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Evaluation of trigeminal nociceptive processing in horses

PhD Dissertation

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Abbreviations

BR: blink reflex
EMG: electromyogram
ION: infraorbital nerve
NRS: numerical rating scale
NWR: nociceptive withdrawal reflex
RS: repeated stimulation
SON: supraorbital nerve
SS: single stimulation
TCR: trigemino-cervical reflex
$T_{RS}$: trigemino-cervical reflex threshold intensity after repeated stimulation
$T_{SS}$: trigemino-cervical reflex threshold intensity after single stimulation
VAS: visual analogue scale
DNIC: diffuse noxious inhibitory control
1. Summary

As a result of a general increased knowledge and sensibility toward pain in humans, successful pain management in equine patients became recently an important issue. Pain recognition and assessment are the first steps to provide adequate analgesic therapy. As mechanism-based treatment options facilitate successful pain management, the investigation of the pathophysiology of neuropathic pain disorders is essential. This latter necessitate substantial understanding of physiologic function of peripheral and central nervous system. While nociceptive neurophysiology of the equine locomotor apparatus is well established, information about nociceptive processing of the trigeminal system in horses has been lacking.

Electrically induced reflexes can be used to investigate the physiology and pathophysiology of the trigeminal system in humans. Similarly, the assessment of the trigemino-cervical (TCR) and blink reflexes (BR) may represent a new diagnostic tool in horses.

Therefore, the first aim of our investigations was to evoke nociceptive trigeminal reflexes and describe their electrophysiological characteristics in non-sedated horses. The infraorbital (ION) and supraorbital nerves (SON) were stimulated transcutaneously in 10 adult Warmblood horses in separate sessions using train-of-five electrical pulses. The current was increased gradually until the TCR threshold was found. The stimulus–response curve of the TCR was evaluated. At the same time as TCR, the BR response was also assessed. Surface electromyographic (EMG) responses were recorded from the orbicularis oculi (OO), splenius (SPL) and cleidomastoideus (CM) muscles. Latency, duration, amplitude of the reflexes and behavioural responses were analysed.

We found that noxious electrical stimulation of the ION or SON evoked reflex EMG responses, with similar features regardless of the nerve that had been stimulated. Stimulations of increasing intensity elicited reflexes of increasing amplitude and decreasing latency, accompanied by stronger behavioural reactions, therefore confirming the nociceptive nature of the TCR.

Our second aim was to evaluate the effect of repeated stimulation on this nociceptive withdrawal reflex, the TCR. It is known that repeated sub-threshold nociceptive electrical stimulation results in temporal summation of the limb nociceptive withdrawal reflex and it is a well-established non-invasive model to investigate the wind-up phenomenon in horses. Due to structural similarities of the trigeminal sensory nucleus to the dorsal horn of the spinal
cord, temporal summation should be evoked by repeated transcutaneous electrical stimulation of trigeminal afferents.

To evaluate this hypothesis repeated transcutaneous electrical stimulation was applied to the SON and ION of 10 horses. Stimulation intensities varied between 0.5 and 1.3 times the TCR threshold defined for single stimulation (T_{SS}). Evoked EMG activity of the OO, SPL and CM muscles was recorded and the signals analysed in the previously established epochs typical to the early and late component of the BR and to the TCR. Behavioural reactions were evaluated with the aid of numerical rating scale.

We found that the nociceptive late component of the BR and the TCR were not elicited by sub-threshold intensity repeated transcutaneous electrical stimulation. Furthermore, the median reflex amplitude for the 10 horses showed a tendency to decline over the stimulation train so temporal summation of afferent trigeminal inputs could not be observed. Therefore, the modulation of trigeminal nociceptive processing attributable to repeated Aδ fibre stimulations seems to differ from spinal processing of similar inputs as it seems to have an inhibitory rather than facilitatory effect. Further evaluation is necessary to highlight the underlying mechanism.

In horses affected by trigeminal pathology, altered nociceptive modulation and DNIC deficiencies could modify the neurophysiological profile observed in the healthy subjects. Therefore, our results regarding to the trigeminal nociceptive reflexes are representing a novel non-invasive tool for a mechanism-based approach to diagnosis.
2. Introduction

In the last decade successful pain management became one of the main goals of the veterinarians. Fortunately equine clinicians recognized that the benefits of analgesia surmount the side effects of the treatment (Taylor, Pascoe et al. 2002). Untreated pain has several undesirable consequences as it provokes sympathetic stimulation, alterations in the neuroendocrine system and development of pathological pain conditions. All these factors lead to increased stress level and even distress, leading to a deterioration of the patient’s quality of life which raises welfare issues (Stafford and Mellor 2007; Lerche and Muir 2008).

Consequently, the necessity of proper pain management is beyond doubt and continuous research helps to understand the underlying mechanisms of different types of pain. This enables mechanism-based treatment approaches (Vinuela-Fernandez, Jones et al. 2007), although equine studies are still lacking in this field (Muir 2010). Besides the tools to alleviate pain, the second cornerstone of successful pain management is the effective and accurate pain assessment (Stafford and Mellor 2007). Recognition and assessment of pain in horses is challenging as they are nonverbal prey animals hiding their symptoms. Furthermore, pain is known to be a subjective, multidimensional experience with high individual variability lacking objective measures (Taylor, Pascoe et al. 2002; Lerche and Muir 2008).

Equine idiopathic headshaking is one of those syndromes where pathologic pain conditions could be responsible for the clinical signs. The symptoms of the disease, including nasal rubbing, snorting and sudden flipping of the head, may deteriorate during exercise and make the horse unrideable. Many affected animals are euthanized on humanitarian grounds as they are in enormous distress and evidently suffering. Although the pathophysiology behind the clinical signs is not known (Newton, 2005) neuropathic pain originating from the trigeminal nerve could be responsible for the symptoms observed (Newton et al., 2000; Roberts et al., 2009). Further, success in the treatment of headshaking in some horses has been obtained using a therapeutic regimen that is commonly used to treat trigeminal neuralgia in humans (Newton et al., 2000).

To investigate the physiology and pathophysiology of the trigeminal system in humans, electrically induced reflexes can be used. As a trigeminal neuropathic disorder is suspected in headshaking horses, investigating the trigeminal nerve function with electrophysiological methods might disclose new perspective into the understanding of the pathophysiology of this disease.
Therefore, our aim was to facilitate successful trigeminal pain management in horses by reviewing the different methods to recognize and assess pain in horses in general, and by evaluating the physiologic function of the trigeminal nerve. The latter could open the door for the development of diagnostic methods and mechanism based treatment options for horses with trigeminal disorders, like equine idiopathic headshaking. According to our expectations, our results and methods might be applied in the preclinical assessment of different analgesics to evaluate their efficacy and the duration of their effect.
3. Literature review

First of all we list the actual definitions of pain taxonomy published by the International Association for the Study of Pain (IASP), which are related to our investigations. As these definitions are used throughout the dissertation, we would like to provide clear reference for the reader.

Secondly, we review the different possibilities of pain assessment in horses reported in the literature to provide a complete overview about available methods. Successful pain management is based on the recognition of pain and mechanism-based treatment approaches, which are equally important and cannot stand without each other. Unfortunately, there are no specific and validated methods to evaluate facial (trigeminal) pain in horses; therefore we discuss the topic in general, covering also the methods we have selected to evaluate behavioural reactions of our horses throughout the experiments.

Thirdly, we review how electrical stimulation can be used to evaluate physiologic and pathologic nerve functions in general and justify our choice of methods to evaluate trigeminal function in horses.

Finally, we provide an overview what is known about the anatomy and function of the trigeminal nerve in horses and discuss the methods used to evaluate its function in humans.

3.1 Pain taxonomy as definitions by the International Association for the Study of Pain (IASP) (www.iasp-pain.org, 2012)

**Pain**

An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

**Allodynia**

Pain due to a stimulus that does not normally provoke pain.

**Causalgia**

A syndrome of sustained burning pain, allodynia, and hyperpathia after a traumatic nerve lesion, often combined with vasomotor and sudomotor dysfunction and later trophic changes.
Dysesthesia

An unpleasant abnormal sensation, whether spontaneous or evoked.

Hyperalgesia

Increased pain from a stimulus that normally provokes pain.

Hyperesthesia

Increased sensitivity to stimulation, excluding the special senses.

Hyperpathia

A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold.

Hypoalgesia

Diminished pain in response to a normally painful stimulus.

Hypoesthesia

Decreased sensitivity to stimulation, excluding the special senses.

Neuralgia

Pain in the distribution of a nerve or nerves.

Neuropathic pain

Pain caused by a lesion or disease of the somatosensory nervous system.

Neuropathy

A disturbance of function or pathological change in a nerve: in one nerve, mononeuropathy; in several nerves, mononeuropathy multiplex; if diffuse and bilateral, polyneuropathy.

Nociception

The neural process of encoding noxious stimuli.
**Nociceptive neuron**

A central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli.

**Nociceptive pain**

Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors.

**Nociceptive stimulus**

An actually or potentially tissue-damaging event transduced and encoded by nociceptors.

**Nociceptor**

A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli.

**Noxious stimulus**

A stimulus that is damaging or threatens damage to normal tissues.

**Pain threshold**

The minimum intensity of a stimulus that is perceived as painful.

**Pain tolerance level**

The maximum intensity of a pain-producing stimulus that a subject is willing to accept in a given situation.

**Paresthesia**

An abnormal sensation, whether spontaneous or evoked.

**Sensitization**

Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs.
Central sensitization

Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input.

Peripheral sensitization

Increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields.

3.2 Recognition and assessment of pain in horses

As no golden standard exists for pain evaluation in horses, our goal was to provide an overview of the different methods to assess pain in horses discussing both their advantages and limitations.

3.2.1 Behavioural and physiological indicators of pain in horses

As horses cannot tell how much pain they are experiencing the – subjective – human observer judge the severity of their discomfort. The observer must be familiar with normal equine behaviour, however, this may be altered with breed and age (Lerche and Muir 2008). Furthermore several physiological parameters have been incorporated into equine pain scales, advantages and disadvantages are discussed in the following paragraphs.

