

## Validation of a Gottingen Minipig Model of Post-Operative Incisional Pain

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### Abstract

**Background:** Evaluating potential new therapies to treat post-operative pain (POP) is highly dependent on successful translation from animal models to humans. Pigs have several benefits over rodents including similar skin innervation to humans, which makes them a more rationale choice. The aim of this present study was to validate a POP model in Gottingen minipigs, as well as address some of the limitations reported using domestic pigs.

**Methods:** Twenty-four female adult Gottingen minipigs underwent full skin and facia incision in the hind leg. Post-incision, the animals were treated with either an increasing dose of morphine and saline using the intramuscular (IM) route of administration or ropivacaine and saline by ultrasound guided perineural (PN) injection. Assessment of pain included evaluation of mechanical allodynia by the von Frey method, pain-like spontaneous behaviour by the human approach test (HAT), and changes in locomotor function using the open field test. Intraepidermal nerve fibre (IENF) density was also measured using anti-PGP9.5 antibody staining technique.

**Results:** Surgical incision induced pain-like behaviours as determined by an increase in the animals' sensitivity to mechanical stimulation, which was reversed with morphine (IM) and ropivacaine (PN) in a dose-dependent manner. Changes in spontaneous behaviour and anaesthesia-induced locomotor impairment were noted. IENF density was 40% higher in the hind leg than in the flank ( $4.8 \pm 1.3$  vs.  $3.4 \pm 1.4$  nerve fibres/mm<sup>2</sup>, respectively;  $p < 0.05$ ).

**Conclusions:** These results suggest that the hind leg POP model in the Gottingen minipig is a reliable and sensitive model for studying incisional pain mechanisms and evaluating new analgesic therapies.

**Keywords:** Gottingen minipig; Post-Operative Pain; Translational Model; IENF; Perineural Injection

## Introduction

The use of animal models for the research of post-operative pain (POP) has been well described [1]. Most POP studies that have been performed to date used male rodents following plantar incision and focusing on the withdrawal response from a noxious thermal, chemical, or mechanical stimulus [2]. Although rodent models have contributed significantly to our understanding of surgical pain over the years and have the added advantage of being readily available for anatomic manipulation, there are some significant limitations, especially for assessing local therapies. For example, rodent skin structure [3], innervation [4-6] and penetration [7] is very different from human cutaneous tissue. Despite major efforts to evaluate spontaneous changes in rodents, [8] such as facial recognition and grimace scale, [9] dynamic weight bearing [10] and heat gradient place preference, [8,11] pain status is still predominantly evaluated using the withdrawal response method [12]. In contrast to the rodent, there are striking similarities between pig and human skin structure in terms of general structure, thickness, hair follicle content, pigmentation, collagen and lipid composition [13]. Indeed, pigs have been used for many years for the study of skin penetration with topical therapies because of the high prediction of permeability compared to humans [7]. The pig model for post-operative pain (POP) is therefore a rationale choice.

It has been suggested in our previously published studies, as well as in studies from other researchers, that the pig POP model allows for better translation to humans [14-18]. However, even porcine skin incision (SI) [19], skin muscle/incision retraction (SMIR) [14] and pararectal laparotomy (PL) [20] models have limitations. Due to their propensity for rapid weight gain and growth, only very young domestic pigs (approximately 6-8 weeks old) can be used in the POP models; [14] hence, studying chronic conditions using domestic pigs is extremely difficult if not impossible. In addition, standardisation of domestic pig models is challenging owing to strain variations [21]. An incisional POP model using the Göttingen minipig might overcome these latter limitations with domestic pigs. Göttingen minipigs have been inbred to gain weight slowly, which in contrast to domestic pigs, allows studies to be conducted using adult animals [22]. For this reason, Göttingen minipigs are considered the gold standard for pharmacology and toxicology studies [22-24], as well as for skin permeability [25] and pharmacokinetic studies [26]. This paper describes the characterisation of a Göttingen minipig model of post-operative incisional pain.

## Materials and Methods

### Animal housing and habituation

Female Göttingen minipigs aged 4 months and weighing  $8 \pm 1$  kg (Ellegaard, Denmark) was used in this study. Prior to study commencement (14 days) and for the duration of the study, all animals were housed in pens (1.4 x 2.4 m) and maintained on a 12 h:12 h light-dark cycle. Feeding occurred twice daily using special pig food (Dry Sows; Ct # 5420; Milobar, 7880, Oshrat, Israel). Fresh drinking water was provided ad libitum by an automated system. All procedures and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) (License number: IL-20-6-213), and were designed to reduce numbers and undue suffering in accordance with the International Association for the Study of Pain (IASP) recommendations.<sup>27</sup>In order to reduce the level of stress and discomfort for the animals, the following steps were taken:14 (i) three animals were housed per pen for companionship; (ii) all tests, except the open field test, were performed within the home pen to avoid any stress of moving the animal to a new environment; (iii) enrichment was added to all pens, including chewing materials, food hiding spots and opportunities to root; (iv) the animals were first acclimatised to the test procedures prior to the study; (v) the same animal technicians were involved throughout the entire study period; and (vi) during the habituation period, the same technician entered the animals' home pen twice daily and played with the pigs for 15 minutes each visit.

During habituation, the animals were acclimatised to all test procedures. For the von Frey test, a technician approached each animal and bent towards them but did not apply the von Frey filaments. The animals were acclimatised to the open field procedure by walking once-daily from their home pen towards the open field test arena without entering the arena. Part of the habituation process also included training the animals to accept the anaesthetic facemask. Once daily, a technician entered the home pen and gently picked up each animal and carried it to the surgery room. The technician held the animal and scratched its head and neck whilst a second technician placed the anaesthetic facemask over its mouth and nose without administering any anaesthesia. The facemask was left on the animal's face for 1 to 2 minutes and then removed before the animal was carried back to their home pen.

