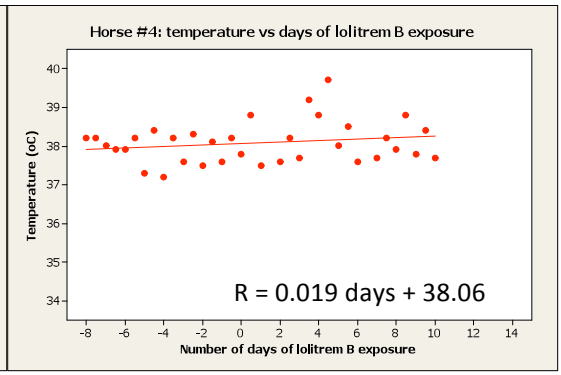
B.



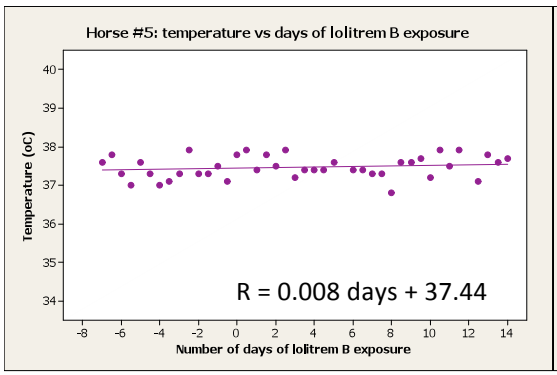
C.



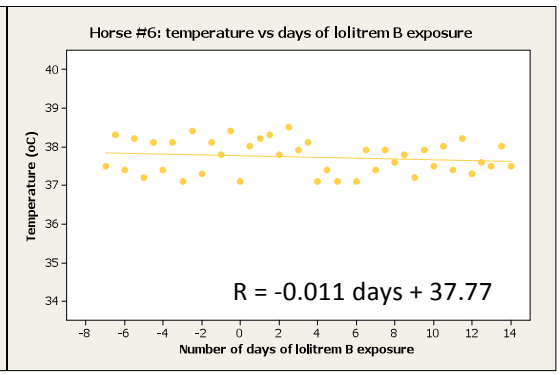
D.



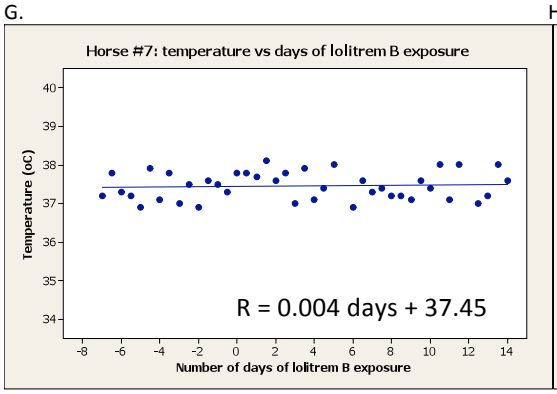
E.



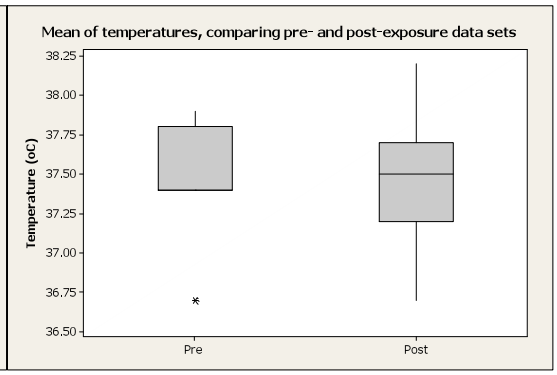
F.



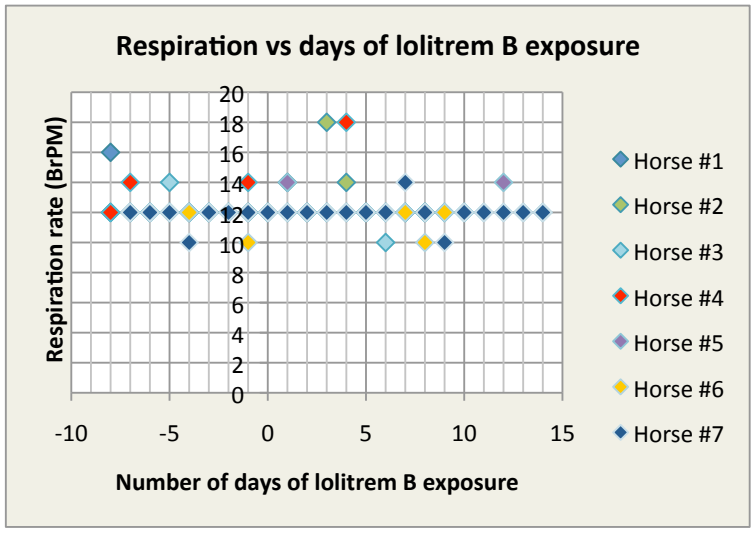
G.



H.



4.3: Respiration



Respiration rate was not affected by exposure to lolitrem-B (p = 0.348, paired t-test). The modal respiration rate was 12 BrPM. Horses 2 and 4 showed an increased respiration rate, which corresponded with an increase in body temperature **(2.3 B & D).** These were judged not to be related to lolitrem-B intoxication as temperature and respiration rate returned to normal values the following day despite continued intake of lolitrem-B.

Appendix 5: Complete blood count and biochemistry raw results and tests of statistical significance

The following tables contain the raw data values and the statistical tests performed on complete blood count and serum biochemistry values. Statistical tests include the following: paired t-test for pre and post data values from each individual horse; the unpaired t-test was used to test for differences in the mean change in values from pre and post data sets (Δ) for horses in group 1 and 2; ANOVA tested the difference in the mean change of values from pre and post data sets (Δ) for groups according to the severity score assigned to each horse; ANOVA was also used to test whether there was a difference between severity groups in post-lolitrem-B values. Values that are red indicate that they exceed the reference range, while those in blue are below the reference range. P-values that are lightly shaded indicate a strong trend ($p < 0.1$), while those in bold and shaded dark indicate significant differences ($p < 0.05$).

