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Antinociceptive effects of levomethadone in standing horses sedated with romifidine

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1 **Anti-nociceptive effects of levomethadone in standing horses sedated with romifidine.**

3 **Abstract**

4 **Objective** To evaluate the antinociceptive effect of a bolus of intravenous levomethadone
5 administered during romifidine constant rate infusion (CRI).

6 **Study design** Prospective, randomized, masked, cross-over experimental study

7 **Animals** Eight adult warmblood horses (seven geldings, one mare) aged 6.6 ± 4.4 years,
8 weighing 548 ± 52 kg.

9 **Methods** Levomethadone 0.1 mg kg^{-1} or an equivalent volume of saline (control) was
10 administered intravenously to standing horses 60 minutes after starting a romifidine CRI.
11 Blood samples to quantify romifidine and levomethadone plasma concentrations by capillary
12 electrophoresis were collected up to 150 minutes after levomethadone administration. The
13 nociceptive withdrawal reflex threshold (NWRT) was determined continuously using an
14 automated threshold tracking device. A pharmacokinetic-pharmacodynamic (PK-PD) model
15 was elaborated.

16 **Results** Horses exhibited higher NWRTs after receiving levomethadone compared to saline
17 ($123 \pm 9 \%$ *versus* $101 \pm 9 \%$ relative to baseline, $p < 0.05$). The PK-PD model identified a
18 contribution of levomethadone to the NWRT increase. Effect size was variable among
19 individuals. No adverse reactions to levomethadone administration were observed. A slight
20 effect of levomethadone on sedation scores was evident for the sixty minutes following its
21 administration.

22 **Conclusions and Clinical Relevance:** A single injection of levomethadone has the potential
23 to increase the NWRT during romifidine CRI in horses and can be administered in
24 combination with alpha-2 agonists to enhance antinociception in horses. However, individual
25 variation is marked.

26 **Keywords:** antinociception, horses, nociceptive withdrawal reflex, levomethadone, PK-PD

Introduction

In equines, general anaesthesia is associated with a high mortality and morbidity risk (Dugdale & Taylor, 2016) and invasive surgical procedures are sometimes performed under sedation in standing position. While alpha-2 agonists provide reliable sedation and analgesia, the addition of an opioid could contribute to further antinociception and improved conditions and safety in such circumstances.

Due to the fear of adverse reactions such as excitation, increased spontaneous locomotor activity and decreased intestinal motility (Clarke & Paton 1988; Kamerling et al. 1989; Clutton 2010; De Oliveira et al. 2014; Echelmeyer et al. 2019) opioids are used more cautiously in horses than in other species. Moreover, there has been contrasting evidence about their efficacy especially when administered concurrently with other analgesic drugs (Bennett et al. 2004; Knych et al. 2009; Villalba et al. 2011).

Current research in equine pain management therefore focuses on defining ideal opioid dosages to obtain the desired clinical end-point while minimizing the potential occurrence of adverse effects (Linardi et al. 2012; Carregaro et al. 2014; De Oliveira et al. 2014; Dönselmann im Sande et al. 2017). While a significant antinociceptive effect of racemic methadone alone could only be demonstrated at dosages that provoked severe side effects, lower dosages were shown to enhance and prolong detomidine-induced antinociception (Schatzmann et al. 2001; De Oliveira et al. 2014; Lopes et al. 2016; Gozalo-Marcilla et al. 2017; Gozalo-Marcilla et al. 2019a,b). Levomethadone is the effective L-enantiomer of racemic methadone and is licensed for use in horses.

The nociceptive withdrawal reflex (NWR) is a polysynaptic spinal reflex responsible for the nocifensive reaction that protects the integrity of the body against a damaging stimulus. In humans, the NWR has been extensively used as a reliable method to define and quantify spinal nociceptive processing and as a supplement to psychophysical methods for pain assessment (Willer 1977; Willer 1985; Willer & Bussel, 1980; Arendt-Nielsen et al.