### Anaesthesia and Surgery

On the day of surgery, a trained technician carried each animal to the surgery room and placed the anaesthetic facemask

(Stephan Akzent Color, Gackenbach, Germany) on the pig's mouth and nose.<sup>14, 28</sup> Each animal was anaesthetised with a 3% isoflurane/100% oxygen mixture. Animals were prepared for surgery as follows: the pig was placed in the sternal position and the area of injection on the hind leg was shaved and washed with warm water and soap. The animal was then placed on its back and the area of incision was swabbed with antiseptic polydine solution (Polysept solution, Rekah Pharmaceutical Industry Ltd., Israel). To avoid movement during surgery the operated leg was secured to the table, and the non-operated areas were covered with sterile sheets. The temperature in the surgery room was maintained at  $19\pm 1$  °C throughout the procedure.

During surgery, anaesthesia and blood O<sub>2</sub> saturation was monitored (Spacelab Medical, USA). A full skin and fascia incision of 6-8 cm long (proximal to distal) on the dorsal area of the hind leg was cut and sutured with 3-0 silk thread (ASSUT, Europe) using continuous suturing methods (Figure 1). Following the incision, all pigs received the antibiotic marbofloxacin (10% w/v; Marbocyl<sup>®</sup>; Vétoquinol UK Ltd., Buckingham, UK) at a total dose of 0.5 mL per pig through intramuscular (IM) injection into the neck muscle. Animals assigned for perineural (PN) sciatic nerve injection were placed in the sternal position for dosing. Animals assigned for intramuscular (IM) dosing were returned to their home pens for recovery and observation.



**Figure 1:** Location of incision in the hind leg of Göttingen minipigs

### Ultrasound-guided perineural sciatic nerve dosing

While under anaesthetic, the shaved lower back of the animal was swabbed with 70% ethanol and injected with either saline or ropivacaine (Naropin) using a M9 premium hand carried ultrasound system (Mindray healthcare) and L14-6Ns (linear) probe. A 5 mm 22 g PANJUK needle with Facet Nano Line tip (PANJUK<sup>®</sup> GmbH, Germany) was inserted through the skin and the muscle approaching the PN area. After a short nerve stimulation was applied to ensure the correct location, the injection

was administered slowly to avoid mechanical injury to the sciatic nerve.

**Von Frey test:** The response of the animals to mechanical stimuli was assessed using the von Frey method. von Frey filaments (Ugo Basile, Italy) were applied on the surface of the hind leg skin, approximately 0.5 cm proximal to the incision line. Filaments were applied until the animal withdrew from the stimulus; each filament was applied 3 times. If withdrawal was not achieved, a thicker filament was applied up to a maximum force of 15 g. If withdrawal was achieved, a thinner filament was applied. Note: thicker or thinner filaments refer to higher/thicker or lower/thinner gram force. By alternating the filament thickness, the force required to achieve withdrawal reaction was determined and recorded.<sup>14</sup> A withdrawal reaction was considered the act of moving away from the stimulus, by shaking or lifting up the incised leg.

**Human Approach test:** Normal, spontaneous behaviour of pigs when someone enters their pen involves moving away from the person initially and then, after a while, approaching the person and performing exploratory behaviour. The more familiar pigs are with a human entering their pen, the more comfortable they feel and the less time it takes for them to approach the person. In this study, the same technician who entered the pen every day during the habituation period also conducted the human approach test (HAT). The delay in time (latency) for the animal to approach the technician once entering its home pen was measured in seconds with a cut off time of 120 seconds.

**Open field test:** The open field test in pigs has been previously described in detail.<sup>28</sup> Briefly, the test pen area was 2.5 x 4.8 m. The walls of the test pen were smooth and 1.6 m in height. Animals were introduced to the open field test arena for 5 minutes exposure time using a closed-circuit television (CCTV) camera and analysed using ANY-maze behavioural tracking software (ANYmaze; USA). For each animal, the total walking distance in metres (m) and percentage (%) of time spent next to the open field pen walls were recorded.

**Drugs:** Morphine (Teva, Cat number: 111-29122280/A; Israel) was administered by the intramuscular (IM) route (dose: 0.5, 1, 2 or 4 mg/kg) and ropivacaine 10 mg/ml solution (Naropin, BioAvenir Ltd., Israel) was administered by perineural (PN) injection (dose: 25 or 35 mg/pig) in this study. The doses of morphine or ropivacaine used in this study were chosen based on the active doses observed in a previously published peripheral neuritis trauma (PNT)-induced neuropathic pain model using domestic pigs.<sup>28</sup>

**Immunohistochemistry:** At the end of the study and immediately following euthanasia, 5 mm skin biopsies were collected from the dorsum area of the hind leg and the flank of non-operated areas. The biopsies were first immersed in Zamboni fixative for 24 hours at 4 °C, then washed 3 times in 0.08M Sorrenson's buffer for 15 minutes each wash, and finally kept in cryoprotectant until freeze-embedding in OCT (optimal cutting temperature) medium. Frozen sections (20 µm thick) were cut (perpendicular plane) and stained using anti-PGP9.5 primary antibody, followed by AF594-conjugated secondary antibody. Counting of intra epidermal nerve fibres (IENF) was performed for fibres crossing the dermal-epidermal junction as described previously [29,30].

**Study design:** Twenty-four animals were included in this study, with six animals assigned to each treatment group. Day 0 is denoted as the day of incision. Animals treated with saline or IM morphine were dosed on Day 1, i.e., 24 hours post-incision, and on Day 2 (Table 1). Animals treated with either saline or ropivacaine using ultrasound-guided perineural injection were dosed immediately post incision on the same day (Day 0).