5.1: Complete blood count values

Test (reference range)	WBC (5.7 – 12.0 x10^9/L)		RBC (7.0 – 11.8 x10^12/L)		HB (112 – 180 g/L)	
	Pre	Post	Pre	Post	Pre	Post
Horse #1	5.8	11.5	8.8	7.8	146	125
Horse #2	4.4	6.2	6.6	8.2	121	151
Horse #3	9.4	9.8	7.7	8.3	143	147
Horse #4	7.4	10.7	8.4	10.0	137	159
Horse #5	8.5	8.7	7.8	10.1	133	177
Horse #6	7.7	6.2	8.5	8.6	141	145
Horse #7	10.6	9.5	8.6	9.0	133	136
Paired t-test (pre & post)	-1.30		-1.90		-1.52	
p-value	0.241		0.106		0.179	
95% CI	(-3.622, 1.107)		(-1.831, 0.231)		(-32.07, 7.50)	
Unpaired t-test (groups: Δ) p-value	**0.044**		0.811		0.573	
ANOVA (severity: Δ) p-value	0.272		0.803		0.820	
ANOVA (severity: post) p-value	0.927		0.318		0.655	

Complete blood count continued

Test (reference range)	Neutrophils (2.9 – 6.9 x10^9/L)		% Neutrophils		Lymphocytes (1.5 – 6.3 x10^9/L)		% Lymphocytes		Monocytes (0.15 – 0.7 x10^9/L)		% Monocytes	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Horse #1	3.02	8.63	52	75	2.32	1.61	40	14	0.46	1.27	8	11
Horse #2	2.99	4.9	68	79	1.10	1.30	25	21	0.22	0.00	5	0
Horse #3	4.32	4.7	45	48	4.42	4.90	47	50	0.28	0.10	3	0
Horse #4	3.63	6.21	49	58	3.11	4.28	42	40	0.37	0.21	5	2
Horse #5	6.12	5.66	72	65	2.04	2.44	24	28	0.09	0.35	1	4
Horse #6	5.31	4.22	69	68	1.93	1.67	25	27	0.15	0.25	2	1
Horse #7	7.0	5.04	66	53	3.07	3.61	29	38	0.11	0.48	1	5
Paired t-test (pre & post)	-1.02		-0.78		-1.14		0.46		-0.99		0.21	
p-value	0.348		0.462		0.298		0.659		0.360		0.840	
95% CI	(-3.390, 1.399)		(-14.71, 7.56)		(-0.819, 0.299)		(-8.53, 12.53)		(-0.486, 0.206)		(-3.04, 3.61)	
Unpaired t-test (groups: Δ) p-value	**0.049**		0.269		0.905		0.167		0.539		0.158	
ANOVA (severity: Δ) p-value	0.166		0.300		0.436		0.121		0.763		0.655	
ANOVA (severity: post) p-value	0.518		0.074		0.277		0.151		0.673		0.673	

5.2: Serum biochemistry

Test (reference range)	Calcium (2.9 – 3.3 mmol/L)		Phosphorus (0.7 – 1.7 mmol/L)		Magnesium (0.68 – 0.9 mmol/L)		Potassium (3.0 – 4.6 mmol/L)		Sodium (131 – 141 mmol/L)		Chloride (93 – 105 mmol/L)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Horse #1	2.81	2.8	0.95	0.69	0.71	1.34	3.4	4.5	135	135	100	103
Horse #2	2.82	2.86	0.83	0.93	0.71	0.83	4.1	4.4	134	137	101	101
Horse #3	2.97	3.1	1.05	1.00	0.70	0.80	3.9	4.1	135	139	102	102
Horse #4	2.99	3.16	0.93	1.39	0.79	0.94	3.5	4.0	135	139	101	102
Horse #5	2.92	3.01	0.78	0.99	0.71	0.77	4.3	4.0	135	134	101	99
Horse #6	2.89	2.96	0.99	1.25	0.88	0.87	4.2	3.7	135	135	101	99
Horse #7	2.86	3.04	0.89	1.10	0.75	1.06	4.6	4.1	135	135	100	99
Paired t-test (pre & post)	-3.66		-1.51		-2.38		-0.51		-1.76		0.21	
p-value	**0.011**		0.181		0.055		0.627		0.129		0.838	
95% CI	(-0.16, -0.03)		(-0.35, 0.08)		(-0.39, 0.01)		(-0.66, 0.43)		(-3.42, 0.56)		(-1.50, 1.78)	
Unpaired t-test (groups: Δ) p-value	0.636		0.361		0.462		**0.020**		0.054		**0.027**	
ANOVA (severity: Δ) p-value	0.146		0.161		0.410		0.267		0.914		0.340	
ANOVA (severity: post) p-value	0.071		**0.022**		0.585		**0.017**		0.891		0.514	

Serum biochemistry continued

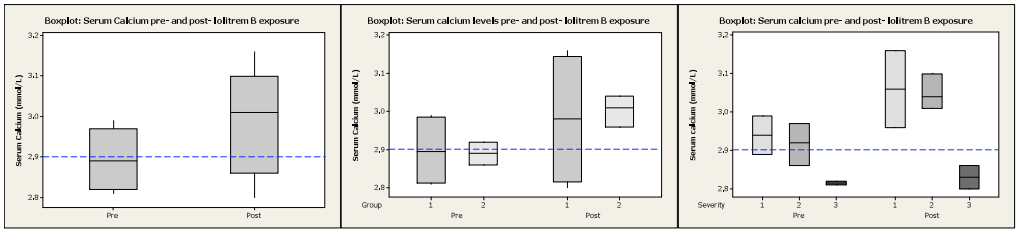
Test (reference range)	CK (63 – 469 IU/L)		AST (0 – 700 IU/L)		GGT (7 – 45 IU/L)		GDH {1 – 8 IU/L)		Bilirubin (8 - 51 μmol/L)		Urea (3.3 – 8.7 μmol/L)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Horse #1	258	147	300	240	11	16	5	1	47	46	7.0	4.7
Horse #2	264	208	264	220	11	14	2	2	36	25	6.8	1.8
Horse #3	281	275	284	299	4	9	2	2	36	24	5.5	5.2
Horse #4	174	357	397	298	21	23	3	2	50	18	7.1	4.7
Horse #5	192	177	287	235	14	14	2	0	41	37	5.3	5.3
Horse #6	146	203	196	175	9	9	1	0	54	30	4.2	1.5
Horse #7	481	599	257	243	7	9	2	0	22	14	6.7	5.1
Paired t-test (pre & post)	-0.63		2.83		-3.10		2.71		3.14		3.23	
p-value	0.551		**0.030**		**0.021**		**0.035**		**0.020**		**0.018**	
95% CI	(-118.5, 69.9)		(5.3, 73.3)		(-4.3, -0.5)		(0.136, 2.721)		(2.89, 23.39)		(0.50, 3.59)	
Unpaired t-test (groups: Δ) p-value	0.533		0.533		**0.027**		0.706		0.834		0.439	
ANOVA (severity: Δ) p-value	0.108		0.449		0.299		0.829		**0.024**		0.094	
ANOVA (severity: post) p-value	0.572		0.803		0.547		0.742		0.586		0.325	