1995; Dincklage et al. 2009). Following electrical transcutaneous stimulations of a sensitive nerve of sufficient intensity, a clearly recognizable and repeatable electromyographic (EMG) burst can be identified that allows definition of the NWR threshold (NWRT). The NWRT has been shown to coincide with the subjective pain threshold in humans (Willer 1985). The same stimulation pattern has been investigated in horses, the time window for the occurrence of the NWRT was defined and the model validated in this species (Spadavecchia et al. 2002; Spadavecchia et al. 2003; Spadavecchia et al. 2004). In equine pain research, the NWR has been described as an alternative to classical behavioural models for investigation of the physiology of nociception and the antinociceptive action of different drugs (Spadavecchia et al. 2005; Spadavecchia et al. 2007; Peterbauer et al. 2008; Rohrbach et al. 2009; Risberg et al. 2015)

Recently, a continuous NWRT-tracking device based on a validated algorithm (Dincklage et al. 2009) has been proposed for use in humans and has not yet been applied in animals. Using a validated electrical stimulation paradigm (Spadavecchia et al. 2002; Spadavecchia et al. 2003; Spadavecchia et al. 2004), this novel automated system allows precise characterization of the time course of drug-related antinociceptive activity, thus representing an interesting tool for pharmacokinetic – pharmacodynamic (PK-PD) modelling because threshold determination can be considered almost continuous.

In order to investigate if the administration of levomethadone could provide additional analgesia during a constant rate infusion of romifidine, the present study aimed at describing the time course of the spinal antinociceptive effect of levomethadone when administered as a bolus during romifidine constant rate infusion (CRI) using the automated NWRT tracking device. We hypothesized that a further increase of the NWRT would take place (primary outcome). Secondary outcomes were the best variable estimates for the pharmacokinetic-pharmacodynamic (PK-PD) model of the NWRT and the drug effect on sedation scores.

Material and Methods

Sample size calculation

Based on previous experience, the NWRT was expected to increase to approximately $400 \pm 50\%$ (4 to 4.5 times) of its baseline during romifidine infusion. Another 50% increase (to 450%) after levomethadone administration was considered to be relevant. Considering a power of 0.85 and a probability of type-I error of 0.05, a sample size of 8 animals per treatment was estimated based on an ANOVA for repeated measures (two treatments, 60 time points, $\eta^2=0.05$) (G*power v.3.1.9.4, University Kiel, Germany).

Study design, ethical statement and animals

This prospective, randomized, masked crossover experimental study was approved by the local Committee for Animal Experiments (Permission number: BE 8/17). It involved eight adult warmblood horses and was part of a parallel trial investigating the antinociceptive effects of a romifidine CRI (Diez Bernal et al. 2019). Government owned horses were recruited from the National Equine Centre and informed consent was provided. Horses had to be healthy and free from any pharmacological treatment during the two months preceding the trial to be eligible for inclusion.

All horses were kept in single boxes under regular housing conditions. Each horse received two treatments (levomethadone or saline solution) in two separated experimental sessions, with a minimum washout period of two weeks. Block randomization of the two treatment options was performed in advance (www.randomization.com) to ensure balanced treatment distribution at each session. Timing of the procedures was standardised and constant environmental conditions were maintained throughout the experimental phase.

Preparation

Before each experimental session, the horses were fasted for 12 hours but had free access to water. To guarantee standardized timing of the study procedures, one horse per day was investigated, starting always in the morning. Prior to the start of the experiment, the horse was

weighed and placed in a standard box, in a stable with other horses. A clinical examination was performed, and a baseline sedation score assigned. The fur was clipped at catheter as well as at the electrode placement sites. Two intravenous (IV) catheters (13 gauge 105 mm catheter, Intranule; Vygon, France) were placed aseptically in the right and left jugular vein respectively after subcutaneous infiltration of lidocaine 2% (Lidocain 2% Streuli; Streuli Pharma AG, Switzerland). One catheter was used for drug and crystalloid fluid administration, the other was dedicated to blood sampling only. After standardized skin preparation, self-adhesive surface stimulation electrodes located 0.5 cm apart (BlueSensor N; Ambu, Denmark) were placed over the lateral digital nerve at the midpoint between the coronary band and the metacarpophalangeal joint of the left forelimb. For EMG recordings, two self-adhesive electrodes (BlueSensor N; Ambu, Denmark) were placed 0.5 cm apart over the deltoid muscle. A ground electrode (BlueSensor VL; Ambu, Denmark) was placed over the greater tubercle of the humerus. Flexible leads connecting the electrodes to the stimulation and recording device were secured with adhesive tape and bandages. The electrode impedance was checked automatically before and during each experimental session. A value up to 2 kOhm was accepted; if the impedance was above this limit the electrodes were replaced after performing a new skin scrub. Ringers Lactate (Ringer-Lactate "Bichsel"; Grosse Apotheke Dr. G. Bichsel AG, Switzerland) infusion was started at a rate of 5ml kg⁻¹ hour⁻¹ after electrode placement. Once instrumented, the horse was left undisturbed for 10 minutes before starting NWRT measurements. During the experimental session, the horse was loosely restrained by a rope attached to the box wall.

