1	Repeated electrical stimulations as a tool to evoke temporal
2	summation of nociceptive inputs in healthy, non-medicated
3	experimental sheep
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21	Running title: repeated electrical stimulations can evoke temporal summation in sheep
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23 1. Introduction

The sheep is widely used as an animal model in biomedical research. Experimental trials are often invasive and can only be ethically justified with a continuous refinement of perioperative pain management according to the 3R principles. Surgical interventions can lead to peripheral and central sensitization, resulting in allodynia and hyperalgesia as a consequence of tissue damage, nerve trauma or inflammation [1, 2].

29 Some years ago, sheep suffering from foot rot, an infectious pododermatitis, have been 30 used as a model of persistent inflammatory pain [3-6]. Various analgesics as opioids, alpha-2 31 agonists [4] and non-steroidal anti-inflammatory drugs [3] have been assessed for their efficacy 32 in modifying the behavioural reaction following a painful stimulation of distal affected limbs. 33 Interestingly, nociceptive thresholds remained lower than in healthy animals even after 34 successful treatment of the inflammatory process. In addition, surgically induced peroneal nerve 35 injury leading to neuropathic pain and thus to a significant persistent increase in skin sensitivity 36 has been promoted as a useful model for evaluating analgesics in sheep [7]. Unfortunately, both models are invasive and non-reversible and therefore not appropriate to be applied in refinement 37 38 studies aiming at improving analgesia in sheep undergoing experimental orthopaedic surgery as 39 animal models.

Until now, a non-invasive model that can be used to evaluate central sensitization as a consequence of orthopaedic surgery or the efficacy of loco-regional and systemic antinociceptive treatments is missing in sheep. The nociceptive withdrawal reflex (NWR) model has been used as a non-invasive neurophysiological tool to assess spinal nociceptive processing in humans and animals [8-12]. In humans, the NWR threshold was found to correlate with the pain threshold [13, 14]. The species-specific characteristics of the NWR after single stimulation have been evaluated in sheep in a previous study [15].

47 Stimuli of intensity below NWR threshold, insufficient to provoke any response when 48 presented as a single stimulus, were found to summate and lead to increased nociceptive 49 reflexes and aversive behavioural reactions when administered repeatedly [16-18]. This 50 facilitation of the nociceptive reflex response, originating from the temporal summation of 51 sensory inputs, has been proposed as a non-invasive measure to quantify the physiology of 52 central integrative and sensitization processes associated with the wind-up phenomenon 53 observed in dorsal horn cells to repetitive nociceptive input [19].

According to our hypothesis, sub-threshold repeated electrical stimulations of the lateral digital nerves of the fore limb and the hind limb will summate facilitating the NWR and it will be possible to quantify this facilitation analyzing specific post-stimulation epochs of the electromyographic signals elicited.

Aim of this prospective study was to first define the individual NWR threshold (I_t) to single stimulation and then to apply series of 10 repeated stimuli at fractions of 0.5-1 x I_t to evaluate the extent of temporal summation of nociceptive inputs in conscious, non-medicated standing sheep. Following each stimulation, the evoked behavioural response was observed and scored to be able to complement the information gained from the neurophysiological data.

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65 2. Methods

66 2.1. Animals

67 Twenty-five adult Swiss Alpine sheep were included in the study (mean weight: $63.1 \text{ kg} \pm$ 6.1 kg (SD); age 2-3 years). All sheep were part of an orthopaedic study starting after this 68 69 experiment. Prior to the experiment, the animals were clinically examined and kept under close 70 clinical surveillance during the entire experimental period. The experiment was approved by the 71 Committee for Animal Experimentation of the Canton Graubuenden, Switzerland. The sheep 72 were housed together in one stable within individual boxes. The room temperature was 16°C. A 73 maintenance diet consisting of a mixture of straw, silage, maize and salt was fed twice daily 74 while water was available ad libitum in an automatic water drinker. Food but not water was 75 withheld in the morning of the experimental session. All measurements were performed during 76 the morning and took place in the stable where the animals had been housed for 4 to 6 weeks. 77 The day before the experiment the sheep were placed into purpose-made suspension slings to 78 guarantee restrain and avoid recumbency during the experiment. Normal standing and limited 79 walking were always possible while being placed in the slings.