Test (reference range)	Creatinine (81 – 164 μmol/L)		Fibrinogen (1.4 – 5.0 g/L)		Total Protein (52 - 72 g/L)		Globulin (21 – 39 g/L)		Albumin (28 – 38 g/L)		A:G (0.9 – 1.7)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Horse #1	85	78	2.1	5.4	65	68	31	37	34	31	1.10	0.84
Horse #2	76	87	1.9	4.9	72	83	41	51	31	32	0.76	0.63
Horse #3	111	113	2.1	4.1	69	72	35	34	34	38	0.97	1.12
Horse #4	106	108	1.7	4.3	68	68	32	34	36	34	1.13	1.00
Horse #5	105	104	4.2	2.1	75	77	41	41	34	36	0.83	0.88
Horse #6	114	101	3.0	2.1	75	74	41	43	34	31	0.83	0.72
Horse #7	136	110	4.2	2.9	72	71	38	36	34	35	0.89	0.97
Paired t-test	1.00		-1.09		-1.55		-1.52		-0.00		0.91	
p-value	0.356		0.317		0.173		0.180		1.00		0.399	
95% CI	(-6.61, 15.76)		(-3.06, 1.17)		(-6.27, 1.42)		(-6.35, 1.49)		(-2.50, 2.50)		(-0.08, 0.18)	
Unpaired t-test (groups: Δ) p-value	0.144		**0.001**		0.196		0.185		1.00		0.145	
ANOVA (severity: Δ) p-value	0.551		0.264		0.158		**0.009**		0.090		0.089	
ANOVA (severity: post) p-value	**0.011**		0.186		0.778		0.531		0.051		0.285	

5.3: Graphical comparisons of serum biochemistry values

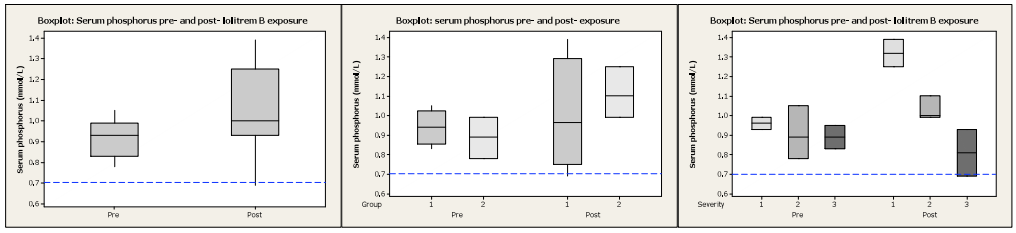
The following boxplots display changes in biochemistry values that occurred following lolitrem-B administration, including graphs that show data grouped according to temporal groups and according to the PRGS severity score that horses were assigned. Severity refers to the grade assigned to each horse on the final day of lolitrem-B exposure.

Total Serum Calcium:



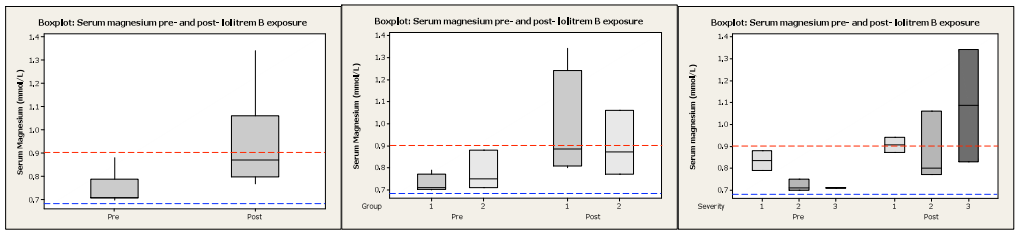
There was a significant increase from the pre- to post- values for total serum calcium (p = 0.011, paired t-test), however values remained below the upper reference range (3.3 mmol/L). There was no significant difference in the change of total serum calcium from pre- to post-exposure between horses with different scores of PRGS (p = 0.146, ANOVA), however there was a trend for total serum calcium post-exposure values to be lower in horses that showed more severe signs and a potential difference in mean post-exposure values between the severity groups (p = 0.071, ANOVA), but this trend was also present prior to lolitrem-B exposure. There was no significant difference between the temporal groups.

Serum Phosphorus:



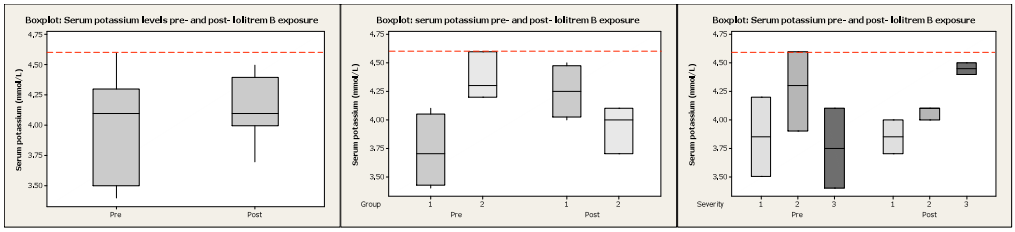
There was a significant difference in the post-exposure values for the severity groups (p = 0.022), but no significant difference was identified between pre- and post-exposure, the mean change in serum phosphorus between each severity group or each temporal group.

Total Serum Magnesium



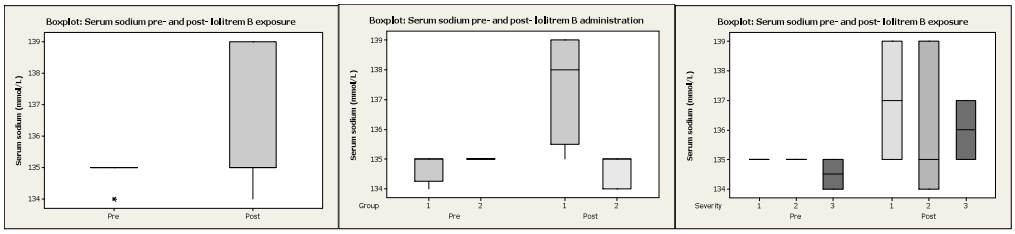
There was a strong trend for total serum magnesium to increase post-exposure (p = 0.055), with some post-exposure values exceeding the upper reference range (0.9 mmol/L, red dotted line). However, there was no correlation between the change in total serum magnesium and the severity of PRGS observed.

