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Adjusting and validating a procedure for parenteral anaesthesia in neonatal mice

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TITLE: Adjusting and validating a procedure for parenteral anaesthesia in neonatal mice

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Abstract

For neonatal pups, parenteral anaesthesia is said to be not reliable as low doses induce no anaesthesia whereas high doses render high mortality rates. In this work we have adapted parenteral anaesthesia procedures approved for pups >7 days of age, to anaesthetize neonatal animals (postnatal days 3-4; P3-P4) for keeping them immobile for a long period. In our first experiment we analysed the behaviour of P3-P4 mouse pups for 70 minutes after i.p administration of low (37.5/3.75 mg/kg) or high doses (50/5) of a ketamine/xylazine anaesthetic mixture, both in the low range as compared to dosages employed in adults. Pups became immobile in ≈7 minutes and remained immobile for ≈45 minutes, irrespective of the age and dose of anaesthesia, younger pups (P3) being apparently more sensitive to the dosage. In the second experiment, we studied the response of P3 pups to mildly nociceptive stimulations, performed with a 4.0g von Frey filament applied to the dorsal aspect of their paws. These stimuli elicited reaction in 100% of the cases in non-anaesthetised pups. The results indicate that the high dose significantly reduced responses as compared to the low dose of anaesthesia. With the low dose, <40% of the pups were unresponsive to nociceptive stimulation, whereas the high dose resulted in 50-60% of the

animals not responding. Mortality was low irrespective of age or dose, suggesting that doses can be further increased if needed for invasive experimental procedures.

Introduction

Mice are macrosmatic animals, and most of their social behaviours are mediated by chemosignals. We are interested in understanding the role of pup-derived chemosignals in parental care, as it is known that neonatal, but not mature pups, emit volatiles (1) that may induce species-specific behaviours in adults, such as pup care or pup-directed attacks (2)(3). To study the role of pup chemosignals in infant-directed behaviours, while avoiding the effects of other sensory cues, some authors use dead pups, which effectively induce pup-directed aggression (4,5) but rarely maternal care. Moreover, these experiments do not avoid somatosensory stimulation (e.g. vibrissae-mediated touch).

To avoid these problems, we plan to analyse the response of adults to pup-emitted volatiles by exposing adult mice to pups enclosed in a stainless-steel strainer that precludes access to pup-derived somatosensory and visual stimuli. To avoid distress calls (auditory stimuli), pups must be anaesthetised. The main difficulty of these experiments is to find a reliable anaesthesia procedure for neonatal pups compatible with the experiment. Hypothermia and inhaled anaesthetics or a combination of both (isoflurane dipping followed by hypothermia; (6)) cannot be employed, thus enforcing us to use parenteral anaesthesia.

Unfortunately, in many institutions parenteral anaesthesia is approved only for pups over 7 days of age. This restriction (see (7)) is due to the reported high mortality rates of parenteral anaesthesia in neonatal rodents at high doses, and its low efficiency at lower doses (8). A second reason is maternal cannibalism following recovery (see (9,10)). However, in many of the papers reporting these results the conditions and specific procedures for administering anaesthesia are not clearly indicated, thus making it difficult to assess whether the procedures, rather than anaesthetic itself, are causing such adverse effects.

Consequently, here we aim at adapting the parenteral anaesthesia doses and procedures approved for pups >7 days of age to anaesthetize neonatal pups to keep them immobile for a long period with reduced mortality rates. We chose dissociative anaesthesia, a combination of ketamine and xylazine usually employed in adults at 65/4 to 100/10 mg/kg doses, depending on the strain, sex, age and procedure (7)(10–12). This is a combination of a non-competitive NMDA antagonist (ketamine), providing analgesia in the face of ischemic and somatic pain, with an α-2 adrenergic agonist (xylazine) producing chemical restraint, analgesia, sedation and muscle relaxation (9) while potentiating the analgesic effects of

ketamine. This combination seems also convenient because it has been used in pregnant mice (13), thus suggesting it being safe for foetuses and neonatal pups.

We hypothesise that this drug combination renders a reliable anaesthesia in neonatal mice if injected with high precision syringes and very thin needles. We assume that a 10:1 ketamine/xylazine (K/X) mixture administered at low doses (≤50/5 mg/kg) will result in a low mortality while providing long periods of immobility and reduced response to nociceptive stimulation.