3.2.1.2 Behavioural assessment

Recently Ashley et al. (Ashley, Waterman-Pearson et al. 2005) reviewed the nonspecific and specific behavioural indicators of equine pain cited in the literature. According to this review, the nonspecific indicators of pain in horses are: considerable restlessness, agitation and anxiety, rigid stance and reluctance to move, lowered head carriage, fixed stare and dilated nostrils, clenched jaw, aggression towards own foal, handlers, horses, objects and self. According to the origin of pain, indicators of abdominal, limb and foot, head and dental pain were also collected. The following behaviours are referring to abdominal pain: vocalization (deep groaning), rolling, kicking the abdomen, flank watching, stretching, dullness and depression. The specific indicators of limb and foot pain are: weight-shifting between limbs, limb guarding, abnormal weight distribution, pointing, hanging and rotating limbs, abnormal movement, and reluctance to move. Headshaking, abnormal bit behaviour, altered eating, anorexia, quidding, food pocketing are usually the signs of head and dental pain.
The observation of these behaviours facilitates the detection of discomfort and pain; however, they are rarely reflecting the severity and the source of pain or the progression of the disorder (Ashley, Waterman-Pearson et al. 2005). Furthermore, unfamiliar surroundings or presence of strangers may suppress these behaviours (Lerche and Muir 2008). As pain is known to be a dynamic process, the amount of time horses spent displaying indicators of pain can be small (Pritchett, Ulibarri et al. 2003). Continuous video recording and analysis of activity budgets could facilitate pain assessment but is impractical for daily clinical use (Price, Catriona et al. 2003). Still, behavioural observation is an important tool to distinguish between pain levels (Hardie 2000), especially if sensitive indicators have been identified and incorporated into validated pain scales.

3.2.1.2 Physiological parameters

The effect of pain on the autonomic nervous system leads to possible alteration in the physiological parameters like heart rate, respiratory rate, non-invasive blood pressure (NIBP), cortisol, catecholamines or beta-endorphine plasma levels. Heart rate and respiratory rate are easy to measure but they are not specific measures of pain as other factors, e.g. anxiety, hypovolemia, drugs, can influence them. The results of Rietmann (Rietmann, Stauffacher et al. 2004) showed that analysis of heart rate variability may represent an alternative method for the evaluation of the autonomic nervous system activity and may be useful in pain assessment. Increase in mean NIBP was reported as specific and sensitive indicator of orthopaedic pain (Wagner 2010) which correlated to the pain scores. When evaluating plasma levels of beta-endorphine, cortisol and catecholamines, only the increase of beta-endorphine correlated to severe orthopaedic pain (Raekallio, Taylor et al. 1997) in horses, the other parameters were found unreliable for pain assessment (Taylor, Pascoe et al. 2002). Anyway, the measurements of catecholamine, cortisol or beta-endorphine levels are invasive, expensive and need serial measurements, therefore not applicable for daily clinical use.

3.2.2 Pain scoring systems

Pain scoring systems are developed to assess the intensity of pain and response to therapy. They use quantitative measures with the aim to enable detection of pain and its progression (Ashley, Waterman-Pearson et al. 2005). The ideal pain scoring system is easy to use even by unexperienced observers, linear and weighted, sensitive to the type of pain, breed and species specific, repeatable and providing consistent results (Ashley, Waterman-Pearson et al. 2005; Wagner 2010). The benefits of using pain scoring system according to a recent
review by Wagner (Wagner 2010) are: promoting the recognition and quantification of pain in individual animals, better inter-observer agreement regarding to the level of pain assessed which improves patient care due to consistent evaluations, raising awareness of animal pain and encouraging treatment, the progress or deterioration of the patient can be followed and documented.

Pain scoring systems are based on elected behavioural indicators of pain and some of them incorporating the evaluation of physiological parameters, too. Pain assessment improves if more indices – possibly weighted with multivariate analysis – are used which are changing over different pain intensities (Molony and Kent 1997). To determine the validity and sensitivity of pain indicators and to remove unreliable indices from scoring systems, every index alone or combined with others needs to be evaluated using groups of animals ranked according to the severity of their pain (Molony and Kent 1997). As it is a rather complex process, the number of available pain type-specific validated scoring systems for horses is limited and the existing ones all need further evaluation.

### 3.2.2.1 Visual analogue scale

Visual analogue scale (VAS) consists of a 100 mm line with "no pain" and "worst possible pain" as two extremities and the observer places a mark over the line representing the severity of the patient's pain according to the observer's impression. The pain assessment relies on the subjective opinion of the observer judging the behaviour of the horse and the experience and training of that person; therefore high inter-observer variability and underestimation of the pain is probable (Taylor, Pascoe et al. 2002; Vinuela-Fernandez, Jones et al. 2011). However, when used to evaluate pain in horses with laminitis, VAS was found to be a reliable measure of lameness over time and between observers even with limited experience. Its amended but also not validated form, the dynamic interactive visual analogue scale (DIVAS) which is evaluating the willingness of interaction of the patient with the observer beside the subjective pain level was reported as a measure of post-operative pain after castration in horses (Love, Taylor et al. 2009).

### 3.2.2.2 Numerical or verbal rating scales

Numerical rating scales (NRS) and verbal rating scales (VRS) assign scores or letters to behavioural descriptions. This approach is also dependent on the observer and usually not linear (Taylor, Pascoe et al. 2002). Probably the most known scales among veterinarians are the ones used for lameness evaluation during the daily clinical work. However, the inter- and
intra-observer variability of these scales is high indicating only moderate reliability (Hewetson, Christley et al. 2006). The degree of lameness in laminitis is often evaluated by the use of Obel-grading system which reflects the effect of analgesic management (Rietmann, Stauffacher et al. 2004). The use of a clinical grading system reported by Taylor (Taylor, Hood et al. 2002) had high overall reliability across times and observers assessing lameness in horses with laminitis (Vinuela-Fernandez, Jones et al. 2011) together with the modified Obel-score (Owens, Kamerling et al. 1995). Despite being some of these scales widely used, none of them is validated in horses. It is worth to mention, that new techniques like force plate analysis can promote the objective assessment of orthopedic pain (Vinuela-Fernandez, Jones et al. 2007).

3.2.2.3 Multidimensional pain scales

Multidimensional pain scales incorporate the most relevant indicators of a certain type of pain, possibly previously presented in the literature, and in some occasion assign a different weight to each indicator according to its clinical relevance. An adequate score is attributed to each parameter and the sum of these scores defines the total pain score. Recently few scales were developed and found to be reliable assessing the severity of orthopaedic, acute abdominal or post abdominal surgery pain. All of them were developed for easy daily clinical use to facilitate the determination and documentation of the level of pain and the assessment of the response to therapy.

The composite orthopaedic pain scale developed by Bussieres et al. (Bussieres, Jacques et al. 2008) was the first multidimensional scale evaluating acute equine orthopaedic pain. When validated in horses affected by acute experimental synovitis, heart rate and respiratory rate were the only physiological parameters found to be at least moderately specific and sensitive. On the other hand, all evaluated behavioural items showed good to excellent reproducibility and most of them were reported as having good-to-excellent sensitivity and specificity, like posture, kicking the abdomen, pawing on the floor, response to palpation at the painful area; head movement showed excellent sensitivity besides its good reproducibility. From the complementary physiological criteria (mean systemic arterial blood pressure, blood glucose, blood cortisol), blood pressure was a specific parameter with excellent sensitivity to indicate the severity of pain. Anyway, the overall sensitivity of the composite pain scale was good, and it was shown to be a valid method to differentiate between three different levels of pain intensity. Revalidation of the scale with adjustment regarding to the parameters with low sensitivity and specificity would possibly improve the performance of the scale (Bussieres, Jacques et al. 2008).
Recently Sutton et al. (Sutton, Dahan et al. 2012) constructed a behaviour-based pain scale via clinimetric approach to evaluate pain severity in horses with acute colic symptoms. First they identified easily recognisable behavioural items among those which are reported typical for colic horses in the literature (mathematical approach) and then experts ranked the selected indicators regarding to the severity of pain they are representing (judgemental approach). The frequency of certain behaviours defines the pain score on the multidimensional scale which was called equine acute abdominal pain scale (EAAPS). The study had couple of limitations as the expert group was small and they forced some parameters into the final scale which had been excluded before due to poor performance by the mathematical and judgemental approaches (Sutton, Dahan et al. 2012). Before the scale can be used widely it needs to be validated.

The first approach to assess postoperative pain in colic horses was a complex numerical rating scale by Pritchett et al. (Pritchett, Ulibarri et al. 2003). This could differentiate between postoperative colic horses and horses underwent anaesthesia for diagnostic imaging or healthy controls (Pritchett, Ulibarri et al. 2003), but had no validation. Later the post abdominal surgery pain assessment scale (PASPAS) consisting of arbitrarily scored physiological and behavioural parameters was presented by Graubner et al. (Graubner, Gerber et al. 2011). They found low inter-observer variability and good correlation of the following indices with the total pain score: general subjective assessment, postural behaviour, response to food. Its clinical validity has been tested on clinical patients and was found to be a reliable tool to use after short training. To further improve the validity of the scale, exclusion or weighting of the physiological parameters was recommended by the authors (Graubner, Gerber et al. 2011).

3.2.3 Quantitative methods to assess pain in horses

Applying a standardized mechanical, thermal or electrical stimulus evoking response from the patient enables an objective and quantitative determination of the nociceptive threshold. Threshold testing can be applied in experimental settings to evaluate the potency and duration of action of analgesic drugs, to investigate the pathophysiology of certain neurological disorders or clinically to diagnose pathologic forms of pain like hyperalgesia or allodynia. In this case, threshold assessment is usually defined as quantitative sensory testing (QST) (Love, Murrell et al. 2011).
3.2.3.1 Thermal stimulation

Recently Love et al. (Love, Murrell et al. 2011) reviewed the application of thermal and mechanical nociceptive testing methods in horses. Latency of response to radiant heat was used to evaluate the efficacy of analgesic drugs in horses but the method has several limitations like different conductive properties of the skin, distance from the heating source, and learning effect. Thermode based systems were developed to measure thermal nociceptive thresholds and used to investigate antinociceptive effects of analgesic drugs in horses. The different rate of increase in temperature enables the separate evaluation of Aδ (fast rate) and C fibers (slow rate) function. However fast cooling probes were developed and even if cut-off temperatures are incorporated in the tests, thermal skin injury can occur. Direct contact of the probe with the skin influences the results via the activation of mechanosensitive efferents (Love, Murrell et al. 2011). Most of the limitation of thermal stimulation could be eliminated by the use of laser as heat source (Garcia-Larrea 2012) but this has not been evaluated in horses until now.

