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Clinical and experimental investigation of the nervous system of neonatal foals. Neuroendocrin transition from foetal consciousness to perinatal life

PhD Thesis

Dr. Balázs Tóth

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Supervisor:
Zoltán Bakos
Associate Professor
Department and Clinic of Equine Medicine
University of Veterinary Medicine Budapest
Üllő, Hungary

Thesis Committee Members:
John Madigan
Distinguished Professor
Department of Medicine and Epidemiology
School of Veterinary Medicine
University of California-Davis
Davis-Ca, USA

Monica Aleman
Professor
Department of Medicine and Epidemiology
School of Veterinary Medicine
University of California-Davis
Davis-Ca, USA

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1.1. General summary and hypotheses

Foals are particularly prone to perinatal disease and the neurologic abnormalities are often the first clinical signs one can appreciate. Many physiologic processes can deviate during delivery since the mare's placenta separates very early and the second stage of labour only lasts about 20-30 minutes (Knottenbelt 2003). While the cattle can deliver live calf after 2-4 hours spent in second stage of labour, a foal will likely be stillborn after 1 hour, and be already very compromised after 30 minutes (Madigan 1997). Additionally, since the mare's placenta prevents passage of immunoglobulins in utero, newborns are highly dependent on colostrum intake and timing of first suckle. Considering the fractious nature of foals, the rigid birth environment, the extreme caloric and fluid needs of newborn foals, it is not surprising that foal mortality has been always the highest among domesticated animals (Platt 1973, Cohen 1995).

There are common, lay rules for assessment of normal behaviour and neurologic status of foals. The 1-2-3 rule is considered one of those: normal foal stands within an hour, suckles within 2 hours and in 3 hours the meconium is passed (Mayhew 1988). On the other hand, it is often

difficult to separate abnormal behaviour from transient findings and from an overt reaction given to external stimulus especially that response to neurological testing of apparently healthy newborn foals differs significantly from the adults (Adams and Mayhew 1984). Among others, premature foals for example (foals born prior to 320 days of gestation) are often hyperreactive and hyperreflexive (Rossdale and Leadon 1979, Madigan 1997). Foals born after prolonged gestation (foals born after 360 days of gestation) are usually weaker, slower and may exhibit various neurological deficits including abnormal behaviour, aimless wondering, inadequate suckle, vocalization, orientation problems, head-tilt and nystagmus. There is another subset of term foals that appear normal in the first 24 hours then become flaccid and obtunded progressively (Bernard et al 1995, Rossdale 1972).

Pioneers in the field of equine neonatal neurology including Rossdale, Ousey, Silver, Palmer, Mayhew, Madigan and a few others have investigated and described the neurologic exam and special neurologic findings in foals (Jeffcott and Rossdale 1979, Adams and Mayhew 1984, Bernard et al. 1995, Palmer and Rossdale 1976). Thus, we now recognize that foals show

stereotypic behaviour patterns that will disappear later in life. The most common stereotypic behaviour of foals is that they predictably collapse and become flaccid during a particular type of physical restraint (Mayhew 1988). This phenomenon previously referred to by authors as “flopping reaction or “reflex relaxation occurs in most newborn foals that are physically restrained (Jeffcott and Rossdale 1979).

The previous explains why normal and abnormal findings can be difficult to discern initially and that every foal, born from dystocia; or exhibit pre-, or dysmaturity, will have a high likelihood to show some form of neurologic deficit. Furthermore, most neurologically abnormal neonates will be categorized as “dummy”, hypoxic-ischaemic or maladjusted regardless of the findings and the inciting cause, which highlights that in reality how little is understood about the pathologic and humoral processes of neurologic diseases in foals.

1. Our first hypothesis was that, physical restraint of neonatal foals will result in vital, electroencephalographic, and humoral changes consistent with sleep and analgesia.

2. Our second hypothesis was that infectious neurologic disease, more specifically bacterial meningitis occurs in neonatal foals secondary to sepsis.
3. Our third hypothesis was that the serum steroid profile of foals with NMS is different than that of healthy control foals.
4. Our fourth hypothesis was that the infusion of allopregnanolone in a healthy neonatal foal would induce clinical signs compatible with NMS.
5. Our fifth hypothesis was that plasma adrenomedullin is increased in septic neonatal foals compared to sick non-septic and healthy control foals, and that it is predictive of survival.

1.2. Materials and methods

In experiment 1, eight foals were included. The criteria were as follows: foals had to be term (>330 days gestation) with a normal, uncomplicated delivery and had to be healthy without apparent neurological deficits based on neurological and physical examination.