Therefore, we have performed two experiments with two doses of K/X. In the first experiment we measured the latency and duration of immobility in otherwise undisturbed pups (postnatal days 3 and 4). In the second one, we evaluated the depth of anaesthesia by assessing the response of P3 pups to standardised nociceptive stimulation (pressing the standard von Frey filaments), and its time course after anaesthetic injection.

Methods

Animals

Mice were housed in the animal facility of the *Universitat Jaume I (Servei d'Experimentació Animal*) with *ad libitum* food and water, under a 12:12 day-night cycle with lights on at 8:00

am and controlled temperature (24 ± 2ºC) and humidity to ensure animal welfare. Animals were treated throughout following the EU Directive 2010/63/EU and, accordingly, experimental procedures were approved by the Committee of Ethics and Animal Experimentation of the *Universitat Jaume I*, and a license was issued by the *Direcció General de Producció Agraria i Ramaderia de la Generalitat Valenciana* (code 2020/VSC/PEA/0118 type 2).

For each experiment the sample size was prospectively calculated by power analysis using GPower software (University of Dusseldorf; (14). Calculations were required by the Ethical Committee of the *Universitat Jaume I*, which approved the procedure for using anaesthetised pups in our behavioural experiments. No criteria of exclusion were needed.

EXPERIMENT 1: Latency and duration of immobility under K/X anaesthesia in neonatal pups

Adult female mice (n=8) of the strain CD1 (RjOrl:SWISS, Janvier) and their progeny were used. Females were housed in pairs, and at 8 weeks of age (reproductively mature) they were stimulated with bedding that had been soiled for a week by an adult, sexually experienced male. The next day, females were introduced in the home cage of a stud male and left undisturbed for 48h, for mating to occur. All females became pregnant.

After delivery, their pups (n=93) were used for the anaesthesia experiment. The complete litters of four randomly selected females were anaesthetized at postnatal day 3 (P3), and pups from the remaining 4 females were anesthetised at postnatal day 4 (P4). Within each litter, about half of the pups were randomly assigned to each anaesthetic dose of ketamine+xylazine (low dose: 37.5 mg/kg+3.75 mg/kg; high dose: 50 mg/kg+5 mg/kg). In total, 47 pups received the anaesthesia at P3 (lower dose n=24; higher dose n=23), and 46 animals were anaesthetised at P4 (lower dose n=22; higher dose n=24).

Procedure

The day of the experiment, pups were weighed and received an i.p. anaesthetic injection using a Hamilton syringe of 50 µL with luer tip (705LT) and 30G needles (ref. 7748-16; Fig. 1A). The concentration of the anaesthetics was adjusted to inject 10µL of the solution per gram of pup weight. Immediately after anaesthetic administration, pups were placed in a cardboard box with many compartments that allow easily identifying each pup (Fig. 1B, C), on top of a rechargeable hand warmer (A ADDTOP; #FSP013) set at 40-42ºC, to ensure comfort and thermal stability of the pups (15). In these conditions, pups were video-recorded for 70 minutes, with time zero being the moment in which the pup was placed in its compartment.

Using the free, open-source software BORIS (15), an observer blind to the identity of the pups (age and dose received) measured the latency to absolute immobility, the latency to the first movement after the anaesthesia period, and the latency of complete awakening (appearance of periods with continuous movement >5 seconds, righting reflex).

For those pups not moving at the end of the experiment, we applied pressure to the paw and registered if they responded (yes/no). After the experiment, pups were returned to their nest with their mother. The next morning litters were revised, pups counted and the number of *exitus* was registered.

Behavioural variables were statistically analysed using IBM SPSS 22 Statistics Software, to compare the effects of the two doses of the anaesthetics on pups of the different ages. Since data did not follow a normal distribution (Shapiro-Wilk) we relayed on non-parametric statistics to compare the latency of pups to become immobile (Kruskal-Wallis test). Concerning the end of anaesthesia, since many pups had not moved or awakened at the end of the video (time 70 min), the latencies for those events were censored. Therefore, we compared them by means of a Kaplan Meier survival analysis.

To check if the doses employed are within the dynamic range of the drugs, we also analysed whether latency to immobility and duration of immobility were correlated, by means of Spearman's rank-order correlation test.