Drug administration

After determination of the NWRT (at least 10 minutes of stable NWRT measurements and the earliest 20 minutes after starting NWRT measurements) a bolus of 0.08 mg kg⁻¹ romifidine (Sedivet; Boehringer Ingelheim, Switzerland) was administered manually IV over 60 seconds. The end of the bolus administration was defined as T₀. Thereafter, a continuous rate infusion

of romifidine ($0.03 \text{ mg kg}^{-1} \text{ hour}^{-1}$ IV) was administered until T_{120} (120 minutes after end of the romifidine bolus) using a syringe pump (Perfusor Space Syringe Pump; B. Braun, Switzerland) that was calibrated yearly by the manufacturer. At T_{60} , the study treatment was administered: either a levomethadone (L-Polamivet; MSD Animal Health, Switzerland) bolus of 0.1 mg kg^{-1} or an equal volume of physiologic saline solution (control) manually IV over 60 seconds. Another person prepared the drug syringes in advance such that the investigators assessing sedation quality and recording NWRT data were unaware of the treatment (masked design).

The commercial formulation tested in the current study L-Polamivet contains 2.5 mg ml^{-1} levomethadone and 0.125 mg ml^{-1} fentanyl, an antimuscarinic drug. A levomethadone dose range of $0.05\text{-}0.1 \text{ mg kg}^{-1}$ is recommended for horses in the information leaflet.

Pharmacokinetics: Romifidine and levomethadone plasma concentrations

A baseline blood sample was obtained during preparation of the horse after placement of the IV catheters. Thereafter 10 mL of venous whole blood were collected directly into heparin containing sampling tubes at T_3 , T_5 , T_7 , T_{15} , T_{30} , T_{55} , T_{63} , T_{65} , T_{67} , T_{75} , T_{90} , T_{120} , T_{150} , T_{180} and T_{210} minutes. Immediately after blood withdrawal, the samples were put on ice and centrifuged within 15 minutes for 10 minutes at $2000 g$ (gravitational acceleration) at 10°C to separate the plasma from the blood cells. Subsequently 2 mL of plasma supernatant were manually removed from the sampling container with a pipette and transferred into a labelled tube in which they were stored at -20°C until further analysis was undertaken.

Plasma concentrations of levomethadone and romifidine were determined by capillary electrophoresis. This method was a modification of assays described for the enantioselective determination of ketamine and its metabolites (Theurillat et al. 2016) and methadone and its main metabolite (Theurillat et al. 2019) in plasma. In order to be able to monitor romifidine, methadone and its main metabolite in one assay, the amount of highly sulfated γ -cyclodextrin in the pH 3.0 running buffer had to be lowered to 0.14 % (Diez Bernal et al. 2019).

Quantification of drug concentrations was based on five-level internal calibration using corrected peak areas. The calibration range for levomethadone was 25 to 500 ng mL⁻¹ and the quantification limit was taken as 12 ng mL⁻¹. For levomethadone levels of 100 and 400 ng mL⁻¹, interday precision ($n = 6$) was 8.41 % and 5.91 %, respectively. Accuracy assessments revealed levomethadone concentrations that varied less than 10 % from the target values. The calibration range for romifidine was 10 – 200 ng mL⁻¹ and the quantification limit was taken as 5 ng mL⁻¹. For romifidine levels of 20 and 80 ng mL⁻¹, interday precision ($n = 6$) was 5.22% and 2.36%, respectively. Accuracy assessments revealed romifidine concentrations that varied less than 3% from the target values.

Pharmacodynamics: NWRT determination

Electrical stimulation and EMG recordings were performed using a commercial unit (PainTracker; Dolosys GmbH, Germany), developed for automated threshold tracking. The NWRT was automatically determined using a bracketing design according to a validated continual NWRT tracking algorithm (Dincklage et al. 2009). Each stimulation consisted of five individual rectangular pulses of 1 ms duration delivered at 200 Hz. Electromyographic activity was recorded from 100 ms prior to until 400 ms after stimulation onset for a total recording time of 500 ms (sampling frequency 1 kHz). The time window between 100 and 10 ms prior to stimulation onset was analysed to quantify the presence of noise (noise range), while the time window between 60 and 200 ms following stimulus onset was analysed to quantify the NWR (NWR range). The reflex was considered positive for an interval peak Z-score above 10, meaning that the difference between the maximum EMG amplitude (within the NWR range) and the mean EMG amplitude within the noise range had to be above the ten-fold of the standard deviation of the EMG amplitudes within the noise range (Rhudy & France 2008). Starting stimulus intensity was set at 1 mA. Measurements were discarded when the EMG amplitude exceeded 15 μ V within the noise range; in this case, the same stimulus intensity was repeated. Step size was set at 0.3 mA. Step size increased to 0.5 mA when the intensity changed in the same direction for three consecutive times and decreased

back to 0.3 mA as soon as the intensity reversed in the other direction. The interstimulus interval was set at a mean of 10 seconds with randomization set at 30%. Stimulation could be paused at any moment if necessary.