A 20-foot-long, 0.5-inch diameter soft linen rope was used to construct the restraint device. A modified rope squeeze technique was applied, adopted from an earlier publication (Leahy and Barrow, 1953). As the first step, a bowline knot was used to secure the rope around the neck to prevent tightening of that segment which could result in pressure on the trachea and/or the jugular veins. Two half-hitch knots were used to loop the rope around the thorax and abdomen 5-10 inches from each other perpendicular to the vertebral column. The half hitch knots were positioned directly on the dorsal thoracolumbar area. To propagate the phenomenon, a designated person stood behind the foal and pulled on the rope producing a generalized squeezing of the foal, while a second person was holding the foal and assisted with lying it down. Tension was maintained on the rope (by the person holding the rope) until the experiment was completed.

‘Vital parameters’ were recorded on the foals before and during restraint. Heart rate and respiration rate were determined following auscultation with a stethoscope. Behaviour was recorded based on categorical visible changes in mentation and body position (bright and active or sleepy and recumbent).

Electroencephalographic (EEG) recordings were performed on a 32-channel telemetry unit (WEE-1000 AirEEG, Nihon-Kohden Corp, Tokyo, Japan) an acquisition system (EEG-9100 Neurofax, Nihon-Kohden Corp, Tokyo, Japan.) and a notebook computer (Dell Inspiron, Dell Corp, Round Rock, Texas, USA) with acquisition, review and file utility software installed (EEG-9200, Nihon-Kohden Corp, Tokyo, Japan). Stainless steel needle electrodes were placed subcutaneously using a modified (reduced number of electrodes) protocol used in foals at the Veterinary Medical Teaching Hospital of UC Davis (VMTH) as described in detail elsewhere (Aleman el al. 2006). Three central electrodes (C3, left; Cz, midline; and C4, right), 2 auricular electrodes (A1, left; A2, right), and a ground electrode (Z) were applied. The EEG study was performed continuously for a minimum of 15 minutes. The recordings were visually examined to determine state of consciousness using previously described criteria in humans and horses (Williams et al. 2008, Mysinger et al. 1985).

Pain threshold and analgesic effects of the restraint device were tested with a square pulse stimulator (S48 square pulse stimulator, Grass Technologies, West Warwick, RI, USA). For stimulation, 27 gauge 5/8-inch

needle electrodes (Safelead F-E3-48, Grass Technologies, West Warwick, RI, USA) were positioned with one 50 mm lateral to the base of the tail and the other 50 mm towards the ground over the semitendinosus muscle. The stimulation rate was 50 pulse per second (pps) with pulse duration of 10 msec each. Voltage range was 0.1 V - 20 V with a ramp rate of 0.2 V/s. The experimental session was started at a stimulus intensity of 0.1 mV, with the anodal electrode positioned distal to the cathodal electrode; if no response could be elicited, the current was gradually increased in steps of 0.2 mV/s until a response was observed. Positive conscious pain responses as dichotomous variables (yes or no) were either defined as purposeful avoidance movements of tail, limbs, trunk, head and neck, attempts to kick or turning the head toward the site of electrical stimulation at the time the stimulation was applied (Natalini and Robinson 2000).

Venous blood was collected into a 3 mL previously heparinized (1000 U/ml) syringes, closed with a plastic stopper and placed on ice immediately. Venous blood gas analysis was performed within 2 hours of collection with a commercially available blood gas machine (ABL-705 blood gas analyzer, Radiometer, Copenhagen, Denmark). Blood

was collected and transferred to serum, glass and plastic EDTA, and heparin tubes and then placed on ice until the experiments were finished. Samples were centrifuged for 10 minutes at 3400 rpm and stored at -80 °C (Revco ultra–low-temperature freezer, Thermo Fisher Scientific Inc, Franklin, MA, USA) until processing.

Measurements for ACTH and cortisol were performed with a solid-phase, two-site sequential chemiluminescent immunometric assay (IMMULITE 2000, Siemens AG, München, Germany) as described by others (Perkins et al. 2002).

Samples for other circulating steroids were analysed by liquid chromatography mass spectrometry (LC-MS) utilizing on-line sample extraction by turbulent flow chromatography (TFC) and detection by Single Reference Material (SRM) on a triple quadrupole mass spectrometer (Fisher Scientific Inc, Franklin, MA, USA). This method has been described in detail elsewhere (Yu et al. 2008).

Samples for β-endorphin measurements were collected in heparinized tubes. Tubes also contained 2.5 UI/ml of Bacitracin (Sigma B-0125, 50,000 U/g, Sigma-Aldrich Corp, St. Louis, MO, USA) and 1000 IU/ml Trasylol in 0.1

M PBS (pH=7.0 with 0.01% Thimerosal, 10,000 kIU/ml). Measurements were performed with β-endorphin radioimmunoassay (RIA) kits (S2013, Bachem Inc, St Helens, Merseyside, UK) that have been previously validated for use in horses (Bossut et al. 1983).