Finally, we analysed if *exitus* was due to the direct effect of anaesthesia and animals of both ages were similarly vulnerable, by comparing mortality between doses and ages by means of a Chi-square. Level of significance was $p<0.05$.

EXPERIMENT 2: Response of P3 pups under anaesthesia to standardized nociceptive stimulation

Females (n=6) and progeny were treated as in experiment 1. A total of 78 pups were used in this experiment at postnatal day 3, according to two different procedures.

a. Determination of the nociceptive threshold on P3 pups using von Frey filaments

A litter of 9 pups was used in a pilot study to determine the threshold of nociceptive stimulation following an adaptation of the SUDO method (16). Pups were gently restrained and von Frey filaments (Ugo Basile, code 37450-275) were pressed against the dorsal aspect of their paws until filaments bent. Each fibre was applied five times to the different paws in

a random order, and retraction of the paw and/or vocalisations in response to the stimulation were registered. This allowed assigning each animal a score of 0-5 reactions for each filament. Filaments were applied to each animal in order of increasing pressure (e.g. thickness) according to the sequence: 1.0 g, 1.4 g, 2.0 g and 4.0 g.

b. Level of anaesthesia in P3 pups: response to nociceptive stimulation after K/X injection

Since 4.0 g von Frey fibres rendered a 100% of responses in non-anaesthetised P3 pups, we used this fibre as a mild nociceptive stimulus to control the level of anaesthesia in P3 pups (complete litters of 5 females, n=69 pups) after administration of either low (37.5/3.75 mg/Kg; n=34) or high (50/5.0 mg/Kg; n=35) doses of K/X. After i.p. K/X injection, animals were videorecorded while stimulated four times with 4.0 g von Frey filament, once to each paw. An observer blind to the experimental condition of the animal (dose), recorded whether pups responded to nociceptive stimulation 20, 30, 40, 50 and 60 minutes after anaesthetic injection. Pups were considered to be responsive when they retracted the paw in response to 2 or more stimulations with the von Frey 4.0 g filament. This allowed studying the efficiency and dynamics of anaesthesia under the two doses of K/X.

Since data did not display a normal distribution (Shapiro-Wilk tests), nonparametric Mann-Whitney tests were used to compare the response of the animals having received the two doses, 20, 30, 40, 50 and 60 minutes after injection. We also compared responses at different times within each dose of anaesthesia by means of a Friedman test with multiple pairwise comparisons. Moreover, we compared the proportion of animals not responding to nociceptive stimulation (showing response to ≤1 stimulations) between doses, using a Chi-square test. Level of significance was $p<0.05$.

After experiment 2, anaesthetised pups were returned to their nest with their mother. Like in experiment 1, litters were revised the next morning and pups counted to register *exitus*.

Results

EXPERIMENT 1: Latency and duration of immobility under K/X anaesthesia in neonatal pups

a. Latency to become immobile after K/X administration in neonatal pups

Mann-Whitney U test for independent samples revealed no effect of age (P3 vs P4 pups; U=922; p=0.222) or dose (high vs low; U=1235; p=0.237), on the latency to immobility. Therefore, at the doses employed, K/X mixture (10:1) provides a quick anaesthesia with

immobility after less than 7 minutes (378.8±32.1 seconds; global mean ± SEM), irrespective of the age and tested dose of anaesthetic (Fig. 2A).

b. Latency to first movement and awakening after K/X administration in neonatal pups

In our experiment 31 pups did not move and 41 pups did not awake (continuous movement for >5 seconds) until the end of the movie and they were assigned a latency of 4200 seconds. Since data were censored, we evaluated the effects of the age and dose on the duration of anaesthesia using a Kaplan-Meier survival test with these two variables.

When pups of both ages were analysed together, there were no statistical differences between doses in the survival curves of the latency to move for the first time (X^2 (1, N=93)=0.281, p=0.596). Conversely, when doses are pooled, no difference between ages (X^2 (1, N=93)=0.281, p=0.596) were found. Similarly, when ages were analysed separately, effect of the dose on the latency to move was observed neither in P3 (X^2 (1, N=47)=2.022, p=0.155) nor in P4 pups (X^2 (1, N=46)=0.004, p=0.947).