In order to reduce the number of animals required, and since the animals showed low withdrawal thresholds for several days post-incision, a dose escalating approach was used for the morphine groups. On Day 1 post-incision, 12 animals were dosed with saline (n=6) or 0.5 mg/kg morphine (n=6), then 6 hours later, the same animals were re-dosed with either saline or 1 mg/kg morphine, respectively. An alternating approach was used on Day

2: animals that were previously dosed with morphine (0.5 mg/kg or 1 mg/kg) on Day 1 were dosed with saline and, vice versa, the animals that were dosed previously with saline were dosed with 2 mg/kg morphine, then 6 hours later, these animals were re-dosed with either saline or 4 mg/kg morphine, respectively (Table 1).

Another 12 animals were dosed with either saline (n=6) or ropivacaine (n=6) at a dose of 25 mg/kg immediately after incision (Day 0). On Day 1, the animals dosed previously with ropivacaine were dosed with saline and, vice versa, the animals that were dosed previously with saline were dosed with 35 mg/kg ropivacaine using ultrasound-guided perineural injection.

All tests, including IENF analyses, response to a mechanical stimulus, HAT and the open field test were performed blind, i.e., the technician(s) performing the tests were kept unaware of the animals' individual treatment.

**Statistical Methods:** All data is presented as mean ± standard deviation (SD). The choice of the number of animals per group in this study was based on previous knowledge gained working with domestic pigs in the area of pain.<sup>28</sup> Between-group comparisons were performed by using ANOVA followed by Tukey's multiple comparison test (using GraphPad Prism® software, GraphPad Software Inc., San Diego, CA, USA) assuming a normal distribution of data. Comparison of repeated measurements was performed using repeated measures ANOVA. A p-value of <0.05 was considered significant.

**Table 1:** Treatment groups and administration schedule

Route of administration	Dosing days	Dosing schedule	Treatment
IM	1 day post-incision (Day 1)	1 <sup>st</sup>	Saline (animals # 1-6)
			Morphine 0.5 mg/kg (animals # 7-12)
	2 days post-incision (Day 2)	2 <sup>nd</sup> *	Saline (animals # 1-6)
			Morphine 1 mg/kg (animals # 7-12)
	2 days post-incision (Day 2)	1 <sup>st</sup>	Saline (animals # 7-12)
			Morphine 2 mg/kg (animals # 1-6)
2 days post-incision (Day 2)	2 <sup>nd</sup> *	Saline (animals # 7-12)	
		Morphine 4 mg/kg (animals # 1-6)	
US-guided PN	Immediately post-incision (Day 0)	1 <sup>st</sup>	Saline (animals # 13-18)
			Ropivacaine 25 mg/animal (animals # 19-24)
	1 day post-incision (Day 1)	2 <sup>nd</sup>	Saline (animals # 19-24)
			Ropivacaine 35 mg/animal (animals # 13-18)

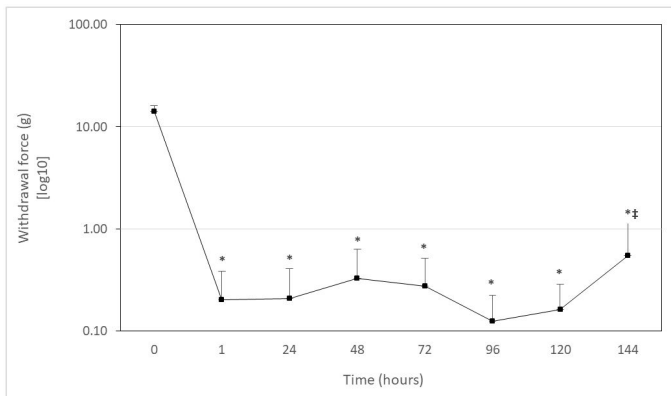
\*Note: Second IM dose was performed 6 hours post-1st dose

# = designated number of individual pigs.

IM: intramuscular; PN: perineural; US: ultrasound.

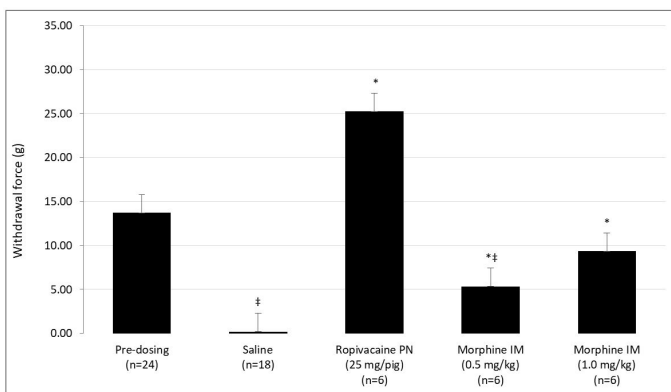
## Results

### Mechanical Allodynia



**Figure 2:** Effect of incision on mechanical nociception in saline-dosed animals. Results are reported as mean±SD. A low withdrawal force (g) [log10] was detected for ≥144 hours (6 days).

\* $p < 0.001$  vs. baseline, ‡ $p < 0.05$  vs. 1-hour post-incision. SD: standard deviation.



**Figure 3:** Effect of incision on mechanical nociception (pre-dosing and 1-hour post-dosing)

Results are shown as mean±SD 1hour post-dosing (Day 0 for saline and ropivacaine 1st dose PN, and Day 1 for saline and morphine 1<sup>st</sup> and 2<sup>nd</sup> doses IM).

\* $p < 0.001$  vs. saline.

‡ $p < 0.05$  vs. pre-dosing.