3.2.3.2 Mechanical stimulation

Equine clinicians are using a simple form of mechanical sensory testing in their daily routine when they apply the hoof tester. These devices lack the ability to measure the applied force objectively therefore they can be used only to provide an estimate of the increased hoof sensitivity. Quantitative mechanical testing tools described for use in horses include: a cuff with a blunt ended pin applying pressure over the dorsal surface of the cannon bone, a similar device with a sharp-ended pin, a hand-held pressure algometer, spring-loaded pinprick instruments and von-Frey filaments to evaluate somatic pain and balloon models to evaluate visceral pain (Driessen, Scandella et al. 2008; Love, Murrell et al. 2011). These methods were used mainly in research settings to define mechanical nociceptive thresholds and evaluate analgesic effect of drugs. Only the hand-held pressure algometer has been reported to quantify muscle or joint pain in clinical or research conditions (Varcoe-Cocks, Sagar et al. 2006; Haussler, Behre et al. 2008; van Loon, Menke et al. 2012) and von-Frey filaments to quantify cutaneous sensitivity after tissue damage (Redua, Valadao et al. 2002).

3.2.3.3 Electrical stimulation

When electrical stimulation is applied to produce a nociceptive input, not only the visual behavioural response can be monitored easily but electromyographic (EMG) activity of the efferent muscles can be recorded serving as quantitative measure of nociception. The
threshold of nociceptive withdrawal reflex (NWR) and the magnitude of the EMG activity strongly correlate with the subjective pain sensation in humans (Price 1989; Curatolo, Petersen-Felix et al. 2004). Electrical stimulation directly depolarizes the afferent nerves bypassing the receptors, which makes it a non-selective modality to evaluate nociception (Curatolo, Petersen-Felix et al. 2004). The activation of the different afferent sensory fibers (Aβ, Aδ and C fibers) are frequency dependent (Curatolo, Petersen-Felix et al. 2004), so the stimulation paradigm can be designed according to the research interest. Repetitive electrical stimulation evoking temporal summation is a model of a long-lasting nociceptive input (Curatolo, Petersen-Felix et al. 2004) which may reflects better clinical pain conditions. Clinical application of nociceptive reflexes for pain assessment is limited because it requires special equipment and reported reference values for each reflex. However, it is a valuable and widely used non-invasive tool to investigate the efficacy of analgesic drugs or techniques in horses using either behavioural observation of avoidence behaviour (Kerr, McDonell et al. 1996; Natalini and Robinson 2000; Natalini and Linardi 2006; Natalini, Polydoro Ada et al. 2006; Driessen, Scandella et al. 2008) or reflexes measured by EMG as end-point. (Spadavecchia, Levionnois et al. 2006; Spadavecchia, Arendt-Nielsen et al. 2007; Peterbauer, Lenza et al. 2008; Rohrbach, Korpivaara et al. 2009; Levionnois, Menge et al. 2010).

3.2.3.4 Quantitative sensory testing (QST)

Physiologic pain has an important protective function in the adaptation to environmental conditions and survival; however, the proper function of the nociceptive system is mandatory to fulfil this role. Pathologic alterations of the nociceptive system results in the development of neuropathic pain syndromes, like, for example, allodynia, hyperalgesia or spontaneous pain (Woolf and Salter 2000).

The commonly used quantitative sensory testing methods consist in applying certain thermal and mechanical stimulation methods to evaluate the change in the sensitivity of skin areas innervated by different nerve branches via observation of visible responses. Unfortunately, only few normal values for QST are reported in the literature (Haussler and Erb 2006; Haussler, Behre et al. 2008) therefore their clinical use is limited to diagnose altered nociceptive function in horses if baseline measurements before the painful condition arose are not available. However, measurements on the contralateral side of the body can serve as useful comparison if the presence of painful area is suspected. The combination of different stimulation modalities provide a more comprehensive picture about the condition of the
patient due to the involvement of different nociceptors and type of afferent fibres which facilitates mechanism-based pain treatment (Love, Murrell et al. 2011).

3.3 The evaluation of nerve function by electrical stimulation

Nociceptive withdrawal reflexes (NWR) are the most relevant and widely used non-invasive methods to investigate nociceptive processing in humans and in animals. A potentially harmful stimulus generates afferent inflow and after spinal procession of the sensory input it may provoke motor response to escape from the painful impact. Therefore the NWR not only serve as direct measure of peripheral and spinal nociception but also provide indirect measure of supraspinal modulation of spinal transmission (Andersen 2007). The standard method to evaluate NWR is by electrical stimulation as this modality bypasses the peripheral receptors and the response can be recorded and analysed quantitatively by EMG. Other neurophysiological tools like electroencephalogram (EEG) and sensory evoked potentials (SEP) show poor sensitivity as an indicator of nociception (Curatolo, Petersen-Felix et al. 2004; Murrell and Johnson 2006).

The typical single stimulation (SS) paradigm used in the evaluation of nociceptive reflex responses in humans (Sandrini et al. 2005) is the train-of-five constant current square wave pulses delivered at the frequency of 200 Hz. The duration of each stimulus in the train is 1 ms which will result in 21 ms duration of the complete stimulus. Compared to a single-shock (one pulse of 1 ms) the train of five allows to decrease the intensity and length of stimulation necessary to evoke the reflex (thus decreasing the occurrence of possible stimulus-related tissue damage); being its frequency (200 Hz) so high the 5 stimuli are perceived as a single stimulus in humans.

The frequency of the stimulation is thought to be fibre selective; 2000 Hz stimulation is used to evaluate the function of Aβ-fibres, 250 Hz stimulation induces responses from Aδ-fibres and 5 Hz pulses stimulate C-fibres when used in constant current perception testing (Liao et al, 2010). However, at higher stimulation intensities, Aδ fibres are also recruited along with the Aβ fibres when 2000 Hz stimulation is applied; Aβ and Aδ fibres are producing tonic firing above the same threshold stimulating with 250 Hz; Aβ and Aδ fibres are stimulated at much lower intensity than C fibres when 5 Hz stimulation is used (Koga et al. 2005). This shows that intensity of the stimulation plays a more important role in the recruitment of the sensory fibres than the frequency of stimulation itself.

As horses are non-verbal species, objective measures are needed to separate perception threshold and pain threshold, because reflex activity can be present before the subject
actually senses the stimulation as painful. The latency of the muscle compound action potential reflects the conduction velocity of the fibres, which is representative to the type of fibres activated (Andersen 2007). This helps to confirm the nociceptive origin of the given reflex components.

Several recent studies have focused on the determination of the physiological characteristics of the NWR to investigate spinal nociceptive processing linked to Aδ fibres activity in different species (Spadavecchia et al., 2002; Bergadano et al., 2006) and to assess the antinociceptive effectiveness of pharmacological interventions (Spadavecchia et al., 2005, 2007; Bergadano et al., 2009a,b; Rohrbach et al., 2009; Levionnois et al., 2010; Lervik et al., 2012). Using a standard electrical stimulus, the NWR threshold can be defined as the minimal current intensity needed to evoke a NWR in a given subject. As part of the NWR model, the repeated stimulation paradigm can be applied to evaluate the modulation of temporal summation, an important phenomenon most likely resulting in wind-up and central sensitisation (Arendt-Nielsen et al., 1994). Subthreshold repeated stimulation elicit temporal summation of synaptic potentials at the level of the secondary wide dynamic range (WDR) neurons of the afferent pathway thus facilitating the NWR (Arendt-Nielsen et al., 2000). Originally, wind-up was believed to result from repetitive C-fibre stimulation only (Li et al., 1999). Later on, it was proved in humans that the wind-up phenomenon involved the Aδ fibres as well, when these were stimulated at a minimum frequency of 0.3 Hz (Andersen et al., 1994). Stimulation of C-fibres would necessitate a high stimulus intensity and long duration due to the characteristics of C-fibres, which would be neither acceptable nor tolerable for subjects in research settings (Arendt-Nielsen et al., 2000), especially if the subjects are non-medicated animals.

However, as pain is a multidimensional sensory and emotional experience, nociceptive stimulations only serve as pain models but fail to evoke the same pain experience as in clinical, especially chronic pain conditions (Love, Murrell et al. 2011). It is also known that mental status of the patient can influence the test results which necessitate standardized environment for the evaluations (Yarnitsky and Granot 2006).

3.4 Trigeminal nerve

The nervus trigeminus provides the sensory innervations of the head surface; therefore, it is playing an important role in the protection against external, harmful impacts. In humans, the blink reflex (BR) and the trigemino-cervical reflex (TCR) are the most common used reflexes to evaluate the function of the trigeminal system and to localize pathologic alterations within the reflex pathway. Both reflexes have a nociceptive component in humans, which is
confirmed by verbal feedback from the tested subjects. This type of communication is not possible in animals; however, the presence of the particular reflex components indicates reaching the nociceptive threshold. As the intensity of the nociceptive stimulus is increasing, the characteristics of the reflex components (amplitude, latency, etc.) are changing, which properties can be measured by the evaluation of electromyographic (EMG) recordings. Therefore, nociception can be measured objectively in non-verbal animal species. Based of human literature we decided to evaluate the blink reflex and trigemino-cervical reflex contemporaneously to describe the function of the trigeminal system in horses (Di Lazzaro et al. 1995; Milanov et al. 2001; Liao et al. 2010). The choices of nerve branches for stimulation and muscles for recording the responses were also relying on human experience (Sartucci et al. 1986; Ertekin et al. 1996; Milanov et al. 2001).

Percutaneous electrostimulation of the trigeminal afferents, the supraorbital (SON), infraorbital (ION) and mental nerve (MN) evokes activity of the muscles innervated by the efferent neurons in the reflex pathway. Evaluating the electrophysiological characteristics of the reflexes may help to localise a lesion within the reflex pathway (Cruccu and Deuschl, 2000; Aramideh and Ongerboer de Visser, 2002; Valls-Sole, 2005). For diagnostic and research purposes, the blink reflex (BR) evoked by stimulation of the SON is probably the most commonly elicited trigeminal reflex. In both humans and horses, three components, generated by different pathways, have been described, with the late components being commonly interpreted as nociceptive in origin. In addition to the BR, the trigemino-cervical reflex (TCR) has been investigated and described in humans (Sartucci et al., 1986; Ertekin et al., 1996, 2001; Leandri et al., 2001; Milanov et al., 2001; Serrao et al., 2005) (Figure 1). By stimulating the SON, late EMG activity in the neck muscles is induced when the stimulus becomes painful, which was suggestive of a nociceptive nature of the TCR. Furthermore, increasing stimulation intensities corresponded to enlarged reflex size and stronger pain sensation (Serrao et al., 2003). Consequently, both the late component of the BR and the TCR appear to have a primary nociceptive physiological function that might enable them to be used for the investigation of neuropathic pain syndromes (Ertekin et al., 1996).
Direct motor fibres activation of the facial nerve, and indirectly, the transmission of the action potentials to the cutaneous branches of the cervical nerves may interfere with the responses evoked in the muscles of the face and neck. Furthermore, direct muscle stimulation may evoke short latency H-waves on the EMG recording as representation of mono/oligosynaptic proprioceptive reflexes originating from the same muscle or its tendon (stretch reflex) (Kandel et al. 2000). Therefore, careful selection of the subjects of investigation is necessary. The supraorbital nerve (SON) is a branch of the purely sensory ophthalmic division of the trigeminal nerve. Some terminal branches of this division have anastomosis with the motor fibres of the facial nerve, but not the SON. Similarly, the infraorbital nerve (ION) is originating from the maxillary division, which also does not contain motor fibres. Only the third, mandibular division has motor fibres next to the sensory ones and therefore we decided to avoid its evaluation later on in our study (Table 1).
Table 1