A mean NWRT was calculated for each minute of recording; the calculated mean values were then used for further analysis. $\text{Baseline}^{\text{nodrug}}$ and $\text{baseline}^{\text{rom}}$ were calculated as the mean NWRT from the five minutes preceding romifidine and treatment administration, respectively. The relative NWRT used for PK-PD modelling was calculated as absolute NWRT divided by the individual $\text{baseline}^{\text{rom}}$.

The measurements were stopped when the absolute NWRT returned to $\text{baseline}^{\text{nodrug}}$ or at T_{210} . Data was smoothed by calculating the central moving average for a window of 5 minutes, and peak values as well as time of occurrence were recorded from T_0 to T_{60} (Peak NWRT_{0-60}) and from T_{60} to T_{120} (Peak NWRT_{60-120}).

PK-PD modelling

Based on measured plasma concentrations for romifidine (Diez Bernal et al. 2019) and levomethadone, a pharmacokinetic model was performed using a commercially available software (Phoenix 64 v.8.0.0.3176 2017, WinNonLin/NLME application; Cetara Inc, USA).

The best suitable compartmental model was chosen from observation of data fit and diagnostic variables. A PK-PD model had been defined for romifidine alone in the previous study (Diez Bernal et al. 2019). The contribution of levomethadone plasma concentrations to the modulation of the NWRT was added to the existing model. Several models were assessed and the best fitting model chosen from observation of data fit, diagnostic variables and objective function values.

Sedation scores and vital variables

Sedation quality was scored before (baseline^{nodrug}) and every ten minutes after drug administration using a predefined multifactorial sedation scale ranging from 0 (no sedation) to 10 (heavily sedated) (Table 2) (Rohrbach et al. 2009). A score of ≥ 5 was considered to represent effective sedation. Heart rate, respiratory rate and borborygmi sounds were evaluated at regular intervals. Horses were observed for occurrence of adverse effects. After the end of the experimental session, animals were returned to their stables. All horses underwent a clinical examination in the evening after the experiment to assure normal vital variables as well as adequate gut sounds. The next day they went back to their routine activity.

Statistical analysis

Statistical analysis was performed using Sigma Plot for Windows (Sigma Plot v. 14; Systat Software GmbH, Germany). Data were tested for normal distribution by visual inspection and confirmed by a Shapiro-Wilk test. Normally distributed data are presented as mean $\pm$ standard deviation or mean $\pm$ standard error of the mean for repeated measures. Non-normally distributed data are presented as median (interquartile range 25%-75%). Paired t-test or Wilcoxon rank sum test were applied accordingly to compare data between treatments, and two-way repeated measure ANOVA was performed followed by Holm-Sidak tests for pairwise multiple comparisons to detect the effect of time and treatment on repeated measures. One-tailed tests were applied where treatment with levomethadone was expected to exhibit higher values. Linear correlations between plasma concentrations (AUC), peak NWRT and sedation scores were tested with Pearson product moment analysis.

Results

Horses

A total of seven geldings and one mare were included in the trial. Median age was 4.5 (4–16) years and mean weight was 548 ± 52 kg. All horses completed the study.

Pharmacokinetics: Romifidine and levomethadone plasma concentrations

Pharmacokinetic details for romifidine CRI determined during this study are reported elsewhere (Diez Bernal *et al.*, 2019). Mean plasma concentration of romifidine during the second hour of the CRI (after treatment administration) was $25.4 \pm 0.5 \text{ ng mL}^{-1}$. There was no difference in the plasma concentration of romifidine before or after the administration of levomethadone ($p = 0.431$) and drug levels were stable. Plasma concentrations of levomethadone best followed a standard two-compartment pharmacokinetic model (Fig. 1, Table S1). Maximal plasma concentration of $481 \pm 77 \text{ ng mL}^{-1}$ was reached at the first blood sample after bolus administration.