IM: intramuscular; PN: perineural; SD: standard deviation

Prior to incision and pre-dosing (Day -1), all animals in the saline-dosed group showed a baseline withdrawal force ranging from 10 to 15 g (Figure 2 and 3). One hour post-dosing, all saline dosed animals experienced increased sensitivity, expressed as low withdrawal force of the incised hind leg to a relatively thin von Frey filament (<1 g) (Figure 2 and Figure 3). Mean group withdrawal force 1 hour post-dosing was  $0.2 \pm 0.2$  g ( $p < 0.001$  vs. pre-dosing). On Day 1, 24 hours post-dosing, saline dosed animals continued to express increased withdrawal sensitivity to von Frey filaments. The low withdrawal threshold was still noticed in saline dosed animals 6 days post-dosing  $0.5 \pm 0.6$  g ( $p < 0.001$  vs. pre-dosing;  $p < 0.05$  vs. 1 hour post-dosing). However, the variability on Day 6 was higher with a significantly higher withdrawal threshold than observed 1 hour post-dosing (Figure 2). Notably,

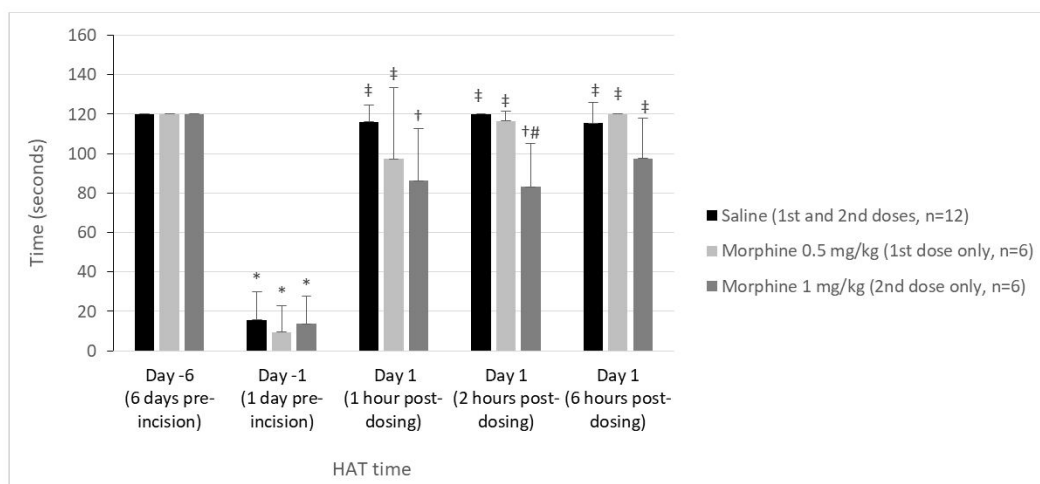
there was no difference in withdrawal threshold between animals receiving saline on Day 1 with those dosed with saline on Day 2, even though those animals had been dosed with morphine (0.5 mg/kg and 1 mg/kg) on Day 1.

After 1 hour post-dosing, animals treated with ropivacaine (PN) showed an increase in withdrawal force to  $25.3 \pm 7.9$  g ( $p < 0.001$  vs. saline), which surpassed the mean pre-dosing withdrawal force ( $13.7 \pm 2.1$  g) (Figure 3). The effect of ropivacaine (25 mg/pig) was no longer observed 24 hours post-dosing (mean withdrawal force  $0.5 \pm 0.3$  g). A higher dose of ropivacaine (35 mg/pig) was also administered post-incision on Day 1 but caused impaired locomotor function in the pigs; mechanical allodynia results are therefore not presented at this dose.

Systemic treatment with morphine (IM) at 0.5 mg/kg and 1 mg/kg doses on Day 1 post-incision resulted in an increase in withdrawal force in a dose-dependent manner ( $5.3 \pm 1.2$  g and  $9.3 \pm 1.2$  g, respectively;  $p < 0.001$  vs. saline) 1 hour post-dosing (Figure 3). von Frey testing in pigs administered with higher doses of morphine (2 mg/kg and 4 mg/kg; IM) was compromised by a change in the pigs' locomotor activity (see open field test results); hence the von Frey test results with 2 mg/kg and 4 mg/kg morphine are also not presented.

### HAT

During the acclimatisation period, HAT time decreased in all IM treated animals ( $n=12$ ) from 120 seconds (cut off HAT threshold) on Day -6 (pre-incision) to  $15.7 \pm 14.3$ ,  $9.4 \pm 13.5$  and  $13.6 \pm 14.3$  seconds on Day -1 (pre-incision) for saline-, morphine- 0.5 mg/kg and morphine- 1 mg/kg dosed animals, respectively ( $p < 0.001$  vs. pre-incision Day -6) (Figure 4). Following incision and 1hour post-dosing (Day 1; 1st and 2nd doses), HAT markedly increased in all IM groups for example in the saline treated group the HAT was  $116.2 \pm 8.6$  seconds;  $p < 0.001$  vs. pre-incision Day -1. Notably, IM dosing with 0.5 mg/kg morphine was not effective in reducing HAT 1, 2 or 6 hours post 1ST dose on Day 1. Animals dosed with 1 mg/kg morphine post-2nd dose on Day 1 showed a moderate decrease in HAT at 1 and 2 hours vs. pre-incision Day -1 ( $p < 0.05$ ). At 2 hours post-2nd dose on Day 1, animals dosed with 1 mg/kg morphine also showed a moderate decrease in HAT vs. saline-dosed animals ( $p < 0.05$ ) (Figure 4). HAT results from animals administered with higher doses of IM morphine (2 mg/kg and 4 mg/kg) on Day 2 are not shown due to impairment of locomotor function, as described in the open field test results section.



**Figure 4:** Results of the Human Approach Test (HAT)

Results are shown as mean±SD. HAT cut off during the 6-day acclimatisation period was 120 seconds. Incision was performed on Day 0.

\*p<0.001 vs. Day -6 (first day of acclimatisation, 6 days pre-incision).

#p<0.05 vs. saline-dosed animals.

†p<0.05 vs. Day -1 (pre-incision).

‡p<0.001 vs. Day -1 (pre-incision).

HAT: human approach test; SD: standard deviation.