**Divisions of the trigeminal nerve and their areas of innervation (Feher 1980).** S: sensory, M: motor, SY: sympathetic PS: parasympathetic, VII: facial nerve, anast: anastomosis, IX: glossopharyngeal nerve

<table>
<thead>
<tr>
<th>Division</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ophthalmic division</strong></td>
<td></td>
</tr>
<tr>
<td>S: lacrimal nerve</td>
<td>lacrimal gland and upper eyelid (anast: n. zygomaticus, VII PS, SY)</td>
</tr>
<tr>
<td>S: frontal or supraorbital nerve</td>
<td>forehead and upper eyelid (anast: zygomatic nerve, n. auriculopalbebralis VII)</td>
</tr>
<tr>
<td>S: naso-ciliary nerve</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S: ethmoidal nerve</td>
<td>mucosa of nasal septum and dorsal turbinate</td>
</tr>
<tr>
<td>S: infratrochlear nerve</td>
<td>skin of the medial canthus, conjunctiva, third eyelid, lacrimal ducts and sac</td>
</tr>
<tr>
<td><strong>Maxillary division</strong></td>
<td></td>
</tr>
<tr>
<td>S: zygomatic nerve</td>
<td>lower eyelid and surrounding skin (anast: n. lacrimalis, n. frontalis, VII PS, SY)</td>
</tr>
<tr>
<td>S: sphenopalatine nerve</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S: caudal nasal nerve</td>
<td>medial: nasal septum, Vomer-nasal organ</td>
</tr>
<tr>
<td></td>
<td>lateral: mucosa of ventral turbinate and middle and ventral meatus</td>
</tr>
<tr>
<td>S: major palatine nerve</td>
<td>hard palate and gums, mucosa of ventral meatus</td>
</tr>
<tr>
<td>S: minor palatine nerve</td>
<td>soft palate</td>
</tr>
</tbody>
</table>
infraorbitale nerve

*nasal branches* dorsal nasal area and diverticulum

*anterior nasal branch* nasal mucosa, upper lip and nostrils

*superior (dorsal) labial branch* skin of the anterior cheek, skin and mucosa of upper lip (*anast: dorsal buccal branch of VII*)

**Mandibular division (S and M and SY)**

masticatory nerve

*masseteric nerve* masseter muscle

*deep temporal nerve* temporalis muscle

buccal nerve (PS ggl. Arnoldi, IX) mucosa and gland of the lips at the commissure, temporal, lateral pterygoid muscles, mucosa of the cheek, buccal glands

pterygoid nerve medial pterygoid muscle

superficial temporal nerve (n. auriculotemporalis)

*transverse facial branch* skin of the cheek (temporomandibular joint, temporalis region, crista faciei)

*ventral branch* (*anast: ventral buccal nerve of VII – n. auricularis caudalis*) guttural pouch, parotid gland, external ear, skin of external auditory meatus, tympanic membrane

mandibular alveolar nerve mandibular teeth, lower lip and chin,

*mylohyoid nerve (S and M)* mylohyoid, digastrics muscle, skin of the anterior part of the mandibular space

*mentalis nerve* lower lip and chin
lingual nerve

\textit{superficial branch} (chorda tympany VII PS, SY) mucosa of the tongue

\textit{deep} tongue, bottom of the oral cavity

The facilitation of limb NWR by repeated electrical stimulation has been successfully evaluated in horses (Spadavecchia et al., 2004) and dogs (Bergadano et al., 2007), thus providing indirect insight into the role of the wide dynamic range (WDR) neurons in the dorsal horn of the spinal cord. In the trigeminal system, the subnucleus caudalis, which is considered to be the nociceptive specific part of the trigeminal brainstem sensory nuclear complex, resembles the dorsal horn in its structure and basic function (‘medullary dorsal horn’) and also contains WDR neurons (Sessle, 2000). So far there have been no investigations evaluating whether repeated electrical stimulation of trigeminal afferents is able to evoke temporal summation in the trigeminal system. We hypothesised that due to structural similarities of the trigeminal sensory nucleus with the dorsal horn of the spinal cord, temporal summation should be evoked by repeated transcutaneous electrical stimulation of trigeminal afferents and could be quantified by the measurement of the EMG activity recorded from muscles involved in the reflex arch.

To the best of our knowledge, TCR has not yet been described in horses and BR has only been described in sedated horses, although assessment of the related electrophysiological parameters may provide a diagnostic tool for diseases affecting the trigeminal system in non-sedated horses. A better understanding of the trigeminal nociceptive physiology could facilitate the treatment of diseases associated with trigeminal nerve dysfunction, too.

Therefore, the aims of this thesis were as follows: (1) to assess if noxious transcutaneous electrical stimulation of the ION or SON would be suitable to evoke TCR in horses as in humans; (2) to describe its electrophysiological characteristics and stimulus–response function in non-sedated horses; (3) to evaluate the BR while eliciting the TCR; (4) to define the trigeminal temporal summation threshold, and (5) to evaluate the electrophysiological properties of trigeminal nociceptive reflexes evoked by repeated electrical stimulation in non-medicated horses.
4. Material and methods

4.1 Animals

Ten adult Warmblood horses (6 geldings, 4 mares; 7 Swiss Warmblood, 2 Freiberger and 1 Hanoverian) were included in the study. The horses, aged 14–23 years and weighing 540–640 kg, were judged to be clinically healthy with no known neurological disorders. The experiments were performed between the 2\textsuperscript{nd} and 28\textsuperscript{th} of February 2009 with the approval of the Bernese Committee for Animal Experimentation, Switzerland (Tierversuche/Bewilligung 92/08). The measurements were taken either at the Vetsuisse Faculty of the University of Bern or at the National Equine Centre in Bern (NPZ Bern - Nationales Pferdezentrum Bern).

4.2 Definitions of experimental objectives

The BR is the response of OO muscles to the stimulation of the cutaneous area innervated by the trigeminal nerve. Its afferent pathway is formed by the sensory branches of the trigeminal nerve and the efferent arch is provided by the facial nerve motor fibres. According to Anor et al. (1996) the BR in horses consists of three components, called R1, R2 and R3. The TCR is the response of the neck muscles to the stimulation of the cutaneous area innervated by the trigeminal nerve. Its afferent pathway is formed by the sensory branches of the trigeminal nerve and the efferent arch is provided by cervical nerve motor fibres. In human studies, the reflex induced by superficial noxious electrical stimulation occurs quite late after stimulation onset, in the mean range of 40–50 ms (C3) (Ertekin et al., 1996, 2001; Serrao et al., 2003), whereas the earlier components (C1, C2) are usually elicited only by mechanical, percutaneous or non-noxious electrical stimulation (Di Lazzaro et al., 1996; Ertekin et al., 2001; Leandri et al., 2001).

4.3 Instrumentation

Experiments were performed between two feeding times and after daily exercise to reduce the stress for the animals and in order to standardise the procedure. A venous catheter was placed into the left jugular vein to allow prompt sedation if necessary since the horses were restrained in stocks for stimulation and recording. The same investigators (CS and KV-Ny) performed the electro-physiological recordings and scored the behavioural reactions. The nerve stimulator was a purpose-built battery-powered optoisolated constant-current stimulator with a maximum voltage of 200 V.
Table 2

Numerical rating scale (NRS) to evaluate behavioural reaction to stimulation

<table>
<thead>
<tr>
<th>Score</th>
<th>Observed behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no reaction</td>
</tr>
<tr>
<td>1</td>
<td>blinking, but no other reaction</td>
</tr>
<tr>
<td>2</td>
<td>blinking and mild retraction of the head</td>
</tr>
<tr>
<td>3</td>
<td>blinking and powerful retraction of the head</td>
</tr>
<tr>
<td>4</td>
<td>sudden violent reaction of the whole body to the stimulus</td>
</tr>
<tr>
<td>5</td>
<td>unmanageable reaction of the whole body</td>
</tr>
</tbody>
</table>

4.4 Scoring the behavioural reactions

One observer (KV-Ny) judged the behavioural reaction to stimulation using a numerical rating scale (NRS) (Table 2) and a visual analogue scale (VAS). The VAS was consisting of a 100 mm line, where no reaction corresponded to the zero on the left end and worst possible reaction on the right end of the line. Measuring the distance in millimetres of the assigned score on the scale from its left end gave a measure of the intensity of the reaction observed (Figure 2).

![Visual analogue scale](image)

Figure 2

*Visual analogue scale*

4.5 Stimulating technique

In order to evoke the TCR and the BR, either the SON or the ION was transcutaneously stimulated using two pairs of self-adhesive electrodes (Ambu 700 05-J). The electrode sites above the left supraorbital and infraorbital foramen were shaved and cleaned with alcohol (Softasept N, B. Braun Medical). The stimulating electrodes were applied with the cathode over the respective foramen and the anode 20 mm dorsally (Figure 2).
4.6 Recording technique

In order to simplify the experiments as far as possible, and to provide the minimum discomfort for the horses, both BR and TCR were only recorded ipsilateral to the stimulus. To record the BR from the OO muscle, one pair of self-adhesive electrodes was placed on the shaved and degreased skin of the lateral canthus of the left eye over the OO. To record the TCR, two 7.5 cm long purpose-made stainless steel un-insulated needle electrodes (0.35 mm diameter) were placed approximately 5 cm apart subcutaneously above the middle of the left SPL while another pair was placed above the middle of the left CM as perpendicular to the muscle fibres as possible (Figure 3). The ground electrode was fixed on the left side behind the withers on previously moistened hair (Figure 4). Flexible leads were connected to the electrodes and secured to the skin and the mane to prevent displacement of the electrodes and disturbances of the horses. The distances between electrodes and the base of the ear, considered as the projection of the trigeminal nerve origin on the skull surface, were measured for estimation of conduction time. The resistance of the stimulating electrodes had to be lower than 5 kΩ during the whole experiment.
Figure 3