Pharmacodynamics: NWRT determination

General results for baselines and peak NWRTs are presented in Table 1. Overall, there was no difference in NWRTs between treatments at timepoints T_0 through T_{60} (romifidine bolus followed by CRI) for both absolute values ($p = 0.263$) or relative to baseline^{no drug} ($p = 0.600$). Following treatment, from T_{69} to T_{101} , the horses exhibited higher NWRTs after receiving levomethadone compared to saline ($123 \pm 9 \%$ versus $101 \pm 9 \%$ relative to baseline^{rom}, $p < 0.05$, Fig. 2). There was no correlation between levomethadone plasma concentration and peak NWRTs ($r^2 < 0.05$).

PK-PD modelling

An ordinary Emax indirect response model (Fig. S1) best fitted the observed effect of levomethadone on the NWRT values when including following model strategies: 1) Non-competitive interaction of romifidine and levomethadone (Earp *et al.* 2004); 2) Inhibition of loss of effect (indicating a decrease in sensibility as physiological effect, (Sharma & Jusko 1998) by levomethadone; 3) Inclusion of an effect compartment (k_{e0}) to model the response (R) delay compared to the concentration time course (Koch *et al.* 2014). The evaluated

pharmacodynamic fixed variables were K_{in} , $IC_{50\ rom}$, γ_{rom} , $k_{e0\ rom}$, $IC_{50\ lmeth}$ and $k_{e0\ lmeth}$ (Table S2), including random effect ($I_{max} = 1$, $K_{out} = 0.9 - 1.1 \times K_{in}$), according to following equations:

$$\frac{dR}{dt} = K_{in} - K_{out} \times \left[\left[1 - \frac{Ce_{rom}^\gamma}{IC_{50\ rom}^\gamma + Ce_{rom}^\gamma} \right] \times \left[1 - \frac{Ce_{lmeth}}{IC_{50\ lmeth} + Ce_{lmeth}} \right] \right] \times R$$

$$\frac{dCe_{lmeth}}{dt} = k_{e0} \times \left[\frac{C_{lmeth}}{V_{1\ lmeth}} - Ce_{lmeth} \right]$$

The PK-PD model allowed the prediction of the respective contribution of romifidine and levomethadone on the response effect and identified a relevant increase of the NWRT (Peak median additional effect of 102 % at $T_{111 \pm 13}$, Fig. 3) as a result of levomethadone administration.

Sedation scores

There was no difference between treatments for sedation scores before treatment administration ($p = 0.796$). A slight effect of levomethadone on sedation scores for the sixty minutes following its administration [6 (4–7) versus 7 (6–8), $p = 0.031$, Fig. S2] was observed. There was a weak correlation between levomethadone plasma concentration and maximal sedation scores ($r^2 = 0.317$, $p = 0.146$).

Vital parameters and adverse effects

Mean heart rate did not differ between groups before or after treatment administration. No adverse effects such as excitation or increased spontaneous locomotor activity were observed. All horses tolerated the experiment well without signs of discomfort.

Discussion

In the present study, a bolus of 0.1 mg kg⁻¹ levomethadone during romifidine CRI in standing horses led to an increase of the NWRT nine to 40 minutes after drug administration, indicating that an antinociceptive effect was provided by the opioid when combined with the alpha-2 agonist CRI. The elaborated PK-PD model confirmed and highlighted the specific contribution of levomethadone on the NWRT increase. However, marked individual differences in effect size and duration of action were noticed and need to be taken into consideration.

Recently, several studies investigating the antinociceptive activity of methadone administered with detomidine were published (Gozalo-Marcilla et al. 2017; Gozalo-Marcilla et al. 2019a,b). In accordance with our findings, a potentiation of antinociception was observed when the two drugs were co-administered. In this context, our study provides further evidence for a potentially clinically relevant enhancement of antinociception when alpha-2 agonists and opioids are combined.

The current study used the NWRT to evaluate antinociception. The NWR is recognized as an objective method as it relies on neurophysiological recordings. While bypassing peripheral receptors, nerve fibres are stimulated electrically in a non-selective manner (Le Bars et al. 2001). By defining a timeframe of interest after the stimulus onset, the reflex component originating from the activation of A δ afferent fibres (RIII reflex in humans) can be selectively quantified. Therefore, our results indicate an inhibitory effect of levomethadone on A δ -fibres nociceptive processing at the spinal cord level. This is an interesting finding as human and laboratory animal experiments indicate that clinical doses of μ -agonists primarily depress C fibre activity (Jurna & Heinz 1979; Strimbu-Gozariu et al. 1993; Guirimand et al. 1995; Yeomans et al. 1996; Le Bars et al. 2001).