Serum K^+



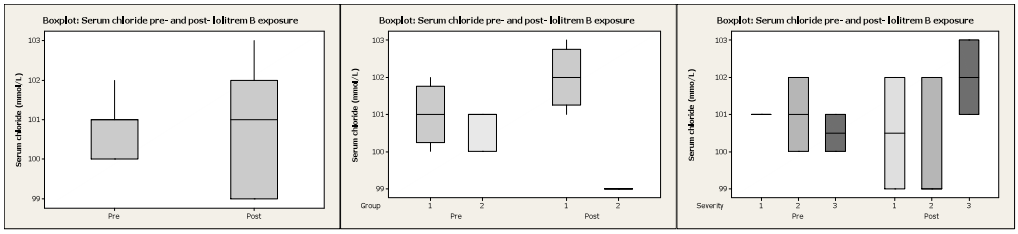
No significant difference between pre- and post- lolitrem B exposure (p = 0.627, paired t-test) occurred, however there was a strong temporal group effect (p = 0.020, unpaired t-test) with group 1 increasing, while group 2 decreased. There was also a significant difference between the post-exposure values when horses were grouped according to severity score (p = 0.017, ANOVA) with a trend of K^+ increasing as severity of clinical signs increased. However, values remained within the reference range (3.0 – 4.6 mmol/L).

Serum Na^+



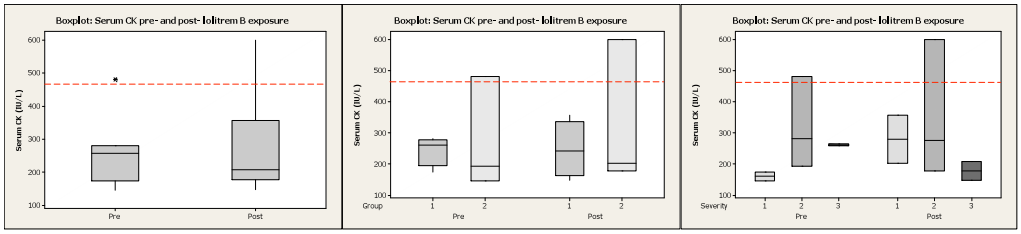
There was a strong trend for a difference between temporal groups (p = 0.054, unpaired t-test), with group 1 increasing, while group 2 decreased.

Serum Cl^-



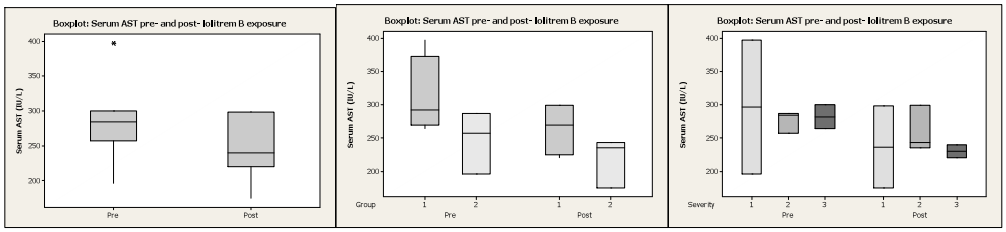
There was a significant difference between temporal groups (p = 0.027, unpaired t-test) with group 1 increasing, while group 2 decreased.

Serum CK:



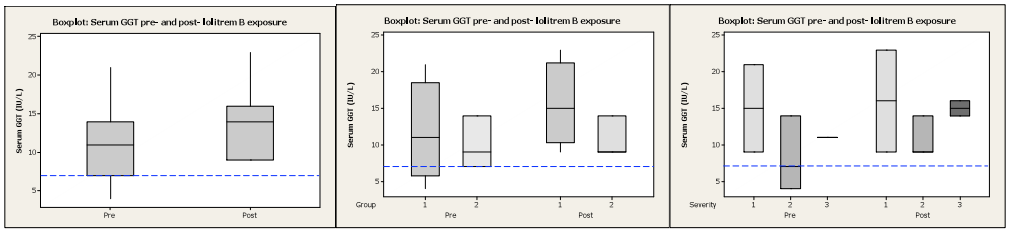
There was no significant differences in CK values.

Serum AST:



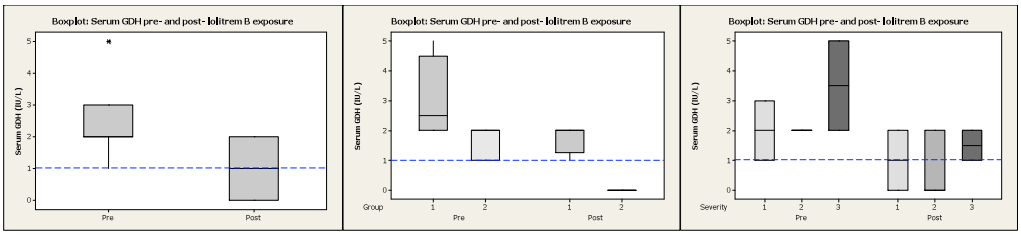
There was a significant decreasing trend from pre- to post-exposure levels ($p = 0.030$, paired t-test), however no significant difference between the change in AST activity for horses in different severity groups occurred ($p = 0.449$, ANOVA).

Serum GGT:



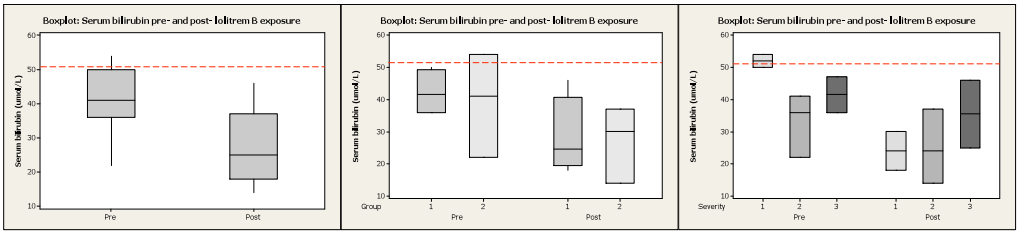
Paired t-test showed a significant increase in GGT values from pre- to post-exposure ($p = 0.021$, paired t-test), however there was no correlation between the change in GGT and the severity of clinical signs displayed ($p = 0.547$, ANOVA). There was a significant difference between the temporal groups ($p = 0.027$, unpaired t-test), with group 1 showing greater increases than group 2.