Positioning of the electrodes: stimulating electrodes over the supraorbital (1) and infraorbital nerves (2); recording electrodes over the orbicularis oculi (3), splenius (4) and cleidomastoideus muscles (5)
4.7 Electrical stimulation

4.7.1 Single stimulus (SS) to define TCR reflex threshold and evaluate stimulus-response curve

The stimulator was activated manually when the behaviour of the horse and the position of the head were deemed adequate, i.e. when there were no movements, a straight neck and minimal background activity of the SPL. Electromyographic activity was recorded from 100 ms before (background activity) to 400 ms after stimulation started. Inter-stimulus intervals were random within the range of 30–60 ms to prevent habituation. A standard SS consisted of a 1 ms train-of-five constant current square wave pulses delivered at the frequency of 200 Hz (Figure 5). Stimulation was always started at the lowest intensity of 1 mA. Then, in order to define the TCR threshold, the current was gradually increased in steps of 0.5 mA until a clear aversive backward movement of the head was elicited, which corresponded to

Figure 4

Ground electrode placement behind the withers onto previously moistened hair
behavioural score of 3 (Figure 6). Adjustments were made by increasing the stimulation intensity in steps of 0.1 mA from the last sub-threshold stimulation to the point at which threshold intensity ($T_{SS}$) for the TCR was confirmed (Figure 7). To be considered a threshold reflex, the amplitude of the EMG activity burst recorded from the neck muscles had to be at least three times the background activity. A second stimulation at $T_{SS}$ was performed to verify reproducibility of the response. Once the threshold was defined, intensities of 0.9, 1, 1.1, 1.2, 1.3, 1.4 and 1.5 x $T_{SS}$ were applied, with a minimum interval of 30 s between stimulations, to evaluate the stimulus–response curve. Stimulations were stopped if NRS score of 4 was reached.

Figure 5

*The standard single stimulus (SS) used (shown here as stimulation artefact on an EMG recording): 1 ms train-of-five constant current square wave pulses. The arrow indicates the stimulation onset after recording 100 ms baseline activity.*
Figure 6

Clear aversive backward movement of the head corresponding to behavioural score 3

Figure 7

An example of TCR threshold confirmation
4.7.2 Repeated stimulation (RS) to evaluate temporal summation

In order to perform the repeated stimulation, the standard SS consisting of 1 ms train-of-five (200 Hz) constant current square wave pulses previously used to define $T_{SS}$ was delivered 10 times with a frequency of 5 Hz over 2 s (Fig. 8). Each RS was delivered at progressively increasing intensities, in particular at 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2 and 1.3 times the previously defined $T_{SS}$ intensity. To rule out relevant changes of the reflex threshold, each RS was evaluated shortly after the assessment of $T_{SS}$ in the same experimental session. To avoid habituation, at least 30 s were allowed between each RS.

**Figure 8**

*The repeated stimulation paradigm used to evaluate temporal summation: 10 standard single stimuli (SS) delivered at the frequency of 5 Hz over 2000 ms (shown here as stimulation artefacts on an EMG recording). The arrows indicate the stimuli onset.*
4.8 Definition of variables and signal analysis

4.8.1 Single stimulation

The latency of the reflex or of its individual components was defined as the time measured from the start of electrical stimulation to the onset of the muscle response. The duration of the reflex or of its individual components was measured as the interval from the beginning of the first deflection to its final return to the baseline. The peak-to-peak amplitude (PTP) was calculated as a difference between the highest and lowest peak of the evoked EMG burst. Moreover, the root mean square amplitude (RMS) of the EMG activity was calculated for the 100 ms epoch before stimulation, and over the individual reflex bursts (Figure 9 and 10).

![Figure 7](image)

Electromyograms recorded from the orbicularis oculi (OO), splenius (SPL) and cleidomastoideus (CM) muscles after supraorbital nerve (SON) stimulation at threshold intensity ($T_{SS}$) of a horse. The X-axis represents the time (ms) and the Y-axis represents amplitude ($\mu$V). The arrows indicate the onset of the electrical stimulus.
4.8.2 Repeated stimulation

The total electromyographic (EMG) recording time was 4000 ms, including 500 ms before stimulation onset. The 500–2500 ms interval was analysed during RS and was divided into ten 200 ms sections to investigate the response to each stimulus separately. The epochs of the reflex components were determined according to the reference values acquired by SS: (1) 10–40 ms and 40–140 ms after the onset of each stimulus to analyse the early (BR early) and late components (BR late) of the blink reflex, and (2) 50–200 ms interval after the onset of each stimulus to analyse the trigemino-cervical reflex (TCR SPL and TCR CM). The root-mean-square (RMS) amplitudes of these epochs were calculated to quantify the magnitude of the electromyographic responses. To reduce the inter-individual variability, the reflex size was normalised to the baseline activity of muscle. The post-stimulation activity was recorded for the interval 2500–4000 ms (Fig. 11-14). The reflex threshold was defined as the intensity
of each SS necessary to elicit electromyographic reflex activity with amplitude of at least three times the baseline activity and a behavioural score of $BR = 1$ and $TCR = 3$. Reflex threshold of TCR SPL, TCR CM, BR early and BR late ($T_{RS}$) were defined for each nerve and the reported values were normalised to the single stimulation threshold ($T_{SS}$) of the TCR. In addition, the stimulus number able to evoke the first reflex and the maximal reflex response were recorded.

**Figure 11**

*Sample electromyographic activity of the orbicularis oculi muscle after 0.5 x $T_{SS}$ and 1.0 x $T_{SS}$ repeated stimulation. I, II and III represent baseline (0-500 ms), repeated stimulation (500-2500 ms) and post-stimulation (2500-4000 ms) intervals, respectively.*
Figure 12

Sample electromyographic activity of the neck muscle after $0.5 \times T_{SS}$ and $1.0 \times T_{SS}$ repeated stimulation. I, II and III represent baseline (0-500 ms), repeated stimulation (500-2500 ms) and post-stimulation (2500-4000 ms) intervals, respectively.
Figure 13

Sample electromyographic activity of the orbicularis oculi muscle after 0.5 x $T_{ss}$ and 1.0 x $T_{ss}$ repeated stimulation. Root mean square (RMS) amplitudes were analysed over the following epochs: 0-500 ms, baseline activity; 10-40 ms after the onset of each stimulus, early blink reflex component (E); 40-140 ms after the onset of each stimulus, late blink reflex component (L). The arrows indicate the stimuli onset.
Sample electromyographic activity of the neck muscle after 0.5 x $T_{SS}$ and 1.0 x $T_{SS}$ repeated stimulation. Root main square (RMS) amplitudes were analysed over the following epochs: 0-500 ms, baseline activity; 50-200 ms after the onset of each stimulus, trigemino-cervical reflex (TCR). The arrows indicate the stimuli onset.

4.9 Analysis of the data

4.9.1 Single stimulation

In order to describe the reflex characteristics when stimulating at $T_{SS}$, medians and interquartile ranges (IQR) were calculated for reflex latency, duration, PTP, RMS, stimulation intensity, NRS and VAS scores. Three EMG records per horse were analysed both in case of ION and SON stimulation. Wilcoxon Signed Rank Test was used to compare the records obtained while stimulating the two nerves (ION and SON) from each muscle and from the two muscles (SPL and CM) for each stimulated nerve. To evaluate the stimulus–response
curve, the Friedman Repeated Measures Analysis of Variance on Ranks was applied. The Spearman Rank Order Correlation was used to evaluate correlation between parameters.

4.9.2 Repeated stimulation

Non-parametric tests were used for statistical evaluation of the data. Descriptive statistics were used to define individual reflex threshold to repeated stimulation. One sided exact Wilcoxon Signed Rank Test was applied to investigate the effect of repeated stimulation on the reflex threshold intensity. The Brunner–Langer model was used to analyse the effect of different stimulation intensities on trigeminal reflexes evoked by repeated stimulations. The Mann–Whitney Rank Sum Test was used to compare the electrophysiological characteristics of the two nerves.
5. Results

All horses tolerated the experimental procedure easily and without discomfort. Stimulation sites were regularly inspected after the experiment but no signs of skin reaction or damage were detected.

5.1 Reflexes at $T_{SS}$ after single stimulation

Surface electrical stimulation of the trigeminal afferents ION or SON evoked clear EMG reflex responses in the OO, SPL and CM. The horses tolerated the stimulation series without any sign of discomfort or need for additional sedation until $T_{SS}$ was reached. Technical problems (computer failed to record the measurement) were responsible for the loss of 1 out of 3 threshold EMG recordings from one horse for ION and SON stimulation, so only 29 sets of data at $T_{SS}$ for each nerve could be analysed. The occurrence of the different components of BR (Anor et al., 1996) was inconsistent when stimulating at $T_{SS}$. The R1 component (BR early) was recorded in 14 cases (48%) after SON stimulation and 20 cases (69%) after ION stimulation. The R2 and R3 components (BR late) were present in 19 (66%) and 25 (86%) cases after SON stimulation and in 19 (66%) and 27 (93%) cases after ION stimulation. Blink reflex evoked by stimulating either the ION or the SON showed similar PTP and RMS, latency and duration (Table 3).
Table 3

Median parameters and interquartile ranges [IQR] of the 3 components of the BR at T$_{SS}$ stimulation of SON and ION recorded from the orbicularis oculi muscle

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SON Median [IQR]</th>
<th>ION Median [IQR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA OO RMS (µV)</td>
<td>2 [2;6]</td>
<td>3 [2;9]</td>
</tr>
<tr>
<td>PTP (µV)</td>
<td>20 [15;39]</td>
<td>24 [11;49]</td>
</tr>
<tr>
<td>latency (ms) R1</td>
<td>13 [10;16]</td>
<td>16 [15;21]</td>
</tr>
<tr>
<td>R2</td>
<td>46 [36;57]</td>
<td>40 [35;50]</td>
</tr>
<tr>
<td>R3</td>
<td>78 [70;92]</td>
<td>81 [74;92]</td>
</tr>
<tr>
<td>RMS (µV) R1</td>
<td>192 [68;462]</td>
<td>197 [119;348]</td>
</tr>
<tr>
<td>R2</td>
<td>87 [48;160]</td>
<td>127 [80;169]</td>
</tr>
<tr>
<td>R3</td>
<td>98 [39;225]</td>
<td>80 [55;109]</td>
</tr>
<tr>
<td>PTP (µV) R1</td>
<td>869 [170;1577]</td>
<td>785 [436;1219]</td>
</tr>
<tr>
<td>R2</td>
<td>315 [157;694]</td>
<td>471 [307;623]</td>
</tr>
<tr>
<td>R3</td>
<td>430 [172;659]</td>
<td>425 [272;551]</td>
</tr>
<tr>
<td>duration (ms) R1</td>
<td>25 [24;32]</td>
<td>23 [19;27]</td>
</tr>
<tr>
<td>R2</td>
<td>26 [19;36]</td>
<td>27 [23;39]</td>
</tr>
<tr>
<td>R3</td>
<td>61 [38;88]</td>
<td>57 [37;77]</td>
</tr>
</tbody>
</table>

BA OO: baseline activity of the orbicularis oculi muscle
RMS: RMS amplitude
PTP: PTP amplitude

Higher current was necessary to elicit the TCR while stimulating the ION compared to the SON (P < 0.001), but similar VAS and NRS scores were attributed at T$_{SS}$ stimulation for the two nerves. No significant difference was found in the TCR latency, duration and size when the two nerves were compared except that the duration of the reflex recorded from CM was longer after ION stimulation compared to SON (P = 0.018). No reflex EMG activity was observed in the first 30 ms epoch after stimulation onset where C1 and C2 component would have been expected, which suggested that the TCR recorded in our study probably corresponded to the human C3. After stimulation of the SON, the evoked muscle potentials recorded from the SPL were comparable to those recorded from the CM as to latency and duration. However, reflex amplitudes were significantly different, with records from the SPL having approximately double RMS and triple PTP than those from the CM (P < 0.001 for both
parameters). Evaluating responses to ION stimulation resulted in similar observations. In particular, both RMS and PTP from the SPL were higher than those from the CM (P = 0.001 and P = 0.004 respectively). The amplitude of the background activity recorded from the SPL was higher than the one recorded from the CM while stimulating both nerves (P < 0.001) (Table 4).