For animals dosed with ropivacaine (25 mg/pig, PN) 1 hour post-incision (Day 0), the HAT also increased despite some of the animals experiencing changes in locomotor activity (see open field test results); data are not presented because they may not accurately reflect the effect of ropivacaine on HAT.

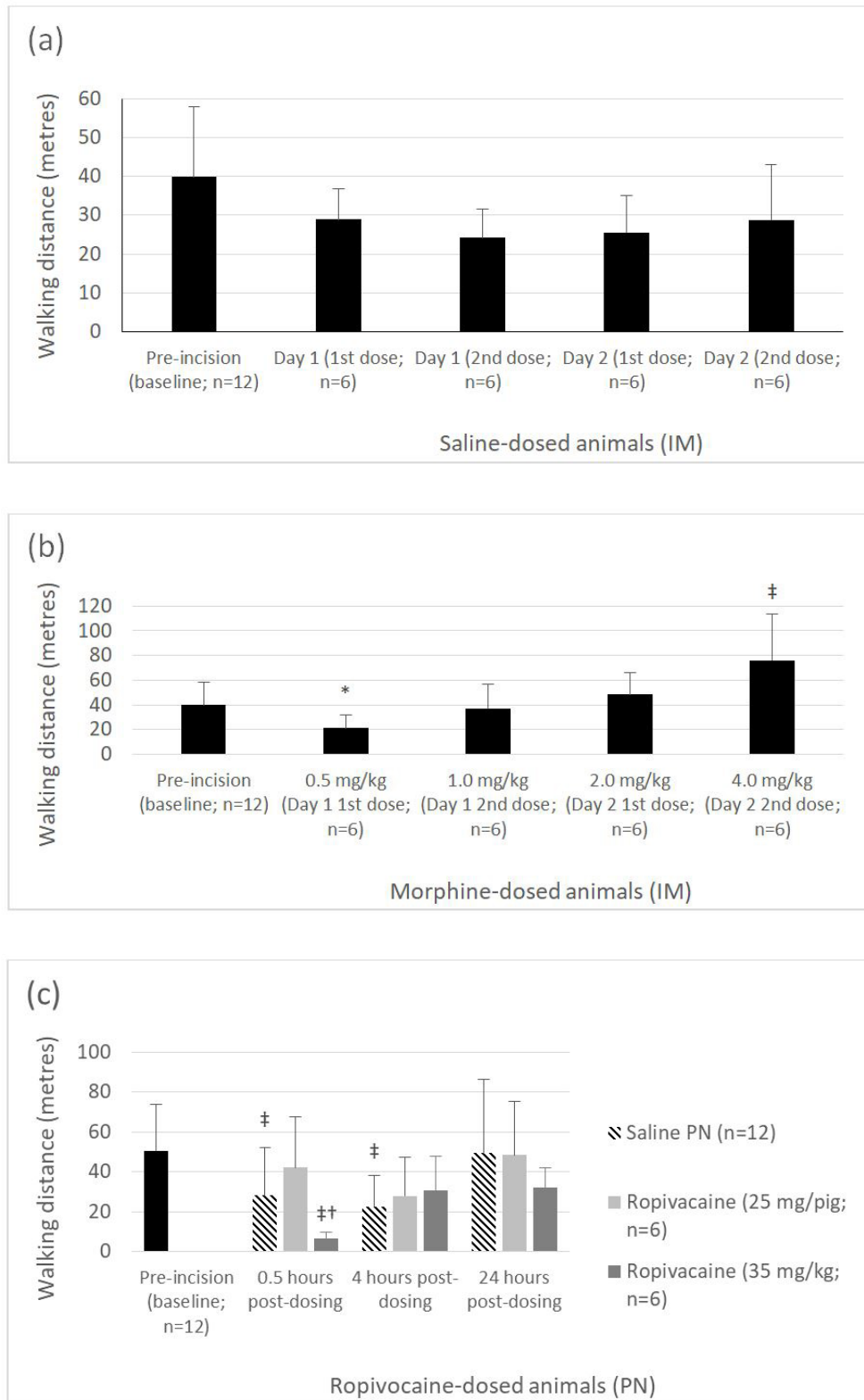
### Locomotor function (open field test)

Prior to incision, all animals in the IM group walked around all areas of the open field arena and showed no location preference. On Day 1 post-incision, there was no significant change in animal walking distance vs. pre-incision in the saline dosed (IM) animals (Figure 5a). Following an interval of 6 hours, repeated exposure to the open field arena on the same day (Day 1) did not affect the saline treated animals' behaviour (Figure 5a: Day 1 1st and 2nd dose vs. baseline). On Day 2, the animals that were dosed with morphine on Day 1 were dosed with saline, but there was no difference in the locomotor function of these animals vs. the saline dosed animals on Day 1, even though these animals had been exposed to morphine (0.5 and 1 mg/kg) on Day 1.

In animals treated with morphine, walking distance increased in a dose related manner (Figure 5b). A dose related increase in the animals' locomotor activity was also observed (Figure 5b). This was expressed as an increase in the total distance that the animals walked in a period of 5 minutes in the open field test arena. Animals treated with 0.5 mg/kg morphine (IM) walked 21.4±10.6 m, which was not statistically different than the walking distance

measured pre-incision (39.9±18.1 m). Animals treated with 1 mg/kg or 2 mg/kg walked 37.1±19.8 m and 48.5±17.8 m, respectively, which was also not statistically different to pre-incision baseline values. Animals treated with 4 mg/kg of morphine showed an increase in walking distance, 76.1±37.1 m (p<0.05 vs. pre-incision).