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81 2.2. Electrical stimulation and electromyography

82 Each experimental session was started at the thoracic limb (deltoid muscle in 25 animals). 83 First, the nociceptive threshold (I_t) was determined as previously reported [15]. In brief, 84 nociceptive withdrawal reflexes were evoked in sequence by electrical stimulation of the lateral 85 digital nerves of the thoracic and the pelvic limb. Electromyographic recordings from the 86 deltoid muscle of the thoracic limb and from the biceps femoris and the peroneus tertius of the pelvic limb were used to objectively quantify the reflexes. The behavioural reaction following 87 88 each stimulation was observed and scored by the same investigator (HR) on a scale from 0 (no 89 reaction) to 5 (strong whole body reaction; Table 1). Threshold intensity (I_t) was defined as the 90 minimal stimulus intensity able to evoke an EMG response of 20 μ V (RMS_A) with a duration of 91 at least 10 ms within the NWR interval and a minimal reaction score of 1/5 (Table 1)[14].

In a next step, 10 pulse trains were delivered over a 2s period with a stimulation frequency of 5 Hz. The stimulation intensities applied were fractions of the individual I_t (0.5, 0.6, 0.7, 0.8, 0.9 and 1 x I_t). If a reflex could be detected after stimulation at 0.5 x I_t evoked by any of the 10 stimuli in the train (EMG, behaviour), the next stimuli were reduced to 0.4 x I_t and then to 0.3 x I_t until no reaction or EMG could be evoked anymore. The full stimulus-response curves were acquired to each sheep so all remaining intensities were subsequently applied. Between two stimulation series, intervals of at least 60s were maintained.

After defining the thoracic limb I_t and the application of the repeated stimulations at the
 corresponding intensities, the procedure was repeated at the pelvic limb.

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102 2.3. Reflex analysis

The pre-stimulation (0-500 ms), the stimulation (500-2500 ms) and the post-stimulation (2500-4000 ms) periods were analysed separately (sampling frequency: 1024 Hz). For the reflex analysis, the stimulation period was divided into the NWR interval (20-130 ms) and the post-NWR interval (130-200 ms) following each pulse train and the nociceptive threshold for repeated stimulations (RS I_t) was determined.

108 The RS I_t was defined as the minimal stimulus intensity able to evoke one EMG response of 109 a minimal RMS amplitude (RMS_A) of 20 μ V with a duration of at least 10 ms within the NWR 110 interval after any of the 10 stimuli and a reaction score of at least 1/5 (Table 1). To better 111 understand the reflex patterns, the amplitude of the EMG activity recorded in the NWR interval 112 after the first stimulus was compared with the amplitude of the maximal NWR within the 113 stimulation series. The post-NWR interval was evaluated to prove the correctness of the NWR 114 interval as only the time window of 20-130 ms was expected to be originated by reflexive 115 activation of A-delta fibres while the window of 130-200 ms could potentially be influenced by 116 supraspinal activation [15].

117 The pre-stimulation period was analysed to prove balanced weight distribution while the 118 post-stimulation-period was used to determine continued movements after cessation of 119 stimulation.

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121 2.4. Statistical analysis

122 Data analysis was performed using statistical software (Sigma Stat, Version 3.5, Systat 123 Software). Nonparametric analysis of data was chosen on the basis of tests for normality of 124 distribution. Friedman repeated measures ANOVA on ranks followed by Tukey test were 125 applied to compare the effects of stimulation intensity on the reflex characteristics (NWR 126 interval, post-NWR interval, pre- and post-stimulation periods and behaviour) if significant 127 overall effects were detected. The Brunner-Langer LD-F2 model [20] was applied to analyse the 128 effects of repetition of the stimuli, the effects of stimulation intensity on reflex amplitude (NWR 129 interval) and the interaction between the two variables at the deltoid and the biceps femoris 130 muscles. Stimulation intensities starting from $0.5 \times I_t$ were considered for all analyses. Only for 131 the determination of RS It stimulation intensities at lower fractions were included. The 132 Wilcoxon Signed Rank Test was used to compare the RMS_A of the EMG activity following the first stimulus with the RMS_A of the maximal NWR within the stimulation series. Significance 133 134 was set at p < 0.05.