Animals served as their own controls in this prospective, interventional, pilot study. Following 15 minutes acclimatization time spent with the foal, pre-restraint blood samples and measurements were taken followed by application of the restraint device. While EEG was being performed, fifteen minutes into the restraint procedure blood samples were collected. Analgesia testing was performed after the EEG electrodes were removed. Paired comparisons were made between pre and post restraint values for each individual animal. Statistics were performed with a commercially available program (Minitab, version 15, Minitab Inc, State College, PA, USA). Distribution of the data was tested with the Kolmogorov-Smirnov normality test. Wilcoxon signed ranked tests were used to compare values pre and post restraint. Fisher's exact test was used to test the difference for dichotomous categorical variables (gender, physiologic vs. supraphysiologic level of ACTH). Spearman rank correlation analysis was performed to reveal any

association between plasma β-endorphin and pain threshold levels, plasma ACTH and androstenedione levels and plasma ACTH and DHEA-sulphate levels. Behaviour was not evaluated statistically. A *P*-value < 0.05 was considered to be significant in all tests.

In experiment 2, horses with a definitive diagnosis of meningitis based on cytological analysis of CSF or postmortem macroscopic and histopathological examination were included in the study. The electronic medical database of the William R. Pritchard Veterinary Medical Teaching Hospital was searched from the years 1985 to 2010, using the words meningitis, meningoencephalitis, and meningoencephalomyelitis under the clinical, laboratory and pathological diagnosis fields. Medical records were reviewed and data extracted included signalment, presenting complaint, history, clinical signs, laboratory work, diagnostic procedures, outcome and histopathological examination. Descriptive statistical analysis was performed on the data using a commercially available statistical software. (Minitab 15, Minitab Inc, State College, PA, USA) Means, medians, ranges, minimums, maximums, standard deviations, and standard errors were calculated.

In <u>experiment 3,</u> foals in the NMS foal group (n = 32; 15 colts and 17 fillies) and the other neonatal disease foal group (n = 12; 4 colts and 8 fillies) comprised foals admitted to the University of California, Davis Veterinary Medical Teaching Hospital in 2008 and Rossdale and Partners, Newmarket, UK in 2010 and 2011. To be included as a foal with NMS other disorders with a similar clinical presentation, such as prematurity and sepsis, were ruled out based on a minimum database (published sepsis score, complete blood count, chemistry panel, blood gases, indirect blood pressure, central venous pressure, blood culture, urinalysis, abdominal ultrasound and carpi, tarsi, thoracic and abdominal radiography) (Brewer and Koterba 1988). Foals with a sepsis score of 11 or greater were additionally classed as septic (Brewer and Koterba 1988). Historical knowledge of pre-, intra- or postnatal hypoxia was recorded. Clinical signs of NMS included altered mentation (obtunded, stuporous, comatose), decreased bonding to the mare, vocalisation, aimless wandering, hyper- or lack of reactivity to stimuli, seizures and abnormal ear position. Foals were subjectively scored by the attending clinician as mild-moderate if able to nurse and ambulate with help or severe if recumbent and unable to nurse, even with help.

Foals in the other neonatal disease group (sick, non-NMS controls) were randomly selected based on client consent and availability of the authors for sample collection.

A third group of healthy control neonatal foals (n = 10; 4 colts and 6 fillies) was recruited from the 2009 and 2010 foal crops at the Center for Equine Health, University of California, Davis. Inclusion criteria for control foals included a term birth (>320 days gestation) with normal, uncomplicated delivery and physical examination. All foals were less than 48 h of age at enrolment into the study. No attempts were made to standardise treatments given to the foals during hospitalisation. Outcome was recorded as survival to discharge. The study was approved by the University of California Institutional Animal Care and Use Committee and client consent obtained prior to enrolment in the study.

Heparinised blood was collected from healthy control foals at 0, 24 and 48 h following birth. Samples were collected from NMS foals and other neonatal disease foals after initial stabilisation and thereafter at the designated 24 and 48 h time points as appropriate. For foals presenting at birth, samples were collected within 2 h of parturition. Whole blood was immediately centrifuged after collection and plasma stored at -80°C until analysed.

Plasma was analysed as by LC-MS as described in Experiment 1.

Descriptive data are reported as median and ranges. Friedman tests were used for repeated measures analysis of steroid concentrations of healthy foals. Kruskal–Wallis tests were used for multiple group comparisons. Following a significant Kruskal–Wallis test, Mann–Whitney tests were used for non-paired 2 group comparisons with Bonferroni–Holm correction. Nonparametric tests were chosen based on the failure of the data to conform to normal distributions using a Kolmogorov and Smirnov test and inability to transform the data using conventional methods. Level of significance was set at $P<0.05$.

In experiment 4, a healthy neonatal 50 kg Quarter Horse colt from the research herd at the School of Veterinary Medicine, University of California, Davis was selected for the infusion.