Following PN dosing with ropivacaine (35 mg/pig), the walking distance 30 minutes post-dosing transiently decreased vs. saline PN dosed animals (6.7±3.0 vs. 28.1±24.0 m, respectively; p<0.01) (Figure 5c). After 4 hours post-dosing, no significant difference between the saline PN dosed animals and ropivacaine PN dosed animals was observed. A slight and reversible decrease in the locomotor activity of the animals dosed with PN saline was observed after 30 minutes (Figure 5c). Animals in the saline PN group walked 50.5±23.4 m prior to incision and 28.1±24.0 m 30 minutes post-incision/dosing (p<0.01; Figure 5c). The walking distance decreased by 44% at 30 minutes and by 55% at 4 hours post-dosing with PN saline vs. baseline. The total walking distance measured at 24 hours post-dosing for saline dosed PN animals no longer differed from the distance measured pre-incision. Importantly, the decrease in walking distance 30 minutes post-dosing with PN saline was not significantly different from the decrease in walking distance measured following systemic saline (IM) (Figure 5a).



**Figure 5a-c:** Results of the Open Field Test

(a) Saline-dosed animals. Note: the saline-dosed animals on Day 2 were previously dosed with morphine (0.5 mg/kg and 1.0 mg/kg IM) on Day 1. (b) Morphine-dosed animals. Note: animals were dosed with 0.5 mg/kg and 1.0 mg/kg morphine (IM) on Day 1, and 2.0 mg/kg and 4.0 mg/kg morphine (IM) on Day 2 (1st and 2nd doses, respectively).

(c) Ropivacaine-dosed animals. Note: animals dosed with 25 mg/pig were dosed immediately after incision on Day 0 (1st dose) whereas the animals dosed with 35 mg/kg were dosed on Day 1 post-incision (2nd dose).

Results are shown as mean±SD.

\*p<0.05 vs. pre-incision.

‡p<0.01 vs. pre-incision.

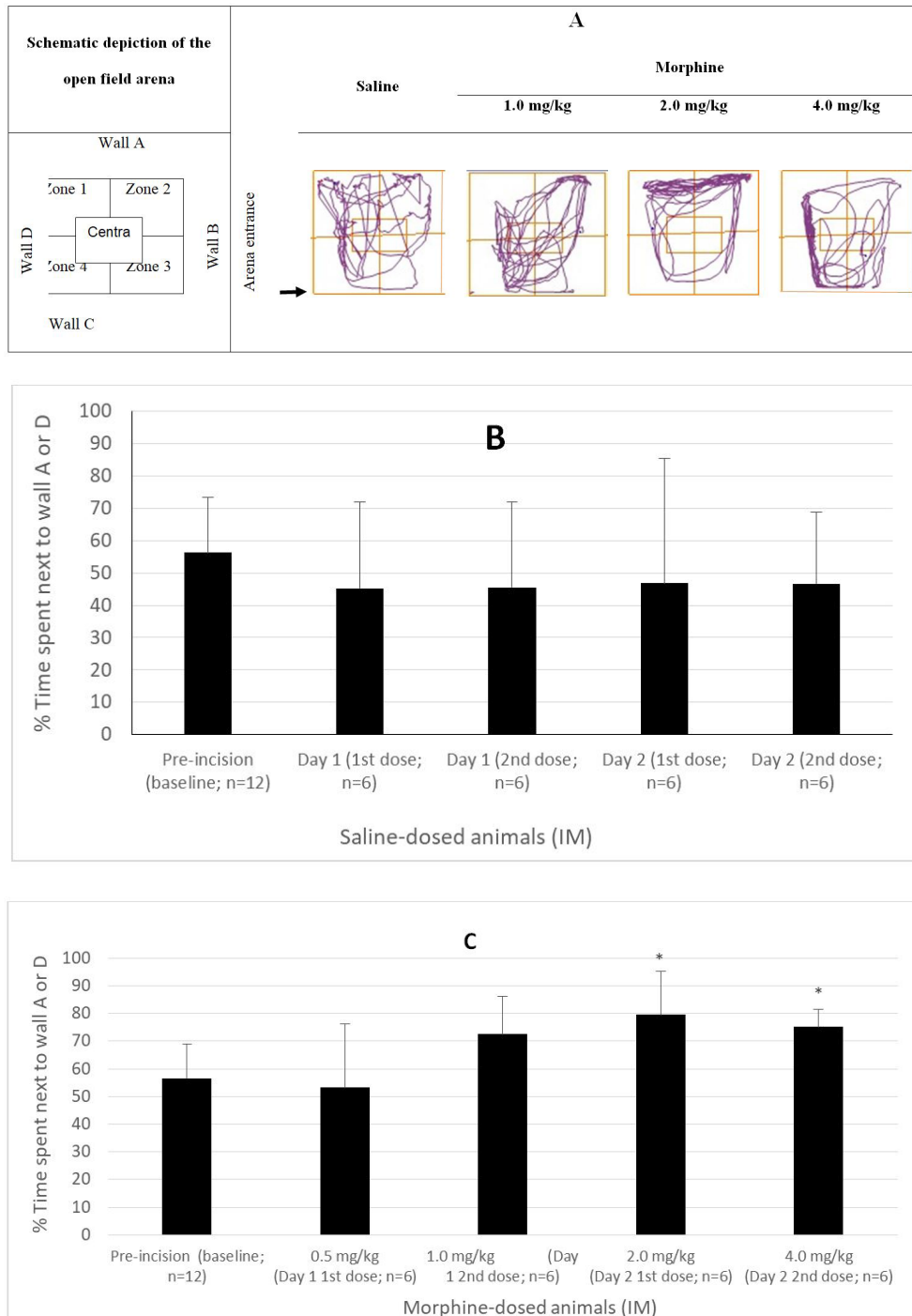
‡‡p<0.01 vs. saline-dosed animals.

IM: intramuscular; PN: perineural; SD: standard deviation.