Serum GDH:



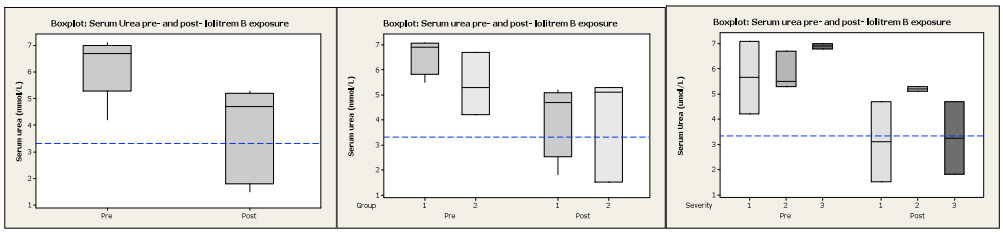
Paired t-test showed a significant decrease in GDH values from pre- to post-exposure ($p = 0.035$, paired t-test), however there was no correlation between the change in GGT and the severity of clinical signs displayed ($p = 0.829$, ANOVA).

Serum bilirubin:



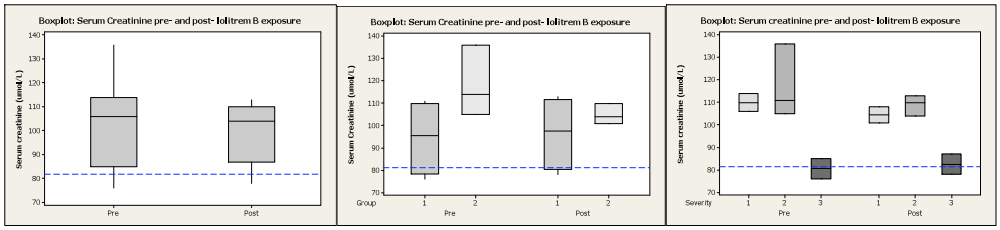
There was a significant trend for the levels of bilirubin to decrease post-exposure ($p = 0.020$, paired t-test). The change was greatest within the group showing the least severe clinical signs (mean difference of -28 ± 11.314, n=2) while the group scoring 3/5 showed the least change (mean of -7 ± 14.142, n=2).

Serum Urea:



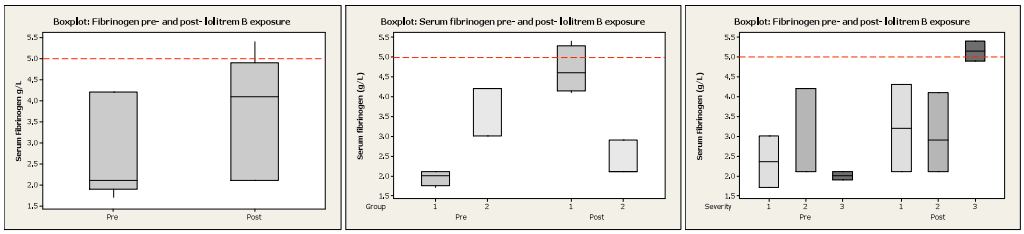
There was a significant trend for the urea values to decrease post-exposure (p = 0.018, paired t-test). There was a strong indication of a difference between the mean change in urea levels from pre- to post-exposure according to the severity of clinical signs displayed (p = 0.094, ANOVA), however the change was greatest in groups that scored 1/5 and 3/5 and least in those that scored 2/5, therefore the decrease in urea is unlikely to correlate with the severity of clinical signs displayed.

Serum Creatinine:



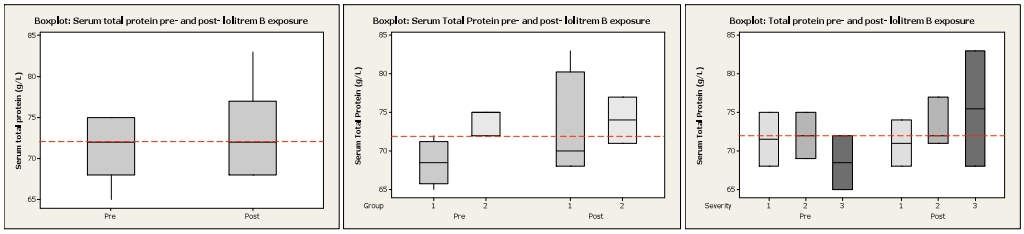
No significant difference was observed between pre- and post-exposure values of serum creatinine. There was a significant difference between the post-lolitrem-B values for the groups of severity (p = 0.011, ANOVA), however these differences were present prior to lolitrem-B administration.

Serum Fibrinogen:



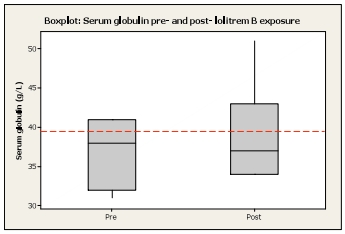
The paired t-test did not demonstrate a significant difference between fibrinogen levels pre- and post-lolitrem-B exposure. The horses that scored 3/5 showed an increase in fibrinogen post-exposure, with horse #1 exceeding the upper limit of the reference range, but the difference between each severity group was not deemed significant by ANOVA (p = 0.186). There was a significant difference between the temporal groups with group 1 increasing and group 2 decreasing, (p = 0.001, unpaired t-test) – this may reflect that the two horses that scored 3/5 were in group 1.

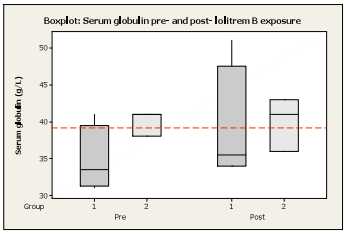
Serum total protein

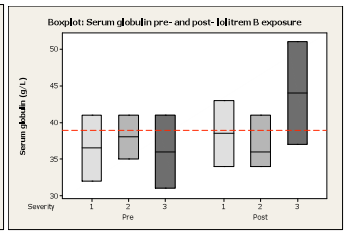


Horses in group 2 had total protein values that exceeded or approached the upper limits of the reference range both prior to and after lolitrem B exposure, while group 1 had levels within the reference range expect for horse #2 had a high total protein value of 83 g/L post-lolitrem B exposure. No significant differences were found.