Intravenous catheters were placed aseptically in the right jugular vein for sample collection and in the left jugular vein for infusion of allopregnanolone. Allopregnanolone (5 alpha-pregnan-3 alpha-ol-20-one) was dissolved in an ethanol-based solution to a total concentration of 9 mg/ml. Infused dose and concentration of allopregnanolone in this foal were determined based on concentrations

reported in *in vivo* studies in the modulation of the HPA axis in male rats (Naert *et al*. 2007). An initial bolus of 0.05 mg/kg bwt i.v. of allopregnanolone was given followed by a constant rate infusion (CRI) of 0.02 mg/kg bwt/min using an infusion pump. Based on clinical effects of the initial dosage, a second bolus of 0.1 mg/kg bwt i.v. was given after 5 min and followed by a CRI of 0.04 mg/kg bwt/min for 5 min. The infusion was discontinued for 30 min to allow observation of any neurobehavioural (NB) alterations, and then a final bolus of 0.2 mg/kg bwt i.v. was given. Neurobehavioural alterations were recorded and graded through a NB scoring system developed by the authors for the assessment of foals with NMS. From preliminary work, foals with NMS had scores >8 from a range of 0 (normal foal) to 20 (comatose with paroxysmal activity). Heparinised blood samples were collected at birth, age 6 h and at 15 min intervals during the infusion. Blood was immediately centrifuged following collection and plasma stored at -80°C until analysed by the same method as in experiment 1 using LC-MS. The study was approved by an Animal Care and Use protocol from University of California, Davis.

In experiment 5, critically ill neonatal foals < 1 week of age were recruited from Purdue University's Veterinary

Teaching Hospital and from Hagyard Equine Medical Institute during the 2011 and 2012 foaling seasons. Jugular venous blood was collected at admission for measurement of p[AM]. Critically ill foals were categorized as septic (sepsis score of > 11 and/or positive blood culture) or sick non-septic (sepsis score ≤ 11 and negative blood culture results when available). Foals euthanized due to financial constraints were excluded from the study. Healthy control foals were recruited from the equine teaching herd at Washington State University's College of Veterinary Medicine and from Gumz Farms, a privately-owned farm in Kentucky.

Information obtained from critically ill foals included signalment (year of admission, age of foal at admission [hours], breed and gender), history of dystocia or caesarean section, evidence of prematurity / dysmaturity, PE findings at presentation including behaviour, mentation, rectal temperature, heart rate, respiratory rate, capillary refill time (seconds), and fecal consistency. Admission mean arterial pressure and blood lactate concentrations were also recorded when they were available. Information obtained from healthy foals included history, signalment, PE findings, and blood IgG concentration at 24 hours of age.

Venous and arterial blood gas analysis (pH, partial pressure of carbon dioxide [PCO_2], partial pressure of oxygen [PO_2], and base excess) and haematologic data (PCV, MCV, MCHC, haemoglobin concentration, total white blood cell count, segmented neutrophil, band neutrophil count, presence of toxic neutrophils, lymphocyte, and platelet count) and plasma fibrinogen, were obtained. Biochemical findings (total CO_2, total calcium, phosphorus, sodium, chloride, potassium, magnesium, anion gap, glucose, creatinine, serum urea nitrogen, total protein, bilirubin, globulin and albumin concentrations and activity of alkaline phosphatase [ALP], creatine kinase [CK], aspartate aminotransferase [AST], gammaglutamyl transferase [GGT]) activity) were also collected. Blood culture results (positive or negative), type of organism (Gram positive, negative, anaerobic or fungal) and species were also recorded when available. Treatments, complications during hospitalization, length of hospitalization, outcome, and hospital bill were also obtained. Additional diagnostic modalities performed in some foals included ultrasonography, radiography, rectal digital palpation, jaundiced foal agglutination testing, fecal bacterial culture, synovial fluid analysis, and cerebrospinal fluid analysis.

Blood samples for culture were obtained after aseptic preparation of the skin and removal of 20 – 30 mL of blood into a sterile syringe from a jugular vein or following sterile placement of an intravenous catheter. The sampling needle was discarded and a separate needle was used to transfer 3 - 7 mL of blood into a commercially available blood culture bottle (BBL SEPTI-CHEK TSB (Tryptic Soy Broth), Becton Dickinson Microbiology Systems, Beckon Dickinson and Company, Cockeysville, MA, USA). For determination of p[AM], 7 - 10 ml blood was transferred into a plastic tube containing ethylenediaminetetraacetic acid (EDTA). Following centrifugation at 3200 rpm for 15 min at 4 °C, plasma was transferred into plastic tubes and placed into a - 20 °C or - 80 °C freezer. Samples in the -20 °C freezer were relocated to a -80 °C freezer within an acceptable time frame based on plasma AM stability (Nishio et al. 1997). Plasma AM concentration was determined using a previously validated, commercially available ELISA assay developed for equine AM (USCN Life Technologies, Wuhan, China).