Table 4

Median parameters and interquartile ranges [IQR] of the TCR at T_{ss} stimulation of SON and ION recorded from the splenius (SPL) and cleidomastoideus (CM) muscles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SON Median [IQR]</th>
<th>ION Median [IQR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA SPL RMS (µV)</td>
<td>18 [14;34]</td>
<td>22 [14;38]</td>
</tr>
<tr>
<td>PTP (µV)</td>
<td>106 [72;174]</td>
<td>114 [84;201]</td>
</tr>
<tr>
<td>BA CM RMS (µV)</td>
<td>7 [2;10]</td>
<td>4 [2;9]</td>
</tr>
<tr>
<td>PTP (µV)</td>
<td>32 [9;45]</td>
<td>17 [11;45]</td>
</tr>
<tr>
<td>latency (ms) SPL</td>
<td>73 [55;86]</td>
<td>65 [51;85]</td>
</tr>
<tr>
<td>CM</td>
<td>75 [50;91]</td>
<td>60 [46;86]</td>
</tr>
<tr>
<td>RMS (µV) SPL</td>
<td>178 [114;256]</td>
<td>196 [130;312]</td>
</tr>
<tr>
<td>CM</td>
<td>99 [57;171]</td>
<td>97 [58;152]</td>
</tr>
<tr>
<td>PTP (µV) SPL</td>
<td>1270 [719;1598]</td>
<td>1260 [725;1907]</td>
</tr>
<tr>
<td>CM</td>
<td>444 [285;827]</td>
<td>626 [286;1046]</td>
</tr>
<tr>
<td>duration (ms) SPL</td>
<td>116 [105;146]</td>
<td>127 [108;148]</td>
</tr>
<tr>
<td>CM</td>
<td>120* [104;148]</td>
<td>144* [119;175]</td>
</tr>
<tr>
<td>I_{t} (mA)</td>
<td>2.70* [1.9;3.5]</td>
<td>4.10* [1.9;6.5]</td>
</tr>
<tr>
<td>I_{t} VAS score</td>
<td>35 [25.5;40.25]</td>
<td>35 [22;38]</td>
</tr>
<tr>
<td>I_{t} NRS score</td>
<td>3 [2.5;3]</td>
<td>3 [2.5;3]</td>
</tr>
</tbody>
</table>

*significant difference between ION and SON stimulation recorded from the same muscle
†significant difference between SPL and CM muscles stimulating the same nerve

BA: baseline activity
RMS: RMS amplitude
PTP: PTP amplitude
5.2 Stimulus–response curve after single stimulation

While stimulating at supra-threshold intensity to describe the stimulus–response curve, a NRS score of 4, that determined interruption of the experiment, was reached in two horses after $1.3 \times T_{SS}$ SON stimulation, in three horses after $1.3 \times T_{SS}$ and in two horses after $1.4 \times T_{SS}$ ION stimulation. At $0.9 \times T_{SS}$ the BR was missing in five horses when stimulating the SON and in three horses when stimulating the ION. None of the measured parameters was affected by increasing stimulation intensity. A significant negative correlation was found between R1 latency and intensity for SON stimulation ($r = 0.29; P = 0.041$) (Fig. 15 and 16).

![Median reflex latency (± IQR) of the BR after ION stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the latency of the reflexes (ms). R1: early component of the blink reflex; R2 and R3: late components of the blink reflex.](image-url)

Figure 15
Figure 16

Median reflex latency (± IQR) of the BR after SON stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{ss}$ and the Y-axis represents the latency of the reflexes (ms). R1: early component of the blink reflex; R2 and R3: late components of the blink reflex.

Because some of the data were missing (6/10 at 0.9 x $T_{ss}$ and 5/10 at 1.5 x $T_{ss}$ ION stimulation and 8/10 at 0.9 x $T_{ss}$ SON stimulation), we only performed statistical analysis of the TCR stimulus–response curve in the range of 1–1.4 x $T_{ss}$ after ION stimulation and 1–1.5 x $T_{ss}$ after SON stimulation. When the stimulus intensity was increased both TCR latencies in the SPL (SON: $P < 0.001$; ION: $P = 0.008$) and the CM (SON: $P = 0.007$; ION: $P = 0.002$) decreased (Fig. 17 and 18).
Figure 17

Median reflex latency (± IQR) of the TCR after ION stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the latency of the reflexes (ms). SPL: TCR recorded from the splenius muscle; CM: TCR recorded from the cleidomastoideus muscle.
Median reflex latency (± IQR) of the TCR after SON stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the latency of the reflexes (ms). SPL: TCR recorded from the splenius muscle; CM: TCR recorded from the cleidomastoideus muscle.

This was also confirmed by a significant negative correlation between intensity and latency (SON: $r = 0.57; P < 0.001$ [SPL] and $r = 0.47; P < 0.001$ [CM]; ION: $r = 0.32; P = 0.02$ [SPL] and $r = 0.33; P = 0.01$ [CM]). On the other hand PTP (SON: $P = 0.005$ [SPL], $P = 0.026$ [CM]; ION: $P = 0.036$ [SPL], $P = 0.036$ [CM]) and RMS (SON: $P = 0.001$ [SPL], $P = 0.017$ [CM]; ION: $P = 0.015$ [SPL], $P = 0.013$ [CM]) increased parallel with the stimulation intensity (Fig. 19).
Electromyograms recorded from the splenius muscle after SON stimulation of a horse (when stimulating at 0.9, 1.0, 1.1, 1.2, 1.3, 1.4 and 1.5 x $T_{SS}$). The X-axis represents time (ms) and the Y-axis represents amplitude ($\mu$V). The arrows indicate the onset of the electrical stimulus.

These later results are reinforced by the positive correlation found between the amplitudes and intensity (SON: $r = 0.47$; $P < 0.001$ for RMS, $r = 0.49$; $P < 0.001$ for PTP [SPL], $r = 0.39$; $P = 0.003$ for RMS, $r = 0.45$; $P < 0.001$ for PTP [CM]; ION: $r = 0.45$; $P < 0.001$ for RMS, $r = 0.42$; $P = 0.001$ for PTP [SPL], $r = 0.48$; $P < 0.001$ for RMS, $r = 0.49$; $P < 0.001$ for PTP [CM]) (Figs. 20-23).
Median reflex RMS amplitude (± IQR) of the BR after ION stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to \( T_{ss} \) and the Y-axis represents the RMS amplitude of the reflexes (µV). R1: early component of the BR; R2 and R3: late components of the BR.
Median reflex RMS amplitude (± IQR) of the BR after SON stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the RMS amplitude of the reflexes ($\mu$V). R1: early component of the BR; R2 and R3: late components of the BR
Median reflex RMS amplitude (± IQR) of the TCR after ION stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the RMS amplitude of the reflexes (µV). SPL: TCR recorded from the splenius muscle; CM: TCR recorded from the cleidomastoideus muscle.
Median reflex RMS amplitude (± IQR) of the TCR after SON stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to TSS and the Y-axis represents the RMS amplitude of the reflexes (µV). SPL: TCR recorded from the splenius muscle; CM: TCR recorded from the cleidomastoideus muscle.

Reflex duration was not significantly affected by the stimulus intensity, even if a positive significant correlation was found between most of them (SON: r = 0.28; P = 0.03 [SPL]; ION: r = 0.33; P = 0.01 [SPL]; r = 0.29; P = 0.03 [CM]). The VAS scores showed a significant increase in the magnitude of the behavioural responses as stimulation intensity increased (SON: P < 0.001, ION: P = 0.002) with strong positive correlation (SON: r = 0.73; P < 0.001, ION r = 0.81; P < 0.001) (Fig. 24). No significant intensity effect was found on NRS scores despite that strong positive correlation was detected between them (SON: r = 0.74; P < 0.001, ION: r = 0.75; P < 0.001).
Median visual analogue scale (VAS) score (± IQR) of behaviour reaction after ION and SON stimulation in 10 horses. The X-axis represents the intensity of stimulation relative to $T_{SS}$ and the Y-axis represents the VAS score (mm). ION: infraorbital nerve stimulation; SON: supraorbital nerve stimulation; $T_{SS}$: TCR reflex threshold intensity

5.3 Repeated stimulation

As the values in Table 5 show, the TCR was not evoked by subthreshold intensity RS and temporal summation of afferent trigeminal inputs could not therefore be observed ($P = 0.5$). The stimulus number evoking the first and the maximal reflex response was noted to check whether temporal summation has occurred.
Table 5

Mean threshold values and interquartile range [IQR] of the trigemino-cervical reflex (TCR) and the blink reflex’s (BR) early and late component after repeated stimulation normalized to single stimulation threshold ($x_{TS}$) measured on the splenius (SPL), cleidomastoideus (CM) and orbicularis oculi (BR early and late) muscles. The normalized threshold showed no difference between the two nerves (P values). ION: infraorbital nerve and SON: supraorbital nerve