Serum Globulin:

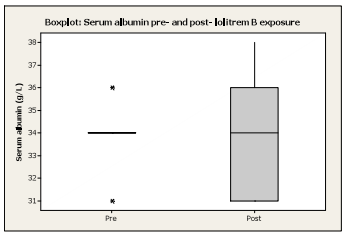


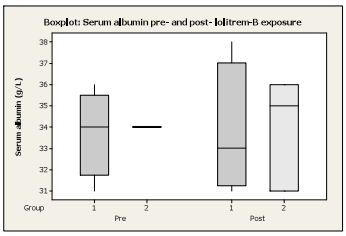


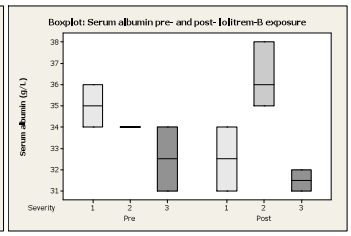


Although no significant difference occurred between pre- and post-exposure values (p = 0.180), there was a significant difference between the values obtained in each severity group (p = 0.009), with horses scoring 3/5 showing the greatest increase in serum globulin levels. Horses #2, #3 and #4 had pre- and post-exposure levels of total protein and globulin that exceeded or equaled the upper limit of the reference range.

Serum Albumin:





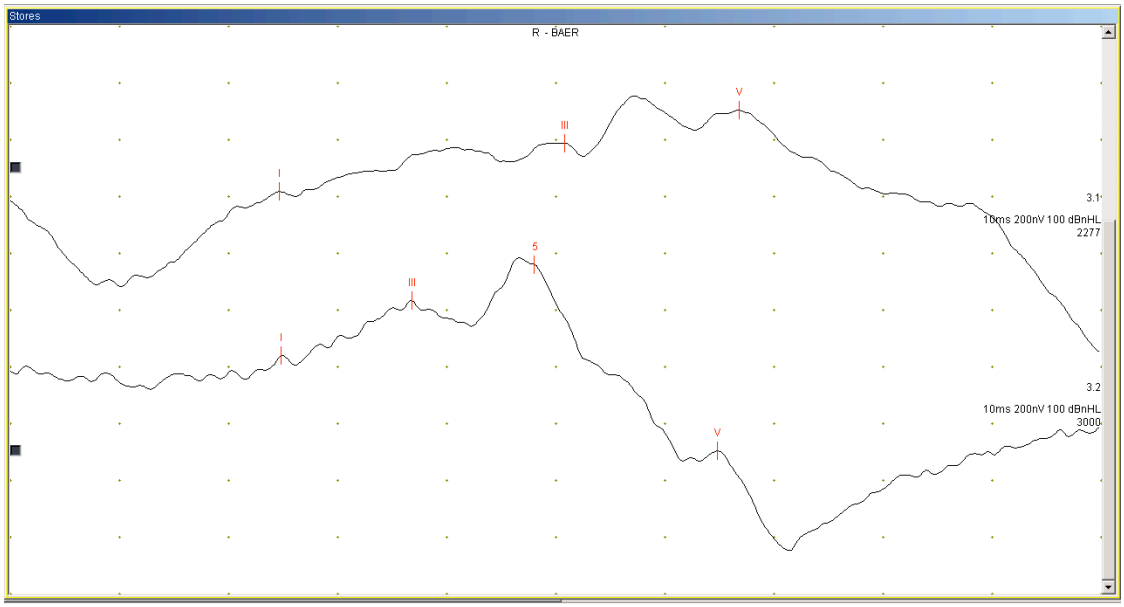


Serum albumin showed no statistical change between pre- and post-exposure values (p = 1.00, paired t-test). However, there was a tentative difference between the change (p = 0.090) and the post-exposure values (p=0.051) for the severity groups.

Appendix 6: Electrophysiology traces

6.1: An example of a Brainstem Auditory Evoked Potential trace that could not be interpreted due to unidentifiable peaks