Concerning the latency to awake, again when pooling animals of both ages no statistical difference between doses was found in the survival curves (X^2 (1, N=93)=1.958, p=0.162). Conversely, when doses are pooled, no differences between ages resulted

(X^2 (1, N=93)=0.375, p=0.541). However, when ages are analysed separately, a significant effect of the dose on the latency to awake was seen in P3 (X^2 (1, N=47)=4.137, p=0.042) but not in P4 pups (X^2 (1, N=46)=0.015, p=0.902) (Fig. 2B-C).

c. Correlation between latency to immobility and duration of anaesthesia

At the doses and pup ages tested, the duration of anaesthesia (latency to move after anaesthesia minus latency to become immobile) was of 2744±131s (mean±SEM), about 45 minutes (P3-low dose: 3039±217s; P3-high dose: 3483±201s; P4 low dose: 3387±169s; P4-high dose: 3432±163s). Spearman's analysis considering all the animals (both ages and doses) revealed a negative correlation between latency to immobility and duration of anaesthesia (ρ_{91}=-0.819, p<0.001). This is also true when groups (doses and ages) were analysed separately (Fig. 3).

d. Analysis of exitus *after anaesthesia*

In all experimental groups a few animals died, global mortality being 6.5% (6 out of 93 pups died). A Chi-Square comparing mortality indicated no statistical differences between doses (high dose: 3 fatalities out of 47 pups, 6.4%; low dose: 3/46, 6.5%; X^2_1=0.978, p=1) or between ages (P3, 3/47 pups, 6.4%; P4, 3/46, 6.5%; X^2_1=0.978, p=1). Finally, similar results

are obtained when comparing doses within ages (X^2_1=0.403, p=0.609 for P3 pups; X^2_1=0.457, p=0.600 for P4 pups).

EXPERIMENT 2: ***Response of P3 pups under anaesthesia to standardized nociceptive stimulation***

a. Determination of nociceptive stimulation threshold using Von Frey filaments

Nine non-anaesthetised P3 pups received stimulation with von Frey filaments. We started with thin filaments representing low pressure (1.0 g) on the paw, which elicited few reactions (<10% of the stimulations). Then we applied progressively higher pressure (1.4, 2.0 and 4.0g). A nonparametric related-samples Kendall's coefficient of concordance indicates a significant effect (W_3=0.864, p<0.001) of the pressure (thickness) of von Frey filaments, on the paw retraction proportion, retractions being more frequent when higher pressure was applied (8,88%, 26.7%, 49.9% and 100%, for 1.0g, 1.4g and 2.0g and 4.0g filaments, respectively). The 4.0 g filament rendered a systematic retraction (100%) of the paws in all 9 pups, and therefore is considered to constitute a reliable nociceptive stimulus for P3 pups.

b. Analysis of responsiveness to a standardised nociceptive stimulus

The reaction to paw stimulation with a 4.0 g von Frey filament was reduced in a large proportion of animals by K/X parenteral anaesthesia (Fig. 4). The results of Mann Whitney's test comparing the distribution of data between doses at different times indicates no differences between doses at 20 minutes (U=694, n1=35, n2=34; p=0.218), but significant differences between doses at 30 minutes (U=812, n1=35, n2=34; p=0.007), 40 minutes (U=785.5, n1=35, n2=34; p=0.016), 50 minutes (U=811, n1=35, n2=34; p=0.006) and 60 minutes (U=810, n1=35, n2=34; p=0.006) (Fig. 4A).

On the other hand, within each dose we performed a non-parametric comparison between times (related samples Friedman's test). The results indicate that the rate of response changes with time in both doses (high dose, X^2_4=11.769, p=0.019; low dose, X^2_4=18.647, p<0.001), although pairwise comparisons between times only rendered significant differences between 20 and 60 minutes in the lower dose of anaesthetic (p=0.045), thus indicating that responses to nociceptive stimulation after K/X anaesthesia, significantly increases with time (Fig. 3A).