<table>
<thead>
<tr>
<th></th>
<th>ION ($x_{TS}$)</th>
<th>SON ($x_{TS}$)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR SPL</td>
<td>1.00 [0.90;1.00]</td>
<td>1.05 [1.00;1.10]</td>
<td>0.144</td>
</tr>
<tr>
<td>TCR CM</td>
<td>1.00 [0.90;1.00]</td>
<td>1.00 [1.00;1.00]</td>
<td>0.233</td>
</tr>
<tr>
<td>BR early</td>
<td>0.90 [0.70;1.00]</td>
<td>0.95 [0.80;1.00]</td>
<td>0.776</td>
</tr>
<tr>
<td>BR late</td>
<td>0.90 [0.80;1.00]</td>
<td>0.95 [0.80;1.00]</td>
<td>0.839</td>
</tr>
</tbody>
</table>

The data presented in Table 6 clearly demonstrate that the first reflex was evoked by the first stimulus in the sequence and this had the highest RMS amplitude. Only the early, non-nociceptive component of the BR had a tendency to increase in size with the stimulus number.
Table 6

The mean stimulus number and interquartile range [IQR] able to evoke the first reflex and the maximal reflex response after repeated stimulation. TCR SPL and CM: Trigemino-cervical reflex recorded from the splenius and cleidomastoideus muscles, respectively. BR early and late: early and late component of the blink reflex recorded from the orbicularis oculi muscle. ION: infraorbital nerve and SON: supraorbital nerve

<table>
<thead>
<tr>
<th></th>
<th>ION</th>
<th></th>
<th></th>
<th>SON</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stimulus number of</td>
<td>First reflex</td>
<td>Maximal reflex</td>
<td>Stimulus number of</td>
<td>First reflex</td>
</tr>
<tr>
<td>TCR SPL</td>
<td>1 [1;1]</td>
<td>1 [1;2.5]</td>
<td>1 [1;1]</td>
<td>1 [1;1]</td>
<td></td>
</tr>
<tr>
<td>TCR CM</td>
<td>1 [1;1]</td>
<td>1 [1;2]</td>
<td>1 [1;1]</td>
<td>1 [1;1]</td>
<td></td>
</tr>
</tbody>
</table>

Likewise with SS, RMS reflex amplitude increased significantly with the stimulation intensity (Figs. 25-28).
Figure 25

Median and interquartile range values of the splenius (SPL) and cleidomastoideus (CM) muscles activity as a function of increasing stimulation intensity after repeated ION stimulation. The dotted line represents 3 times the baseline activity of the muscles.
Figure 26

Median and interquartile range values of the splenius (SPL) and cleidomastoideus (CM) muscles activity as a function of increasing stimulation intensity after repeated SON stimulation. The dotted line represents 3 times the baseline activity of the muscles.
Median and interquartile range values of the early orbicularis oculi (OO) muscle activity (BR early) as a function of increasing stimulation intensity after repeated stimulation. The dotted line represents 3 times the baseline activity of the muscle.
Median and interquartile range values of the late orbicularis oculi (OO) muscle activity (BR late) as a function of increasing stimulation intensity after repeated stimulation. The dotted line represents 3 times the baseline activity of the muscle.

The median RMS amplitude for the 10 horses showed a tendency to decline over the stimulation sequence (Figs. 29-36).
Figure 29

Median and interquartile range activity of the cleidomastoideus (CM) muscle after repeated ION stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Figure 30

Median and interquartile range activity of the cleidomastoideus (CM) muscle after repeated SON stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Figure 31

Median and interquartile range activity of the splenius (SPL) muscle after repeated ION stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Median and interquartile range activity of the splenius (SPL) muscle after repeated SON stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Median and interquartile range of the early activity of the orbicularis oculi (OO) muscle (BR early) after repeated ION stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Median and interquartile range of the early activity of the orbicularis oculi (OO) muscle (BR early) after repeated SON stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Figure 35

Median and interquartile range of the late activity of the orbicularis oculi (OO) muscle (BR late) after repeated ION stimulation over the sequence of ten stimuli. The dotted line represents 3 times the baseline activity of the muscle.
Reflex activity increased significantly with increasing stimulation intensities (ION BR, $P < 0.001$; SON BR, $P = 0.039$; ION SPL, $P = 0.024$; SON SPL, $P < 0.001$; ION CM, $P < 0.001$; SON CM, $P = 0.002$). To exclude long-term effects of RS on muscular function, baseline activity of all muscles was evaluated with respect to increasing stimulation intensity and was found to be stable (ION BR, $P = 0.721$; SON BR, $P = 0.07$; ION SPL, $P = 0.926$; SON SPL, $P = 0.744$; ION CM, $P = 0.635$; SON CM, $P = 0.591$). Behavioural scores increased significantly ($P < 0.001$) with increasing stimulation intensity. Fig. 37 demonstrates that behavioural score 3, corresponding to TCR reflex criteria, was attributed at reflex threshold level in the majority of horses.
Figure 37

Median and interquartile range behavioural scores (of the ten horses) presented as a function of stimulation intensity after repeated stimulation
6. Discussion

Idiopathic equine headshaking shows similar clinical features to the human cluster headache, trigeminal and post herpetic neuralgia (Newton, 2001). As the neurophysiological assessment of the trigeminal system function show typical alterations of the reflexes (Liao et al., 2010; Peddireddy et al., 2009; Truini et al., 2008) in affected humans, it could be expected that the methods described in the present thesis will be helpful in evaluating headshaking horses and possibly gaining further insight into the physiopathology of this disease.

6.1 Single stimulation

First of all, we have found that surface electrical stimulation of the ION or SON induced reproducible reflex responses that could be recorded by electromyography from cervical muscles in horses. The R1 component following SON stimulation could not be elicited consistently. This was in contrast with a previous report (Anor et al., 1996) where the early component was regularly observed in horses sedated with detomidine and could be explained by the fact that α2-agonists facilitate non-nociceptive, tactile reflexes in horses (Spadavecchia et al., 2005; Rohrbach et al., 2009). Another important difference from the Anor et al. (1996) study was the stimulation technique since we used a comparatively long train of five pulses to obtain nociceptive late responses which might have obscured early events.

In contrast to human findings (Sartucci et al., 1986; Milanov et al., 2001), we could not detect differences in latency and amplitude of reflexes evoked by stimulation of the two trigeminal afferents ION and SON. The latency of the TCR as recorded from both muscles, suggested a polysynaptic type pathway of the reflex. In our SS study, reflexes recorded from the SPL were significantly larger in amplitude than from the CM, possibly reflecting their different function in the organization of the final aversive movement or to the higher background activity of the SPL, due to its antigravity function that facilitates the reflex (Di Lazzaro et al., 1995).

The absence of early TCR components in the present study corresponds to human findings where single transcutaneous electrical stimulations were not able to evoke C1 and C2 in active muscles (Ertekin et al., 1996; Leandri et al., 2001). Therefore, the TCR recorded in horses appeared to correspond to the human C3 only.
The decreasing latency of the TCR while the amplitude, VAS and NRS scores were increasing with increasing stimulation intensities agreed with other nociceptive reflexes, such as the limb nociceptive withdrawal reflex in horses (Spadavecchia et al., 2002, 2003).

Horses were kept in a quiet environment during the experimental session to avoid any noise or movement-routines being associated with stimulation, while the time elapsing between the two stimuli was varied randomly (Skljarevski and Ramadan, 2002; Spadavecchia et al., 2002, 2003).

A single pulse stimulation of short duration would have been more appropriate to assess the proprioceptive component of the BR, as the 20 ms post-stimulation epoch could overlap the oligosynaptic early component. As the primary objective of the present study was to focus on the late nociceptive responses, the use of a train of pulses permitted pain threshold to be attained more consistently and at lower stimulation intensities than a single pulse, due to a temporal summation effect (Bergadano et al., 2006). Furthermore, each supra-threshold stimulation was given only once to limit distress of the horses, even though repeating each intensity level two or three times would have resulted in more reliable data.

While a previous study describing the equine blink reflex was performed on horses sedated with \( \alpha_2 \)-agonists, for the present experiments sedative drugs were avoided. Indeed it is known that \( \alpha_2 \)-agonists can profoundly influence nociceptive reflex thresholds and may provoke ataxia and myorelaxation. Thus the physiological properties of the reflexes were maintained, so that the TCR can now be applied as a diagnostic tool to localise pathological lesions in the reflex pathway or the brainstem, similar to human studies (Di Lazzaro et al., 1996; Cruccu and Deuschl, 2000; Aramideh and Ongerboer de Visser, 2002; Valls-Sole, 2005).

A potential weakness of the study design was that we did not evaluate consensual responses to stimulation, but this was because we wished to keep the experimental setting as simple as possible and to minimise any disturbance to the horses provoked by electrodes and cables.

The CM and the SPL muscles were superficial, easy to locate with palpation and possibly involved with different aspects of head withdrawal, all factors which have been considered useful criteria in human studies (Leandri et al., 2001). In horses the CM is the ventral part of the brachiocephalic muscle and has two functions: (1) if the head and the neck are fixed in their position, it elevates the front limb and (2) if the horse puts weight on the limbs, it bends the neck. It is innervated by the ventral branch of the accessory nerve. The SPL is an epaxial muscle of the cervical spine, is innervated by C1–8 and the dorsal branch of the accessory
nerve and has the function of lifting the head (Feher, 1980). In the present study, the two muscles showed different background activity, the SPL being more active during stance in absence of visible movements. This clearly influenced the reflex strength as described in humans (Pearson and Gordon, 2000; Milanov et al., 2001) as higher background activity facilitates the reflex.

The three BR components were evaluated separately using previously published data (Anor et al., 1996) to identify the components. Taking into account the measured distances between the stimulation site and the base of the ear (mean distance: 31.5 cm [ION] and 14.9 cm [SON]), the measured R1 latency in the range of 10–20 ms has to rely on activation of fast conducting fibres. On the other side, the TCR as the R2 and R3 most probably results from activation of slow conducting fibres as it can be inferred from the measured latency. As expected, the reflex latencies found in horses were longer than in humans (Sartucci et al., 1986; Di Lazzaro et al., 1996; Ertekin et al., 1996, 2001; Milanov et al., 2001; Serrao et al., 2003) as the conduction pathways are longer.

6.2 Repeated stimulation

According to our findings, the hypothesis that temporal summation can be evoked by the repetitive sub-threshold stimulation of trigeminal afferents similarly to the limb NWR in horses must be rejected under the present experimental conditions. Repeated stimulation of individually non-painful stimuli is known to possibly provoke pain sensation in humans. This process is due to temporal summation, and can be modelled by repetitive electrical stimulation of Aδ and C fibres of the sural nerve (Andersen et al., 1994; Arendt-Nielsen et al., 1994, 2000) in experimental and clinical settings. The gradual increase in dorsal horn excitability elicits a reflex response at sub-threshold intensity usually after the third or fourth consecutive stimulus and facilitates the subsequent responses until habituation occurs. This phenomenon therefore lowers the pain threshold and increases the magnitude of pain perception and the amplitude of the reflex responses over the stimulation train (Arendt-Nielsen et al., 1994, 2000).