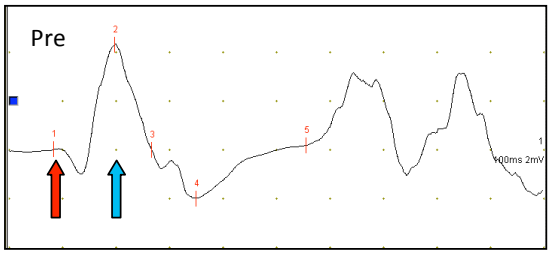
Horse #5 pre-exposure

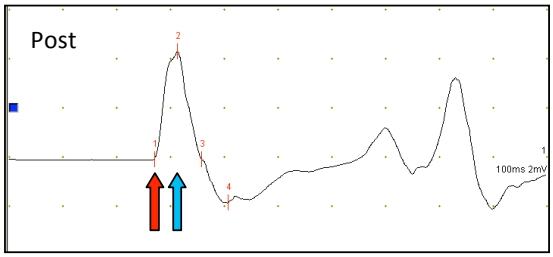


6.2 Magnetic motor evoked potentials:

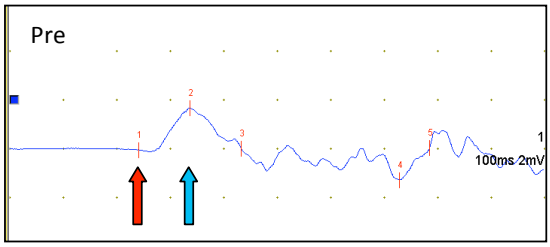
6.2.1. Traces used in analysis

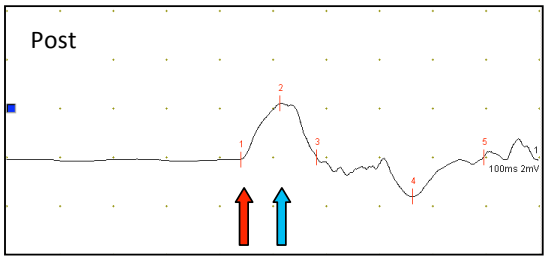
Horse #1



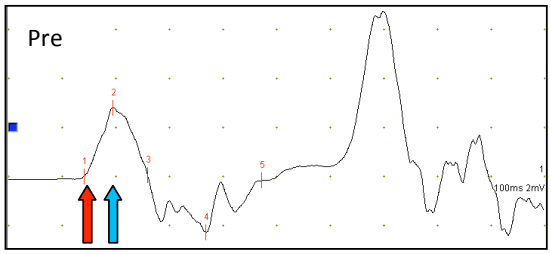


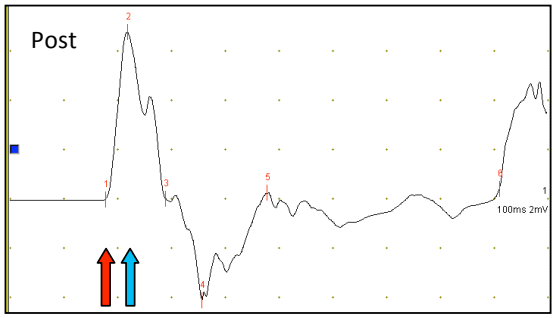
Horse #2



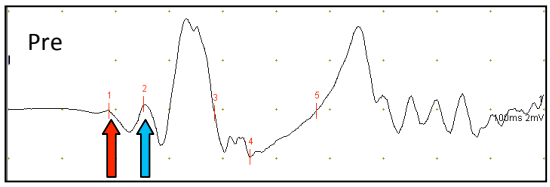


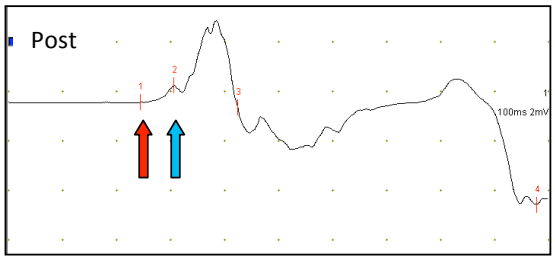
Horse #3





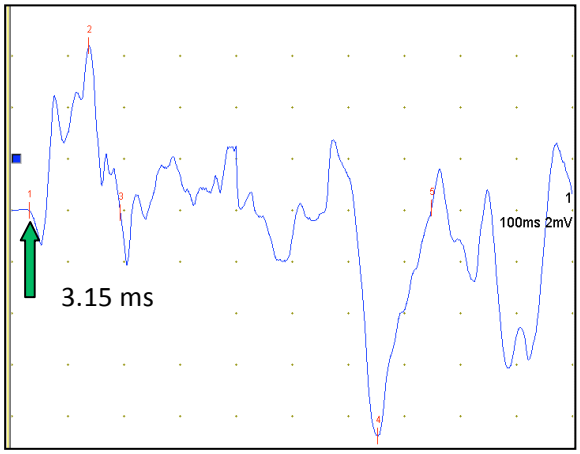
Horse #4



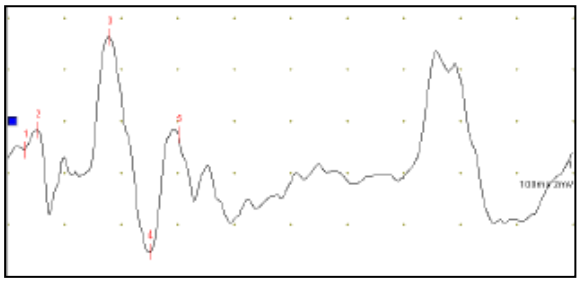


6.2.2 Example of mMEP traces that were discarded from analysis:

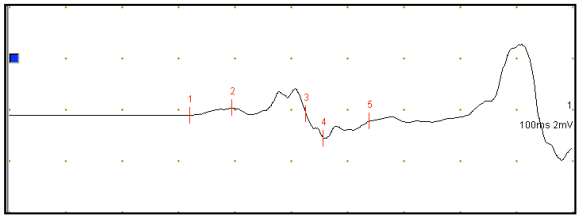
Premature deflection: example Horse #5, extensor carpi radialis, pre exposure.



No clear baseline: example Horse #7, tibialis cranialis pre-exposure.



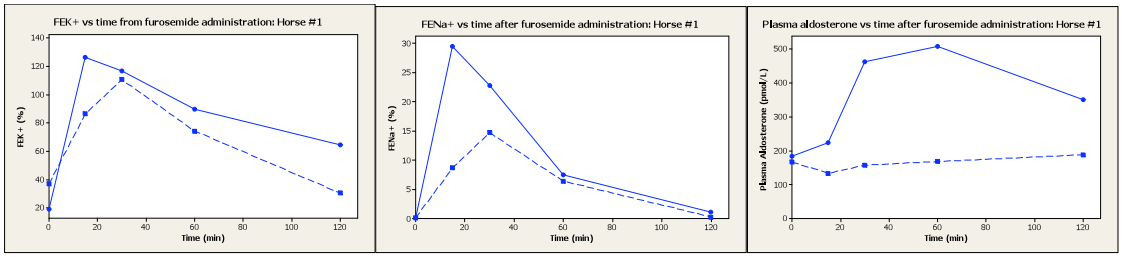
Lack of prominent 1st peak: example Horse #7, extensor carpi radialis, pre-exposure



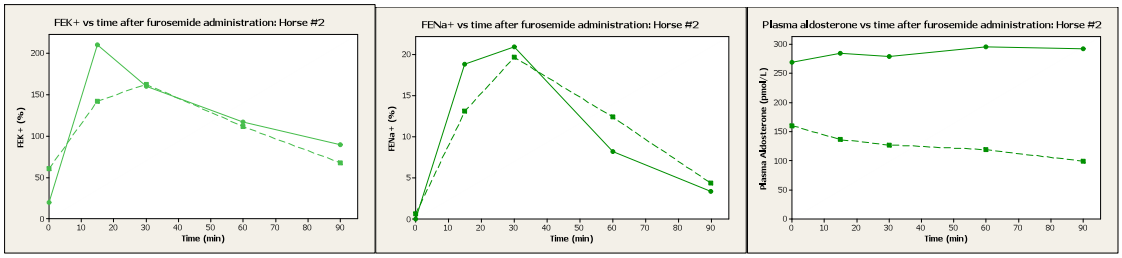
Appendix 7: Graphical representation of frusemide trial data

The following graphs show the change in fractional excretions of K^+ and Na^+ and in plasma aldosterone that occurred with administration of frusemide, before (solid line) and after (interrupted line) lolitrem-B exposure, for each horse.