For routine haematologic and biochemical analyses, blood was either collected from a jugular vein or from a jugular catheter upon admission. In some instances,

blood was also collected into heparinized 3-mL plastic syringes for immediate blood gas analysis. Haemograms were performed with a commercial automated multichannel blood cell–counting system (Cell-Dyn 3500R, Abbott Diagnostics, Abbott Park, IL, USA) with differential counts performed by manual cytologic examination of a blood smear. Serum biochemistry was performed with commercial automated analyzers (Vitros 5,1 FS Chemistry System, Ortho-Clinical Diagnostics, Inc, Rochester, NY, Olympus AU400 Beckman Coulter, Inc, Brea, CA, USA). Statistics were performed with commercially available software (SAS 9.3, SAS Institute Inc, Cary, NC, US). A Distribution of data was tested with the Shapiro-Wilk normality test. Continuous data were expressed as median and range. Mann-Whitney U test was used to test the differences between critically ill and healthy foals, septic and sick non-septic foals, and survivors versus non-survivors. The best cut-offs for p[AM] identified in a multivariate logistic regression model to be associated with health status was performed by use of receiver operating characteristic (ROC) curve analysis. It was considered as non-informative (area under the curve [AUC], 0.50), less accurate (AUC, 0.50–0.70), moderately accurate (AUC, 0.71 – 0.90), or perfect

(AUC, 1.00). For all analyses described above, a P-value < 0.05 was considered significant.

1.3 Results and Discussion

In experiment 1, we have shown that application of the described restraint device induces foals to lie down, and remain in lateral recumbency. Physical restraint appears to decrease the foals' voluntary motor activity; and also triggers somnolence as observed by the authors in all foals along with recorded periods of wakefulness/drowsiness, and late onset slow wave sleep (delta waves, K-complexes) in 3 foals. The latter findings are in agreement with the previous descriptions of SWS in humans, and horses (Berger et al. 1988, Williams et al. 2008, Mysinger et al. 1985). The foals from this study did not exhibit cataplexy as proposed by Adams and Mayhew (Adams and Mayhew 1984). The appearance of sleep spindles, K-complexes, vertex sharp waves and slow waves is not consistent with REM-sleep (Williams et al. 2008). Further, this phenomenon is a well-recognized

event of young foals induced by restraint (squeeze induced somnolence [SIS]).

Although foals exhibited significant increase in pain thresholds during the restraint, there was unlikely sufficient analgesia for surgical procedures causing moderate or severe pain. A study measuring responses to noxious electrical stimuli in horses receiving epidural opioids showed threshold responses higher than 40 V (Natalini and Robinson 2000), in contrast to threshold values <13 V reported during this study. It is unknown whether the analgesia provided would be sufficient for minor procedures. Although circulating β-endorphin was also elevated during restraint, there was no significant positive correlation in the percentile increase of β-endorphin and pain threshold level during restraint, therefore the role of endorphin in this phenomenon remains undetermined and its contribution to pain attenuation is unknown.

In Experiment 2, we have described that meningitis and meningoencephalomyelitis were diagnosed in 28 equine patients from our institution in a 25-year period. Important points from this study include the following: Meningitis is confirmed to be a rare disorder in horses, which is most

commonly associated with infection that can expand from local or haematogenous routes. Penetrating trauma with disruption of the BBB or meninges is also a common way for entry of organisms. Common neurological abnormalities include alterations in mental status, cranial nerve deficits, ataxia and vestibular dysfunction. Leucocytosis, neutrophilia, lymphopaenia, and hyperfibrinogenaemia are the most common haematological abnormalities in the case of infectious meningitis, mainly bacterial. Neutrophilic pleocytosis is characteristic of meningitis. Bacterial meningitis cannot be ruled out in the absence of degenerate neutrophils or organisms in a CSF sample. Polymerase chain reaction for common bacterial organisms and the use of lactate (L- and D-lactate) measurement should be considered as a diagnostic aid in CSF samples when bacterial meningitis is suspected and no bacterial growth is observed. Despite a low yield of bacterial isolation from CSF samples, CSF microbial culture should not be discouraged. In this study, 6 pathogens not previously reported were identified, 3 of which were isolated from antemortem CSF samples. Infectious meningitis or meningoencephalomyelitis is a potentially fatal disease. However, treatment should not be discouraged as cases with successful outcome have

been documented. Early recognition and aggressive treatment may improve survival in affected horses.