None of the horses in our study showed visually recognizable motor reactions in response to stimulation even in the unsedated state during baseline data collection. This is

different to what was reported in previous NWR experiments, in which at least a skin twitch was considered necessary to define the reflex threshold (Spadavecchia et al. 2002; Spadavecchia et al. 2003; Rohrbach et al. 2009) . The automated device used in the present trial applies a very sensitive algorithm to track continuously the reflex threshold, thus remaining always below the current level able to evoke motor responses. This makes this model highly tolerable even in completely awake patients and in flight-animals such as horses.

Individual variation in NWRTs in response to levomethadone was marked in our study population. It is well recognized, that there are many factors potentially influencing individual drug response. While environmental factors might play an important role, also animal related factors like sex, breed, age, individual genetic variations have to be considered. In our setting, the influence of a second drug is an additional relevant factor, as previously discussed (Dutta et al. 2000; Pypendop et al. 2013; Gozalo-Marcilla et al. 2019a,b).

In the present study, levomethadone administration led to a slight increase in sedation scores when compared to a control treatment. This is in accordance with other reports describing enhanced sedation with the co-administration of alpha-2 agonists and equipotent doses of opioids in horses (Schatzmann et al. 2001; Lopes et al. 2016; Gozalo-Marcilla et al. 2017). Our results show, that this is also true for levomethadone and that higher levels of sedation rather than excitation can be expected with the co-administration of levomethadone and romifidine. On the contrary, other equine studies reported an infra-additive synergism or partial antagonism when combining opioids (at equipotent doses) and alpha-2 agonists (De Oliveira et al. 2014; Gozalo-Marcilla, Luna, et al. 2019a,b). These contrasting findings might reflect subtle differences in the experimental environment or an inevitable degree of subjectivity in the methods currently used to assess sedation (Benito et al. 2017). Also, it has been recognized that μ -opioid receptor polymorphisms influence the amount of locomotor response in horses (Wetmore et al. 2016).

In our study, plasma concentrations of levomethadone followed a standard two-compartment pharmacokinetic model. This is in accordance with two previously published studies reporting similar modelling for the racemic methadone in horses (Linardi et al. 2012; Crosignani et al. 2017). A recently published report found best fit with a three-compartmental model for racemic methadone (Gozalo-Marcilla, Luna, et al. 2019b). However, the model was not satisfactory and was improved by implementing an interaction between detomidine and methadone plasma concentrations as methadone clearance was influenced by detomidine concentrations. Another communication on the same data set reported further improvement for modelling non-linearity of methadone pharmacokinetics by including the interaction (Gozalo-Marcilla et al. 2019c). In the present study, levomethadone bolus pharmacokinetics were studied during stable plasma concentrations of romifidine such that an investigation of the influence of **varying romifidine plasma concentrations** on levomethadone was not possible. Thus, the influence of the alpha-2 adrenergic agonist on antinociceptive PK-PD modelling of levomethadone could not be included here, which represents an important limitation. Further studies are required before extrapolating to different dose combinations of the two drugs. Moreover, the levomethadone product used in this study also contains the antimuscarinic drug fentanyl which might influence the pharmacokinetics of levomethadone and romifidine.

This study has other limitations. While the NWRT model is considered as an objective measure of antinociception, it only allows the evaluation of a specific part of the pain pathway. While it gives reliable information about spinal nociception, it bypasses peripheral components and it does not reflect supraspinal processing. While the automated NWRT tracking system is characterized by a particularly high acceptance by the horses, as stated above, it has the disadvantage of not allowing a contemporaneous evaluation of responses to supra-threshold stimulations of progressively increasing intensity. Indeed, a reduction in the steepness of the stimulus-response curve can indicate inhibitory drug effects on central integration of pain processing (Spadavecchia et al. 2007) which would be expected to occur in

response to administration of pure mu opioids (Paalzow & Paalzow 1975). Moreover, it is unknown whether the predefined increase in relative NWRT arbitrarily selected to define levomethadone efficacy in the present study is actually adequate to reflect clinically relevant analgesia in the context of standing surgical procedures.

In conclusion, the present study demonstrates a significant $A\delta$ mediated antinociceptive activity of levomethadone when administered during romifidine continuous infusion. Despite the presence of a considerable individual variability, the use of the PK-PD model distinguished the antinociceptive effect of levomethadone from the effect of romifidine alone.

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