### Walking Pattern

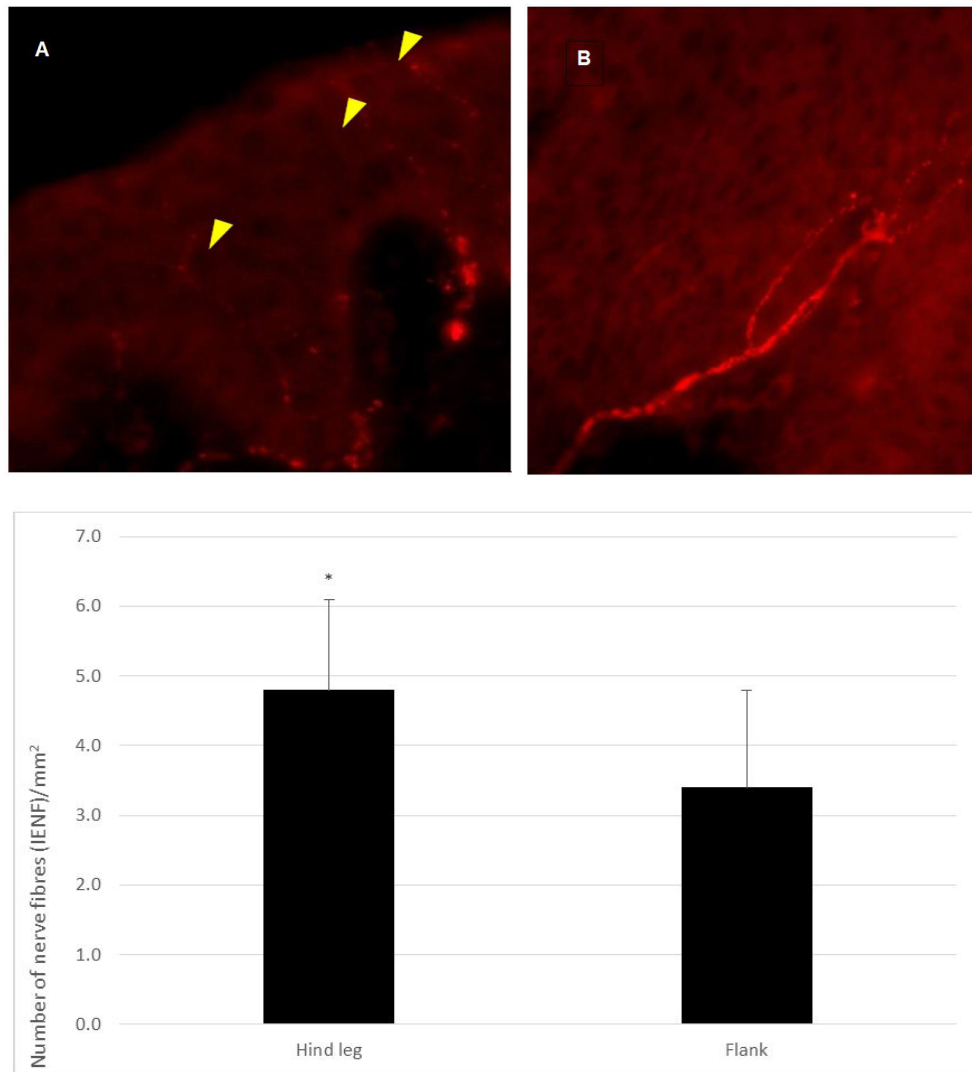
Figure 6a-c shows the post-incision walking patterns for saline and morphine dosed animals. Animals treated with saline or morphine (0.5 mg/kg and 1 mg/kg) on Day 1 walked the entire open field arena. The walking pattern for animals dosed with higher IM morphine doses (2 mg/kg and 4 mg/kg) on Day

2 (1st and 2nd dose, respectively) changed significantly vs. saline dosed animals, with animals showing repetitive behaviour such as walking back and forth next to one of the open field arena walls (A or D) (Figure 6a). These animals showed a clear preference to walk next to either wall A or wall D (Figure 6a and c). Notably, ropivacaine PN dosing did not affect the walking pattern and location preference of the animals (data not shown).



**Figure 6a-c:** Effect of systemic treatment with morphine on locomotor function (a) Walking pattern post-incision (0.5 hours post IM injection); (b) time spent next to wall A or D (saline dosed IM group); and (c) time spent next to wall A or D (morphine dosed IM group) Results are reported as mean±SD. \*p<0.05 vs. saline dosed animals. SD: standard deviation.





**Figure 7a-c:** Results of the intraepidermal nerve fibre (IENF) density analysis

(a) Representative image showing immunodetection of the PGP 9.5 antigen in flank, (b) representative image showing immunodetection of the PGP 9.5 antigen in hind leg, and (c) mean $\pm$ SD IENF recorded in hind leg vs. flank region. Yellow arrows indicate PGP9.5 positive intraepidermal nerve fibres.

\* $p < 0.05$  IENF in the hind leg vs. flank region.

IENF: intraepidermal nerve fibre.

Intraepidermal nerve fibre (IENF) density analysis: IENF are directly associated with functional innervations of the skin. To quantify the IENF density, we carried out an immunodetection of the PGP 9.5 antigen in cryosectioned skin samples. IENF density was higher in the unoperated hind leg than in the unoperated flank ( $4.8 \pm 1.3$  nerve ending/mm<sup>2</sup> vs.  $3.4 \pm 1.4$  nerve ending/mm<sup>2</sup>, respectively;  $p < 0.05$ ) (Figure 7a and b).

## Discussion

We describe the characterisation of a porcine model of incisional post-operative pain in adult Göttingen minipigs. Consistent with reports from previous studies in porcine<sup>14</sup> and rodent<sup>2, 31</sup> POP models, the minipigs used in this present study became more sensitive to mechanical stimulation post-incision, as demonstrated by the significantly lower withdrawal force

compared to pre-incision levels.<sup>32</sup> As expected, ropivacaine (25 mg/pig) injected perineurally acted as a sciatic nerve block and inhibited both motor and sensory functions, fully reversing the low withdrawal threshold observed post-incision. Changes in spontaneous behaviour of the study animals post-incision were monitored using the HAT and open field test. Furthermore, we show for the first time that IENF density is greater in the hind leg compared to the flank region of Gottingen minipigs.