In experiment 3, we confirmed that there are differences in the pregnane profiles of neonatal healthy foals, foals with NMS and foals with other clinical diagnoses. Pregnane concentrations of healthy neonatal foals declined rapidly, to essentially zero, within 48 h of birth in agreement with the study by Houghton et al. (Houghton et al. 1991). The foetal foal is subjected to high levels of progesterone and other progestagens *in utero* (Holtan et al. 1991), deemed important in providing tonic inhibition of foetal central nervous system (CNS) activity and damping movement to prevent maternal damage (Mellor et al. 2005). The loss of placentally-derived precursors at birth and the switch to adrenal or other derived precursors causes this dramatic decline in pregnane concentrations shortly after birth in healthy neonates (Hirst et al. 2006). Apart from epitestosterone concentrations of sick control foals, foals presenting ill to the NICU (i.e. NMS and sick control foals) had higher concentrations of all measurable pregnanes than healthy controls within 2 h of birth. Pregnane concentrations of NMS foals remained increased over the 48 h time period in contrast to those of sick control foals that had significantly lower progesterone

and pregnenolone concentrations at 48 h compared with birth. Serial blood sampling with continued elevation or increasing pregnane concentrations over 48 h of age may therefore prove useful in aiding diagnosis and possibly prognosis of NMS; however, further work is required to validate this possibility. These observations support the hypothesis of a delayed, or interrupted conversion from intra- to extra-uterine life in ill, neonatal foals, particularly those with NMS. This mechanism may be similar to that reported in foals of mares treated with the progestagen altrenogest, which have a slower adaptation to the extra-uterine environment (Neuhauser et al. 2007). These steroids are suspected to be of adrenal origin based on extensive studies of neonatal lamb neurosteroid production (Mellor et al. 2005). Neonatal foals in this study and that of Rossdale (Rossdale et al. 1997) showed endogenous rises in neurosteroid concentrations thus eliminating placental origin. The higher DHEA concentrations in NMS foals compared with foals diagnosed with NMS and another disease suggests different adrenocortical responses in these foal subsets. Pregnane profiles did not appear to differ between mild-moderate and severely affected foals although it is likely that a larger population needs to be sampled to detect

such differences. Furthermore, the categorisation used may have been inappropriate for finding such differences. **In experiment 4,** infusion of allopregnanolone to a healthy foal produced marked neurobehavioural (NB) effects. This is consistent with the clinical use of certain steroidal drugs, such as alphaxalone, as anaesthetic agents in male rats (Naert *et al*. 2007). Allopregnanolone in other species has been shown to cross the blood–brain barrier and is thought to mediate its effects in the central nervous system (CNS) via the GABAA receptor (Zhu *et al*. 2001). Infusion of allopregnanolone in this healthy foal provided evidence that 5-alpha reduced pregnanes can cross the blood–brain barrier and have effects in the CNS. Allopregnanolone concentrations peaked in conjunction with maximum NB effects following infusion. The rapid recovery from NB alterations with no apparent residual deficits once the infusion was discontinued, suggested that allopregnanolone was quickly metabolised in this healthy foal. Similar rapid dampening effects in the CNS and recovery were observed with the use of the neurosteroid anaesthetic alphaxalone in ponies undergoing castration (Leece *et al*. 2009). As allopregnanolone is apparently metabolised rapidly, the clinical signs associated with NMS in foals would be

expected to dissipate rapidly. However, clinical manifestations of NMS can last several days, suggesting ongoing persistent production and release of allopregnanolone or other neurosteroids responsible for such observations. It is also unclear what triggers and stops these events in affected foals. Progestagen levels in this healthy foal decreased with age and are in agreement with the results of previous work (Holtan *et al*. 1991). The rise in DHEA between birth and age 6 h in this foal appeared to be neither testicular nor adrenal in origin as determined by constant levels of luteinising hormone and pregnanes, respectively, and was therefore deemed unlikely to be of biological relevance. The NB alterations induced by the infusion of allopregnanolone support our proposed hypothesis that NMS is in part a manifestation of persistent foetal HPA status mediated and sustained by elevated concentrations of progestagens as occurs naturally in the foetus (Warnes et al. 2004). The foetus must rapidly change from the quiet suppressed state *in utero* to one of arousal, and attempts to rise shortly after birth. A failure of the transition from the foetal HPA status to immediately post birth signals to engage the newborn into normal post foaling neurobehaviour may be the cause or involved in part in the pathogenesis of NMS. We

propose that ongoing production of pregnanes by the foal's brain and adrenal glands causes the clinical signs observed in foals with NMS and that rapid recovery of signs with no apparent residual deficits would be compatible with the decline of pregnane-mediated sedative type effects (Zhu *et al*. 2001). It is unclear how foals that are normal at birth develop NMS within the first 48 h of life. However, we speculate that a similar mechanism reported in neonatal sheep may occur whereby neonatal stress can increase allopregnanolone production by the brain and release of deoxycorticosterone from the adrenal glands, which the brain metabolises into 5a-tetrahydrodeoxycorticosterone (TH-DOC), another CNS depressant (Hirst *et al*. 2008). Obtundation, seizures and hyperaesthesia are common signs of NMS. Whilst the infused neuroactive steroid allopregnanolone has a dampening effect in the CNS, others within the large spectrum of neurosteroids, including metabolites of allopregnanolone, have excitatory effects that may be associated with seizures and hyperaesthesia (Rogawski and Reddy 2004). Neurosteroid concentrations in clinical NMS are likely to be a far more complex condition than that represented by infusion of one compound.