Finally, we calculated the proportion of animals not responding to nociceptive stimulation for each time and dose (Fig. 3B), namely showing retraction of the paw in 1 or none of the 4 stimulations with the 4.0 g filament. More than 50% of the animals having received the

higher dose of anaesthetic were not responsive during the first 50 minutes after K/X administration. With the lower dose, during the first 30 minutes after K/X administration only a 32-45% of the animals did not respond to nociceptive stimulation. Using a Chi square test, we compared the proportion of animals not responding to nociceptive stimulation between doses and the results indicate that the percentage of non-responsive animals was significantly higher in the high K/X dose at 40 min (X^2_1=5.242, p=0.031), 50 min (X^2_1=5.534, p=0.027) and 60 min (X^2_1=6.640, p=0.016) after anaesthetic injection.

c. *Analysis of exitus* after anaesthesia procedure

In Experiment 2, only one pup died after anaesthesia, which belonged to the high dose group, representing a 0% *exitus* (0 out of 34) in the group receiving the low dose and a 2.9% (one in 35) in the group having received the higher dose. A Chi Square analysis rendered no significant differences in mortality between doses (X^2_1=0.986, p=0.321).

Discussion

The present work shows that i.p. administration of a 10:1 mixture of ketamine and xylazine (37.5+3.75mg/kg and 50+5mg/kg of K/X mixture), in similar proportion to its standard use in adult mice, reliably induces anaesthesia with a short latency to immobility and relatively

long duration, if pups are left undisturbed. Moreover, in P3 pups these doses of K/X mixture produce a dose-dependent reduction of the response to standardised mild nociceptive stimulation, namely pressure onto the paws with a 4.0 g von Frey filament.

A. Intraperitoneal injection of low doses of K/X reliably induces immobility in neonatal pups

In our experiments we have been cautious in using doses of the K/X mixture much lower than its standard use in adults (90–150 mg of ketamine with 7.5–16 mg of xylazine per kg of body weight; see (9)). Indeed, we used less than half this dose and first analysed if pups remained immobile for a time after anaesthetic injection, if left undisturbed. Statistical analysis indicates that both doses (high, 50/5 mg/kg; low, 37.5/3.75 mg/kg) render a similar short induction time (latency to become immobile) in neonatal pups (P3 and P4), less than 7 minutes in average (Fig. 2A). Also, the latency to start moving again and to awake (repeatedly exhibiting righting reflex) was similar for animals of both ages receiving both doses of the K/X mixture, rendering an average time of immobility of about 45 minutes.

Our experiment 1 also suggests that younger pups are somewhat more sensitive to anaesthetic dose. Thus, survival analysis (Kaplan Meyer test) of awakening from anaesthesia (latency to exhibit righting reflex after K/X injection), renders differences between doses in P3 pups but not in P4 ones (compare Fig. 2B-C).

These results are interesting by themselves, as they allow a use of K/X mixture for non-invasive experimental purposes in which only sedation is needed to achieve complete immobilisation of the pups for a time.

B. Low doses of K/X reduce the response of P3 pups to standardised nociceptive stimulation

Our experiment 2 was designed to check whether the doses of K/X mixture administered in experiment 1 were also reducing response to noxious stimulation, e.g. increasing nociceptive threshold. In this respect, it is a common practice during animal surgery to pinch the tail or paws of the animal with the fingers or tweezers to check if the depth of anaesthesia is appropriate for starting surgery, but this seems an unreliable procedure to check the level of anaesthesia, as pressure (and therefore activation of nociceptive receptors) may vary depending on the experimenter and/or instrument employed for pinching. Therefore, we decided to use a standardised procedure that reliably provides a specific pressure, e.g. von Frey filaments. In a pilot study, we adapted the SUDO procedure to determine the minimum pressure (thickness of the filament) inducing systematic response (paw retraction/vocalizations) in a set of unanaesthetised P3 pups (n=9). There was an increase of the proportion of animals responding from 1.0 g to 4.0 g of pressure, the

latter showing 100% responses (5 of 5 stimulations) in all 9 animals of our sample. This suggests that 4.0 g von Frey filaments constitute a standard noxious stimulus, eliciting mild nociceptive response in P3 pups. Then, this stimulus was systematically applied to the four paws (random order) of anaesthetised P3 pups (low dose n=34; high dose n=35) and the number of reactions (paw retraction and/or vocalisation) was registered. The results, summarised in Fig. 3A-B, indicate that pups showed an increase of the nociceptive threshold following anaesthesia administration. Globally, 20 minutes after K/X i.p. injection, pups responded to a 45% of the nociceptive stimulations with a high variability. This proportion significantly increased with time (Fig. 4A), thus suggesting a progressive recovery from anaesthesia along the tested period (20-60 minutes post-injection). As a conclusion, the doses of K/X mixture employed induced an increased nociceptive threshold (evaluated by means of analysis of response to stimulation). This effect on nociceptive sensitivity gradually fades away during the first hour after administration of the K/X mixture.