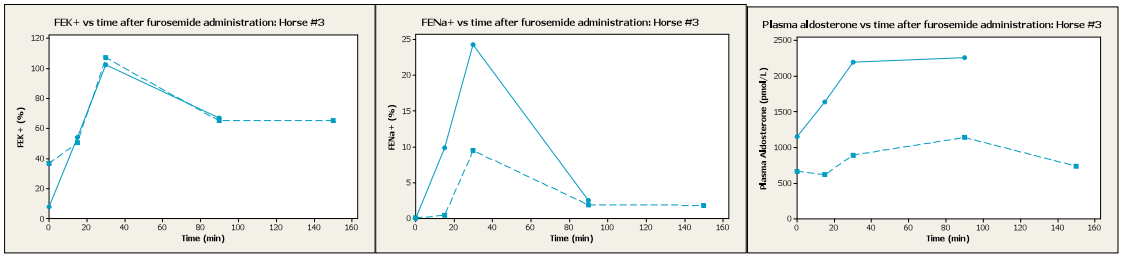
A. Horse #1



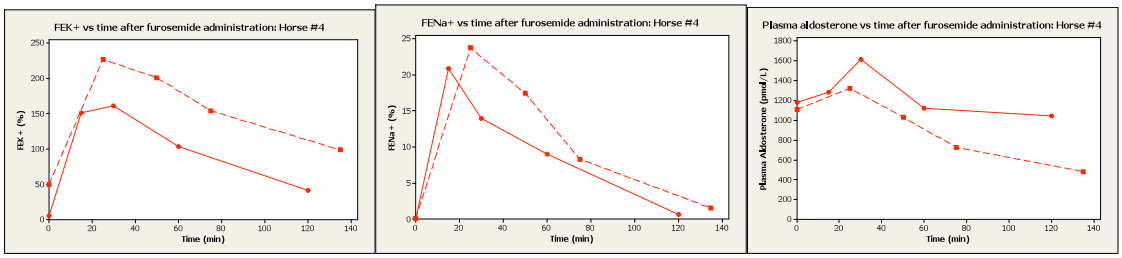
B. Horse #2



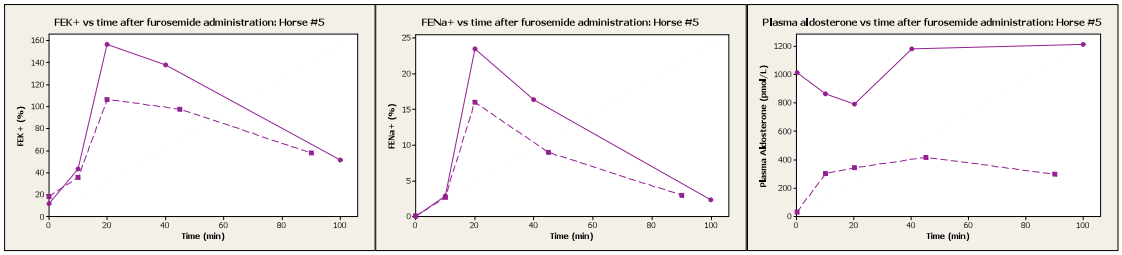
C. Horse #3



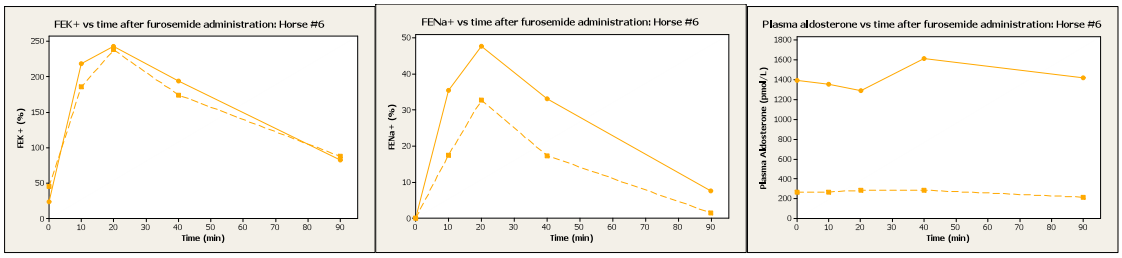
D. Horse #4



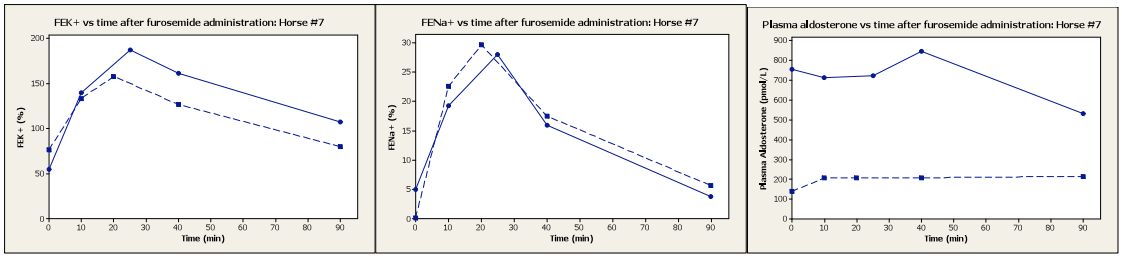
E. Horse #5



F. Horse #5



G. Horse #6



Appendix 8: Analysis of K^+ content of feed

Feed was analysed for K^+ by inductively coupled plasma optical emission spectrometry (ICP-OES) (Nutrition Lab, Institute of food, nutrition and human health, Massey University). The table below presents the mean values of K^+ in each feed and the calculated intake of K^+ by horses with values weighted according to the estimated intake of each feed **(appendix 3).**

	Chaff control	Group 1		Group 2	
		PRG hay	PRG seed	PRG hay	PRG seed
Level of K+ (g/100g DM)	1.15 ± 0.07	1.50 ± 0.3	0.64 ± 0.04	1.50 ± 0.3	0.635 ± 0.04
Level of K+ (g/kgDM)	11.5 ± 0.7	15.0 ± 3	6.35 ± 0.4	15.0 ± 3	6.35 ± 0.4
Amount fed (kgDM)	9	8.1	0.9	8.1	1.8
Total K+ (g)	103.5 ± 6.3	121.5 ± 24.3	5.715 ± 0.36	121.5 ± 24.3	11.43 ± 0.72
Combined K+ dose (g/kgDM)	11.5 ± 0.7	14.1 ± 2.7		13.4 ± 2.5	