In experiment 5, we have found that plasma adrenomedullin (p[AM]) is not associated with sepsis or survival. There is a 6-fold increase in the median p[AM] in critically ill foals compared to healthy controls. Considering that the group of critically ill foals entailed approximately 20 different clinical diagnoses, we conclude that p[AM] is a marker of health rather than an indicator of a specific clinical entity in neonatal foals. There was also no significant correlation between sepsis score and p[AM], which may indicate a lack of true relationship between severity of sepsis and p[AM] in neonatal foals assuming that the conventional categorization using a weighted sepsis score and / or positive blood culture for sepsis in this study population was accurate. Human studies revealed different degree of changes in p[AM] with various clinical diseases (Gibbons 2007), with up to 50-fold increase in septic patients compared to healthy individuals (Ueda et al. 1999). LPS and inflammatory cytokines during sepsis and systemic inflammation induce AM gene expression in various tissues; and during septic shock p[AM] culminates more than in any other pathological condition in human patients (Hirata 1996). Contrary to these facts, there was no significant difference in p[AM] between septic foals and

non-septic foals, which may be due to the inaccuracy of the current sepsis status categorization. It is also possible that the aetiopathogenesis and disease course of equine neonatal sepsis is different compared to sepsis in humans. Experimental studies on laboratory animals show that there is a progressive and significant increase in p[AM] starting around 2 hours following a septic insult (Koo et al. 2001). Nearly half of the septic foals were less than 2 hours of age, which may have resulted in lower p[AM] values at the time of collection. This could explain why increased concentrations were not detected in the septic foal group. The increase in p[AM] is progressive and reaches plateau approximately 20-30 hours after the onset of septic insult (Koo et al, 2001), therefore it is possible that trends during disease course are more representative of diagnosis and prognosis than a single time point sampling.

There were no significant differences in p[AM] values in survivor versus non-survivor foals. This may be due to the lack of direct or indirect effect of p[AM] in survival. However, in human clinical sepsis studies, p[AM] appears to be not only a marker for evaluating disease severity, but also an early predictor correlating with subsequent organ dysfunction and outcome (Ueda et al. 1999). Since

foals subjected to euthanasia due to financial reasons were excluded from the study, it is less likely that euthanasia biased the statistical results. Despite that decisions for euthanasia were based on highly trained and experienced clinicians' discretion, it is still possible that with continuous intensive care a fraction of the euthanized foals would have recovered and survived to hospital discharge.

Plasma [AM] was evaluated within the following subgroups: FTPI, dystocia, caesarean-section, prematurity and NMS). It is noteworthy that none of these entities were associated with a significantly different p[AM]. In human clinical trials, pregnancy complications (preeclampsia, preterm delivery, low birth weight) are associated with significant changes in p[AM] (Lenhart and Caron 2012), while in infants with birth hypoxia, p[AM] is increased (Di Iorio et al. 2004). Plasma [AM] has been linked to numerous other pathologic conditions including cardiovascular, renal and lung diseases (Nishikimi et al. 2003); however, these were not evaluated in this study due to statistically inadequate numbers.

2. Summary of main scientific results

1. We have shown that application of the described restraint device induces foals to lie down, and remain in lateral recumbency.

2. Physical restraint decreased the foals' voluntary motor activity; and also triggered somnolence as observed by the authors in all foals along with recorded periods of wakefulness/drowsiness, and late onset slow wave sleep (delta waves, K-complexes).

3. We named this phenomenon based on its phenotypic characteristics: squeeze induced somnolence (SIS).

4. Meningitis is a rare disorder in horses. The overall prevalence of disease for the study period at this hospital was 0.04% (28/70,000), while in sick neonatal foals, it was 0.2% (2/1,000).

5. Altered mental status, cranial nerve deficits, and gait abnormalities were identified as the most common neurologic signs.

6. Most common clinicopathologic abnormalities included leucocytosis, hyperfibrinogenaemia in the peripheral blood, while increased protein and neutrophlic pleocytosis in the cerebrospinal fluid.

7. Six pathogens not previously described were isolated from a few horses of the present study, 3 of which were isolated from CSF samples obtained before death.

8. There are differences in the pregnane profiles of neonatal healthy foals, foals with NMS and foals with other clinical diagnoses. Pregnane concentrations of healthy neonatal foals declined rapidly, to essentially zero, within 48 h of birth.