C. The doses of K/X mixture employed are within the dynamic range of anaesthesia

If the doses we are testing are within the dynamic range of anaesthesia, higher doses will have stronger effects on the pups. We have tested this hypothesis in both experiments. In experiment 1, in which immobility (probably reflecting sedation) is analysed, our results

show no statistically significant effect on the latency to become immobile, the latency to awake or the duration of the immobility period between doses. In P3, but not in P4 pups, the latency to awake is higher in the high as compared to the low dose of anaesthetic (compare Fig. 2B-C), indicating that younger pups (P3) are somewhat more sensitive to the dose of anaesthetic. This finding also supports the view that, at least in P3 pups, these doses are within the dynamic range relative to sedative effects of the anaesthetic.

In addition, the results of the correlation test confirm that those animals having a quicker anaesthetic-induced immobility are the ones showing a longer effect of anaesthesia (Fig. 3). This also suggests that the concentrations of the drugs are still in their dynamic range (17) in relation to their sedative effects.

Finally, our results of experiment 2 demonstrate a significant effect of the dose on nociceptive threshold, with higher and longer anaesthetic effect of the high dose, as compared to the low dose of K/X (Fig. 4A-B).

Taken together, the results of both experiments suggest that the two doses employed in this work are within the dynamic range of the anaesthetic. Therefore, it is likely that this anaesthetic mixture could be used in even higher doses if potentially harmful interventions are planned, provided that they do not cause a high mortality.

D. K/X injections resulted in low mortality, not dependent on the dose

Considering together both experiments, mortality was low (7 fatalities out of 162 pups; 4.32%), comparable to the figures for adult rodents and other small animals according to The Confidential Enquiry into Perioperative Small Animal Fatalities, CEPSAF ((18)(19)).

In experiment 1, mortality was similar in animals of both ages (P3 and P4) thus suggesting that younger pups are not more vulnerable to deleterious effects of the anaesthetic. In addition, in both experiment 1 and experiment 2, both doses of the anaesthetic resulted in similar mortality (experiment 1: low dose 6.5%, high dose 6.4%; experiment 2, low dose 0% and high dose 1.4%). This suggests that deaths are not caused by direct action of the anaesthetics, in which case the high dose would have shown higher mortality. We can only speculate that fatalities could be due to other factors, e.g. lesions caused by the needle during injection. This can be checked by performing necropsy (20). Due to its small size, new-born pups are difficult to immobilise, and some injections might have affected vital organs (e.g. diaphragm or liver). In this respect, the number of deaths was 6 out of 93 in experiment 1 (6.5% of mortality) and 1 out of 69 in experiment 2 (1.4%), a decrease in mortality that, even if it does not reach statistical significance, can reflect an improvement due to practice.

Using 10-day-old rats Tsukamoto et al. (2017) (21) reported a 100% mortality with a dose of 60/6 mg/kg of Ketamine/Xylazine, just 20% higher than our high dose (50/5). By contrast, the same authors report that a dose of 40/4 mg/kg of this mixture, similar to our low dose, rendered no anaesthesia at all (anaesthetic level zero; pedal withdrawal reflex and tail pinch reflex in all animals). This paper has been very influential and has led to the conclusion that injectable anaesthetics in neonatal mice are unpredictable, have high mortality risk, and should only be considered if gas anaesthesia is not feasible (7).

The findings by Tsukamoto et al. (21) contrast with the low mortality and the reduction in response to nociceptive stimulation of our experiments using the same anaesthetic mixture at similar dosages in neonatal mice. Since Tsukamoto et al. did not specify the kind of syringes and needles employed, we can only speculate that our using a small syringe with a high precision for small injections (±1 µL) and a very reduced dead volume, might have allowed an accurate measurement of the dose, thus resulting in a more reliable anaesthesia. The use of very thin needles (30 gauge) might also have reduced damage to critical organs, maybe further reducing mortality. A short, custom-made needle and puncturing the lower-left quadrant of the abdomen with an angle perpendicular to the skin

(thus avoiding affecting the liver or diaphragm) and some practice may result in virtually nil mortality.