136 **3. Results**

137 Clinical examination revealed no abnormalities in any animal. The sheep tolerated the 138 experiments well as the observed nocifensive reactions stopped immediately after stimulation. 139 With the current intensities used to determine repeated stimulation thresholds in the present 140 study, none of the sheep appeared distressed after termination of the stimulations. Results are 141 reported in Table 2.

Intensities necessary to evoke a NWR varied among individuals and were muscle-specific. Median I_t was 4.4 mA (IQR 2.9-5.7 mA) with a behaviour score of 1 (1-2) for the deltoid muscle, 7 mA (IQR 4-10 mA) with a behaviour score of 1 (1-2) for the biceps femoris muscle, and 3.4 mA (IQR 3.05-4.35 mA) with a behaviour score of 1 (1-1) for the peroneus tertius muscle.

For the deltoid muscle, the first reflex (RS I_t) occurred at 0.6 x I_t (0.5-0.8) corresponding to 2.3 mA (1.6-3 mA) with a reaction score of 2 (1-2). For the biceps femoris muscle, RS I_t was 0.55 x I_t (0.4-0.7) corresponding to 2.9 mA (2.6-4 mA) with a reaction score of 1 (1-2) while for the peroneus tertius muscle RS I_t was 0.8 x I_t (0.8-0.95) corresponding to 3 mA (2.8-3.5 mA) with a reaction score of 1 (1-2). The RS I_t was lower than 0.4 x I_t in 3 animals for the deltoid muscle and in 4 animals for the biceps femoris muscle but for the peroneus tertius muscle the lowest detected RS I_t was 0.6 x I_t .

The mean RMS_A of the NWR interval (20-130 ms) increased significantly (p<0.001) when the relative stimulus intensity was increased from 0.5 to 1 x I_t for all 3 muscles. The reaction score increased with increasing stimulation intensities for all muscles (p<0.001).

157 The RMS_A of the pre- (0-500 ms) and the post-stimulation periods (2500-400 ms) remained 158 low despite stimulus intensity increased from 0.5 to 1 x I_t in all three muscles.

When the stimulus intensity increased from 0.5 to 1 x I_t a significant intensity effect could be detected for the deltoid and the biceps femoris muscles (p<0.001; Fig. 1). The RMS_A increased and a NWR could be detected in an increasing proportion of animals. The stimulus number within the stimulation series had neither any influence on the RMS amplitudes nor on
the proportion of animals with a nociceptive reflex (p=0.45). When both effects were combined,

164 no effect of interaction was found in any muscle (p=0.32).

165 The EMG activity following the first stimulus was significantly smaller than the maximal 166 NWR for the deltoid and the biceps femoris muscle at all stimulus intensities, while for the 167 peroneus tertius the EMG activity following the first stimulus was also the largest NWR at 0.9 168 and 1 x I_t. The proportion of animals in which the first stimulus in the train evoked the largest 169 NWR increased with increasing stimulus intensity for the deltoid and the peroneus muscle, but 170 not for the biceps femoris muscle (Table 3).

172 **4. Discussion**

173 In the present study, electrical stimuli of intensity below the previously determined 174 nociceptive threshold for a single stimulus led to temporal summation with an increase in 175 nociceptive reflex amplitude and aversive behavioural reactions when presented repeatedly at 176 a frequency of 5 Hz in conscious, non-medicated, standing sheep.

177 Repeated stimulation thresholds (RS I_t) and the effects of stimulus intensity on reflex 178 amplitude were determined. Reflex recording was accompanied by a scoring of the 179 behavioural reaction following each stimulation series. For the determination of the 180 nociceptive threshold the appearance of a clear behavioural reaction was considered 181 fundamental to be sure that the animal perceived the stimulation as unpleasant. In humans it is 182 known that the stimulation intensity evoking a nociceptive withdrawal reflex on the 183 electromyogram correlates with pain perception [13, 14].

With the current intensities used to elicit nociceptive reflexes in the present study, none of the sheep appeared distressed as the behavioural reactions stopped immediately on stimulation cessation. Even when high stimulus intensities were used, the reaction pattern remained localized to the limb under evaluation.