9. Pregnane concentrations of NMS foals remained increased over the 48 h time period in contrast to those of sick control foals that had significantly lower progesterone and pregnenolone

concentrations at 48 h compared with birth. These observations support the hypothesis of a delayed, or interrupted conversion from intra- to extra-uterine life in ill, neonatal foals, particularly those with NMS.

10. Infusion of allopregnanolone to a healthy foal produced marked NB effects similar to NMS.

11. Infusion of allopregnanolone provided evidence that 5-alpha reduced pregnanes can cross the blood–brain barrier and have effects in the CNS.

12. The rapid recovery from NB alterations with no apparent residual deficits once the infusion was discontinued suggested that allopregnanolone was quickly metabolised in this healthy foal.

13. However, clinical manifestations of NMS can last several days, suggesting ongoing persistent production and release of allopregnanolone or other neurosteroids responsible for such observations.

14. Plasma adrenomedullin (pAM) was not associated with sepsis or survival.

15. There was a 6-fold increase in the median p[AM] in critically ill foals compared to healthy controls. We concluded that p[AM] is a marker of health rather than an indicator of a specific clinical entity in neonatal foals.

3. Publications in peer-reviewed journals related to the thesis

1. **Tóth B**, Horti K, Bakos Z. Special considerations for the neurological examination of foals and most common perinatal neurologic diseases. *Magyar Állatorv Lapja* 2018:140:259-270 **IF: 0,143**
2. Auth A. K, Rompos L, **Tóth B**. Primary care of neonatal foals II. Literature review. *Magyar Állatorv Lapja* 2017:139:131-142 **IF: 0,196**
3. Auth A. K, Rompos L, **Tóth B**. Primary care of neonatal foals I. Literature review. *Magyar Állatorv Lapja* 2017:139:67-78 **IF: 0,196**
4. **Tóth B**, Jerzsele Á, Horti K, Korenchy L, Bakos Z. Antibiotic therapy in neonatal foals. A literature review. *Magyar Állatorv Lapja* 2015:137:331-342 **IF: 0,212**
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2. **Tóth, B**., Auth, A., Rompos, L., & Bakos, Z. Effect of feed deprivation on selected parameters of lipid mobilisation and hepatic function in healthy Akhal Teke horses. *Equine Vet J*. 2018:50, 98-103 **IF: 2.115**

3. Tuska, P., **Tóth, B.**, Vásárhelyi, G., Hangody, L., Papp, M., & Bodó, G. Evaluation of biomarkers following autologous osteochondral transplantation in the equine stifle joint—An experimental study. *Acta Vet Hung 2*017:64:164-178. **IF: 1,042**

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5. Taylor SD**, Tóth B**, Townsend WM, Bentley TR. Mechanical ventilation and management of an

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19. Tóth B, MacGillivray KC: Cerebellar hypoplasia in a foal. Clinical snapshot. *Compend Cont Ed Pract Vet* 2009:60,62. **IF: -**

5. Scientific meetings (presentations and posters)

1. **Toth. B**, Bakos Z. Effect of enteric coated omeprazol on the calcium homeostasis of horses. ECEIM poster. 2018
2. Kovacs Sz., **Tóth B,** Bakos Z. Weight estimation of foals. BEVA abstract. 2017.
3. Bakos Z, Auth A. Rompos L.,**Tóth B.** Effect of feed deprivation on selected variables of lipid mobilization and liver function in horses. ECEIM oral abstract. Helsinki. 2016.

4. **Tóth B.** Prognostic value of amylase and lipase in proximal enteritis of horses. ECEIM oral abstract. Utrecht 2015

5. Sojka-Kritchevsky JE, **Toth B**. Feeding the dysphagic horse. *ACVIM Proceedings*. 06. 2013.

6. **Toth B**. Plasma adrenomedullin concentration as a predictor of survival in neonatal foals. *ACVIM Proceedings*. 06.2013

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8. Charney VA, Bertin FR, **Toth B**, Couëtil LL, Kritchevsky JE, Taylor SD, Miller MA, Ramos-Vara JA. Ichtysioform dermatitis in camelids. *ACVP Proceedings* 2012

9. **Toth B**, Taylor SD. Rhodococcus equi pneumonia in foals. Indiana Veterinary Medical Association. Indianapolis 02. 2012.

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11. **Tóth B,** Nógrádi N. Hasi és mellkasi ultrahangvizsgálat lovakban. Magyar Lógyógyász Állatorvosok Egyesülete. Gyakorlati előadás. 09.10.11-09.11.11. Budapest. Hungary. 16 óra

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13. Sojka-Kritchevsky JE, **Toth B**, Bertin FR, Messick JB. Progression of parasitemia and clinical course in a splenectomized alpaca with *Mycoplasma haemolamae* infection. *ACVIM Proceedings.* Denver. 2011

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