## Addressing the limitations with domestic pig models

This Gottingen minipig model was developed to overcome some of the limitations using domestic pigs. Previously, we described skin incision (SI) and skin/muscle incision and retraction (SMIR) porcine models POP in Danish Landrace X Large White crossbred domestic pigs [14]. These models were suc-

cessfully used for developing local analgesic treatments, [17,33] as well as validating a new mechanistic approach to treat acute postsurgical nociceptive sensitisation [17]. Yet despite many advantages, porcine POP models using domestic pigs have several limitations. Firstly, there is a lack of inter-farm standardisation of domestic swine since each farm uses its own strain for breeding. Second, domestic pigs rapidly gain weight and require specialised husbandry and expensive research facilities, which can limit the models by using only pre-sexually mature young pigs. The current study was conducted in an internationally standard strain of pigs (Gottingen minipigs), using adult females. Although the dose escalating approach may cause a multiple treatment and testing effect, the advantage of reducing the number of animals used outweighs this possible disadvantage.

### Behavioural assessments

Three behavioural tests were applied in this study, including the von Frey test for sensitivity to mechanical stimuli, HAT for changes in spontaneous behaviour, and open field test for assessment of locomotor function.

The mean withdrawal force measured on Day 6 was higher than the mean withdrawal force measured 1 hour post-incision in saline dosed animals, which suggests a slight recovery or acclimatisation to the repeated von Frey test. Treatment with morphine (IM) at doses of 0.5 mg/kg and 1 mg/kg resulted in a dose-dependent effect during mechanical allodynia testing in the minipigs, as observed previously in human cutaneous pain studies.<sup>34</sup> One-hour post-dosing with 0.5 mg/kg and 1 mg/kg morphine resulted in a respective 42% and 70% reduction in the animals' sensitivity to mechanical stimuli. These findings support previously published data suggesting that doses lower than 0.5 mg/kg are not active in reversing the withdrawal response following incision in young domestic swine [14]. Typically, adult sows show active, non-exploratory behaviour and a low fear of humans.<sup>35</sup> Our results are consistent with other studies in sows that report how HAT can reflect the stress and comfort status of the animal.<sup>36</sup> Following incision, the time it took for the animals to approach the technician entering their home pen increased significantly during the first hour's post-incision. We also showed that treatment with morphine at a dose of 1 mg/kg was effective in partially reducing the post-incision approach time, suggesting a partial relief of stress and discomfort.

Open field testing is commonly used in animal studies to assess locomotor and behavioural changes following perineural injection, and can be correlated with locomotor function.<sup>28</sup> Analysis of response to higher doses of ropivacaine (35 mg/pig) was hindered by locomotor impairment, as observed in the open

field test. The decrease in total walking distance following saline administration is somewhat puzzling. Possible explanations for this finding include: (1) the pigs showed acclimatisation to the test and therefore were less curious so walked less; however, if this was the case then one would expect the pigs to walk less after 24 hours than after 6 hours post-dosing, which was not the case; (2) the animals' locomotor activity was affected by the anaesthetic (isoflurane) administered during surgery; this could explain the reduced walking distance in the saline dosed group 0.5 hours post-surgery but it does not explain the reduced total walking distance after 6 hours post-surgery; and (3) walking distance was reduced in saline dosed pigs due to elevated pain and discomfort during the first few hours post-incision vs. 24 hours post-incision.

There was no difference in the walking distance between ropivacaine (25 mg/pig) dosed animals and the saline dosed animals, which suggests that the corresponding results of the von Frey test were not affected by the animals' ability to move. It has been shown that treatment with ropivacaine injected into the wound area (infiltration) results in partial reversal of mechanical stimuli sensitivity in domestic pigs [16,37]. The differences in the effect of ropivacaine between the studies may be due to the different method of drug administration [16, 37-40]. However, strain differences (domestic pigs vs. minipigs) [41], sex [42], and age [43,44] may also affect the action of ropivacaine.

**IENF density differences:** IENF is widely used as diagnostic tool in human patients as well as in animal models of peripheral neuropathy [29,30]. In patients with sensory neuropathy, the IENF density is consistently and significantly lower than that in healthy controls. Mouraux, et al [45]. showed that the amount of nociceptive information produced by a stimulus in healthy volunteers is dependent on the number of activated IENFs as well as the size of the stimulated surface area and density of IENF.<sup>45</sup> It might be suggested that the higher IENF density found in the hind leg dorsolateral area vs. flank explains the fact that the minipigs used in this study had an increased pre-incision and post-dosing sensitivity to mechanical stimuli than reported in the flank region of domestic pigs used in our previous POP studies [14-16]. Previously, we reported a pre-incision von Frey withdrawal threshold of  $\geq 26$  g in domestic pigs [14-16]. In comparison, the maximum withdrawal threshold on the intact hind leg region was  $\geq 10$  g in our current study. This latter finding might also be related to age and sex IENF differences, since our previous studies used weaned male pigs whereas female adult minipigs were used in this current study. Indeed, Provitera, *et al.* (2016) reported that the age and gender of healthy patients was independent and linearly correlated with IENF density [46]. However, our assumption in minipigs needs to be further investigated.

In conclusion, this study validates the use of the Gottingen minipig as a suitable model for post-operative pain. It also demonstrates for the first time that changes in spontaneous pain-like behaviour of the minipigs can be monitored post-incision. Furthermore, we suggest that the higher IENF density found in the hind leg compared to the flank region of the minipigs may result in increased sensitivity to mechanical stimuli post-incision.

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