188 After determining the I_t after single stimulation, repeated stimuli were applied and the RS I_t 189 was defined. The stimulation pattern of 10 stimuli at a frequency of 5 Hz as used in this study 190 has been previously applied to horses [16] and humans [18]. The relative RS I_t was comparable 191 for the deltoid and for the biceps femoris muscle while it was higher for the peroneus tertius 192 muscle. The lack of reflex facilitation in the peroneus tertius muscle at subthreshold levels 193 might be explained by the functional characteristics of the muscle as a stabilizer rather than as a 194 flexor. As a consequence, recording from the peroneus tertius muscle cannot be recommended 195 for future studies using if the target is to assess spinal nociceptive processing.

Standing equally on 4 limbs was a prerequisite for starting the stimulation procedure. A silent
background EMG (judged visually) reduced the occurrence of facilitation due to increased

198 excitability of the motor neuron pool that might take place in case of spontaneous muscle 199 activity prior to stimulation [21]. Data analysis off-line confirmed that the pre-stimulation 200 period was characterised by a low RMS_A . For the post-stimulation period, the RMS_A had a 201 tendency to increase only for the highest stimulation intensities (0.9, 1 x I_t) associated with more 202 intense and prolonged behavioural reactions.

203 In humans, repeated stimulations at intensities below the individual pain threshold were 204 able to elicit gradually larger NWR [18] most likely reflecting temporal summation in deep 205 dorsal horn neurons due to central integration of the neural responses leading to an increasing 206 pain sensation [18, 22]. Temporal summation seems to reflect the early phase of wind-up, a 207 phenomenon observed in rats [19] which is part of the pathophysiologic basis of clinical 208 persistent pain syndromes [23]. In sheep, the number of stimuli had no influence on the mean 209 RMS_A . Anyway, the amplitude of the maximal reflex was significantly larger than the amplitude 210 of the first reflex which reflects induction of temporal summation as seen in humans. Increasing 211 stimulation intensity towards intensity threshold $(1 \times I_{t})$ increased the number of animals 212 showing the maximal reflex after the first stimulus as presented in Table 3. For the deltoid and 213 the peroneus tertius muscle this phenomenon was evident while for the biceps femoris muscle a 214 further increase of the reflex amplitude was still observed at the highest stimulation intensities 215 even if the first stimulus was already able to evoke a nociceptive reflex. This muscle specific 216 response is probably linked to the topographical organization of the nociceptive reflex in sheep 217 [24].

The epoch of 20-130 ms post-stimulation used in this study to identify the NWR indicates that most probably the reflex originates from activation of A-delta fibres [15]. Wind-up was originally believed to result from repetitive C-fibre stimulation only [25] but when A-delta fibres were stimulated at a minimal frequency of 0.3 Hz an occurrence of wind-up occurs was demonstrated for these fibres as well [26]. C-fibre stimulation would require stimulation for several seconds at an intensity not tolerated by awake subjects [18]. Therefore, the proposed method can be considered as an adequate non-invasive model of wind-up.The goal of this study 225 was to generate reference values for later applications of this model in experimental sheep 226 undergoing orthopaedic procedures. In humans, a segmental sensitization could be detected in 227 the immediate post-operative period [27]. When a local anaesthetic was administered to the 228 epidural space, pain sensation to single stimulation was inhibited in 6/10 human volunteers 229 while it was only abolished in 1/10 of the same subjects if repeated electrical stimuli were 230 applied [28]. This discrepancy has been explained with changes in the excitability of neurons on 231 spinal level induced by repeated stimulations and confirms the importance of a multimodal set 232 of stimulation modalities.

233

234 Conclusions

The repeated application of stimuli at intensity unable to evoke a nociceptive withdrawal reflex if given singularly, led to increasing amplitudes of the nociceptive reflexes and aversive behavioural reactions and therefore temporal summation in healthy standing sheep without inducing persisting stress and discomfort for the animal. Data achieved in this study can serve as reference values for future experimental applications of the model in this species.

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241 **Conflict of interest statement**

None of the authors of this paper has a financial or personal relationship with other peopleor organizations that could inappropriately influence or bias the content of the paper.

244

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249 **5. References**

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