Temporal summation is frequency dependent (Arendt-Nielsen et al., 2000) and 5 Hz RS was found to be appropriate for its electrophysiological evaluation in horses (Spadavecchia et al., 2004). Similar to the current study, Spadavecchia et al. (2004) evaluated the temporal summation of Aδ fibres. Although stimulation of C-fibres would result in a higher magnitude of temporal and spatial summation, the stimulation of Aδ fibres is favoured to investigate temporal summation and wind-up in non-invasive experimental settings. Temporal and spatial summation of nerve action potentials are due to spinal convergence of mainly Aδ and
C afferents (Andersen et al., 1994), but in hyperalgesic conditions Aβ activity (Herrero et al., 2000) also can lead to the wind-up phenomenon. The latter is one of the factors initiating central sensitisation (Woolf, 1996) and consequently contributing to the development of pathological pain syndromes. In the spinal cord, wind-up is the phenomenon of WDR neurons (Herrero et al., 2000) that increase their firing response to low threshold Aβ stimulation, also provoking allodynia, enhancing the NWR and widening their receptive field (Svendsen et al., 1997). Similar to the nociceptive component of the limb flexion reflex in humans, the nociceptive blink reflex component (BR2) is also mediated by WDR neurons (Ellrich and Treede, 1998).

The trigeminal nucleus caudalis was found to be the main relay site for orofacial nociceptive information in rats by single cell microelectrode recordings from nociceptive specific and WDR neurons (Barcelo et al., 2012). Not only does it resemble the spinal dorsal horn in its structure, but it also undergoes similar neuroplastic changes leading to central sensitisation (Sessle, 2000) in mammals. Comparing spinal and trigeminal nerves, one of the clear differences was that the proportion of unmyelinated fibres is lower in the trigeminal compared to the spinal nerves (Sessle, 2000). Furthermore primary afferent fibres terminate not only in the trigeminal brainstem sensory complex, which receives afferent inputs from other cranial and cervical nerves, but also in other parts of the brainstem.

It is noteworthy that the trigeminal brainstem sensory complex has direct projections to the thalamus, cerebellum, superior colliculus, pontine parabrachial nucleus, peri-aquaeductal grey and spinal cord and multisynaptic connections to the reticular formation (Sessle, 2000). On the other hand, the neurons of lamina II of the subnucleus caudalis project only within the subnuclei of trigeminal sensory complex. Moreover, nociceptive responses originating from trigeminal Aδ and C fibres are inhibited, possibly through rostral ventromedial medulla (RVM) mediated mechanisms, as was first demonstrated by glutamate microinjections into the striatum in rats (Barcelo et al., 2012).

These descending modulatory effects on nociceptive neurons are prominent in the trigeminal sensory nucleus (Sessle, 2000). Therefore, the subnucleus caudalis differs from the spinal dorsal horn by the extensive convergence of afferent fibres, direct projection to higher nuclei (Sessle, 2000) and the assumed circuit of descending inhibition. These anatomical peculiarities may partially explain the observed differences between spinal and trigeminal reflexes.

There are some differing outcomes from studies evaluating repeated stimulation in the trigeminal complex. Repetitive stimulation of single neurones in anaesthetised rats evoked a
progressive increase in trigeminal WDR neuronal response even below threshold intensity (Coste et al., 2008; Vernon et al., 2009). Similarly, Griffin (2004) reported temporal summation of the nociceptive blink reflex evoked at 1.5 times the reflex threshold intensity in humans, although much longer inter-stimulus intervals (15–17 s vs. our 0.2 s) were applied compared to the current study. On the other hand, Powers et al. (1997) evaluated the effect of paired SON stimulation with interstimulation intervals between 50 and 2000 ms in rats and humans applying 2 times the intensity evoking the BR and found BR2 component inhibition of the second response in both species, but facilitation of the BR1 in humans. However, when stimulations of different modality were used, the second BR2 was also facilitated. The outcomes from the stimulation studies were explained as a long lasting inhibition of the afferent neurons in the sensory nucleus, short lasting inhibition in the reticular formation and facilitation of the facial motor neurons.

Although sub-threshold intensities were not evaluated in the study by Powers et al. (1997), this theory would support the findings in the current study except that the facilitation of the early BR component was not apparent. Dauvergne and Evinger (2007) described facilitation of the BR in rats after paired ION and SON stimulation, but in contrast with Powers et al. (1997), they explained their findings with the susceptibility of single neurons within the trigeminal sensory nucleus complex to respond to multiple trigeminal branches stimulation and hypothesised the role of reticular formation in long-lasting suppression by paired SON stimulations. Furthermore, a 1 s recovery cycle of the TCR evoked stimulating at 1.2 times reflex threshold reported in humans (Serrao et al., 2005) could explain why the responses to the first stimuli in the stimulation sequence were the largest in magnitude above threshold in our study.

The present study found identical psychophysical (behavioural score) and electrophysiological thresholds (Fig. 37) as has been reported for humans (Arendt-Nielsen et al., 1994). During repeated stimulation, the activity of the limb muscles (Spadavecchia et al., 2004) differed from the neck and OO muscles activity, as progressive increase in the magnitude of RMS amplitude was demonstrated for reflexes recorded from the tibialis cranialis and common extensor digitalis muscles (Spadavecchia et al., 2004). In contrast, a decrease in size of consecutive reflexes over the stimulation sequence was measured in our study, and was possibly due to descending inhibitory modulation (Powers et al., 1997; Dauvergne and Evinger, 2007). A recent publication (Rehberg et al., 2012) also revealed the possible discrepancies between spinal and trigeminal modulation of pain and nociception and confirmed the necessity of further studies in this field.
Finally, the repeated stimulation paradigm in the current study was designed to evoke A fibre recruitment and temporal summation, as previously described for limb sensory nerves (Arendt-Nielsen et al., 1994; Spadavecchia et al., 2004; Bergadano et al., 2007). In humans, the progressive increase in RMS amplitudes reached a plateau in the middle of the repeated stimulation sequence and started to diminish progressively thereafter (Arendt-Nielsen et al., 1994). It appears that habituation due to increased descending inhibitory control affected the magnitude of reflexes towards the end, although the perceived pain intensity did not decrease (Arendt-Nielsen et al., 1994). We therefore hypothesized that the descending inhibition (Sessle, 2000) could be responsible for the diminishing amplitude of reflexes following the first reflex in the current study. Furthermore, as Aβ fibres are activated by lower stimulation intensity, predominating inhibition evoked by these low threshold mechanoreceptor neurons (Sessle, 2000) may be responsible for the lack of sub-threshold temporal summation in the equine trigeminal system.

Other factors could potentially explain the lack of temporal summation observed in our work. For example, the selection of specific neck muscles may have an influence since wind-up may affect different muscles in different ways (Herrero et al., 2000). Furthermore, C-fibre blockade secondary to high intensity/high frequency tetanic stimulation can diminish temporal summation (Herrero et al., 2000), although this was not likely here due to the low stimulation intensity.
7. Conclusions

Improper pain assessment in animals leads to either extra use of drugs with all their side effects or a suffering patient. Unfortunately, no golden standard tool exists to measure pain in horses neither in clinical nor in research settings. All method has its advantages and drawbacks mainly because pain is a dynamic process. Evaluation of single behavioural indicators cannot give exact estimation about the level of pain the animal experiencing and physiological parameters are not reliable indicators of pain. Until now, pain scoring systems suited for the assessment of certain types of pain incorporating sensitive and specific behavioural items are the best way to evaluate quickly and easily the level of pain in clinical patients. Quantitative sensory testing methods used mainly in research settings are valuable tool for objective measurement of nociception in horses.

In conclusion, the trigemino-cervical responses to nociceptive electrical stimulation recorded in this study clearly confirmed the reflex interaction between trigeminal afferents and both brainstem motor centres and cervical spinal cord motor neurons in horses. Our data could provide a reference for TCR performed in healthy non-sedated adult horses so that this reflex can now be used as a new diagnostic tool to assess dysfunction of the trigeminal system in horses.

The present study showed that temporal summation of nociceptive trigeminal afferents evoked by repeated electrical stimulation and recorded by cervical electromyography does not occur in horses. The recovery curves of TCR are significantly faster in humans with migraines and diffuse noxious inhibitory control (DNIC) deficiencies accompany some chronic trigeminal pain syndromes, such as migraines, tension-type headaches and temporomandibular disorders (Proietti et al., 2003; Williams and Rhudy, 2009). Similarly, in horses affected by trigeminal pathology, altered nociceptive modulation and DNIC deficiencies could modify the neurophysiological profile observed in the healthy subjects, representing a novel non-invasive tool for a mechanism-based approach to diagnosis.
8. New scientific results

I. We have found that surface electrical stimulation of the infraorbital (ION) or supraorbital (SON) nerve induces reproducible reflex responses that could be recorded by electromyography from cervical muscles in horses as trigemino-cervical reflex (TCR).

II. We have found that higher current is necessary to elicit the TCR while stimulating the ION compared to the SON.

III. We have found that the TCR evoked by SON or ION stimulation shows similar features regardless of the nerve that had been stimulated.

IV. Stimulations of increasing intensity elicited reflexes of increasing amplitude and decreasing latency, accompanied by stronger behavioural reactions, therefore we could have confirm the nociceptive nature of the TCR.

V. We have found that reflexes recorded from the SPL were significantly larger in amplitude than from the CM, possibly reflecting their different function in the organization of the final aversive movement or to the higher background activity of the SPL, due to its antigravity function that facilitates the reflex.

VI. We have found identical psychophysical (behavioural score) and electrophysiological thresholds.

VII. We found that the nociceptive late component of the BR and the TCR were not elicited by sub-threshold intensity repeated transcutaneous electrical stimulation, so temporal summation of afferent trigeminal inputs could not be observed. Therefore, the modulation of trigeminal nociceptive processing attributable to repeated Aδ fibre stimulations seems to differ from spinal processing of similar inputs as it seems to have an inhibitory rather than facilitatory effect.
9. References


10. The candidate's publications related to the present dissertation

10.1 Full-text papers published in peer-reviewed journals


10.2 Papers in conference proceedings


11. The candidate’s publications not related to the present dissertation

11.1 Full-text papers published in peer-reviewed journals


11.2 Papers in conference proceedings


11.3 Poster presentations