In light of our results, we propose that, for certain experimental procedures, it may be advisable to reconsider the use of parenteral anaesthesia with ketamine/xylazine in neonatal offspring.

Declaration of conflict of interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

Original data of this study are available from the author for correspondence at request.

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Figure Legends

Figure 1. Material and procedure for anaesthesia

A: Hamilton syringe of 50 µL with luer tip (705LT) and 30G needles. **B/C**: Pups in the compartments of a cardboard box, placed on top of rechargeable hand warmers to ensure thermal stability throughout the procedure.

Figure 2. Immobility and recovery after ketamine/xylazine anaesthesia administration.

A: Bar histogram representing the time in seconds (mean±SEM) from administration of K/X anaesthesia until complete immobility, in P3 and P4 animals receiving low (37.5/3.75 mg/kg) and high (50/5 mg/kg) doses of the anaesthetic mixture in experiment 1. Individual values are also represented using coloured dots. **B/C**: Survival curves showing the proportion of individuals of experiment 1 that are awake at any given time point from injection until the end of the record (4200s). Low and high doses are represented in separate lines, using the same colour code as in A. Kaplan Meyer analysis of survival indicates a significant difference in the recovery from anaesthesia between doses in P3 animals (* indicates 0.01<p<0,05).

Figure 3. Correlation analysis between latencies to immobility and awakening after ketamine/xylazine anaesthesia.

Biplots of the latency to immobility after anaesthetic injection (ordinate) and the latency to awake after anaesthesia (abscissa), in P3 (**A**) and P4 pups (**B**) during experiment 1. Data on animals under the low dose of the K/X mixture (37.5/3.75 mg/kg) are plotted as orange dots, whereas blue dots represent animals having received the high dose (50/5 mg/kg). Regression lines illustrate the trend in the correlations, and the results of the Spearman's correlation analysis (correlation coefficient, ρ; associated p value) are indicated for each age and dose, using the same colour code as for the dots and lines.

Figure 4. Response to mild nociceptive stimulation in P3 pups under ketamine/xylazine parenteral anaesthesia.

A: Number of responses (mean±SEM) after 4 nociceptive stimulations shown by P3 pups at different points in time after administration of low (37.5/3.75mg/kg) and high (50/5 mg/kg) doses of K/X anaesthetic mixture. **B**: Proportion of unresponsive pups at different points in time after K/X injection. A pup is considered unresponsive if it responds to ≤1 of the 4 stimulations. In both graphs, Chi square analysis reveal statistically significant differences

between doses at different time points after injection, asterisks indicating the p values: * 0.01<p<0,05; ** p< 0.01.

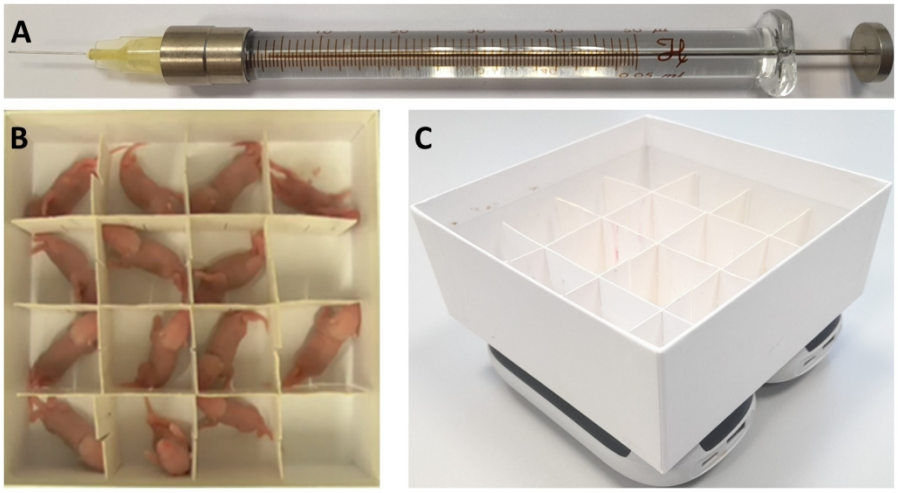

Figure 1. Material and procedure for anaesthesiaA: Hamilton syringe of 50 µL with luer tip (705LT) and 30G needles. **B/C**: Pups in the compartments of a cardboard box, placed on top of rechargeable hand warmers to ensure thermal stability throughout the procedure.

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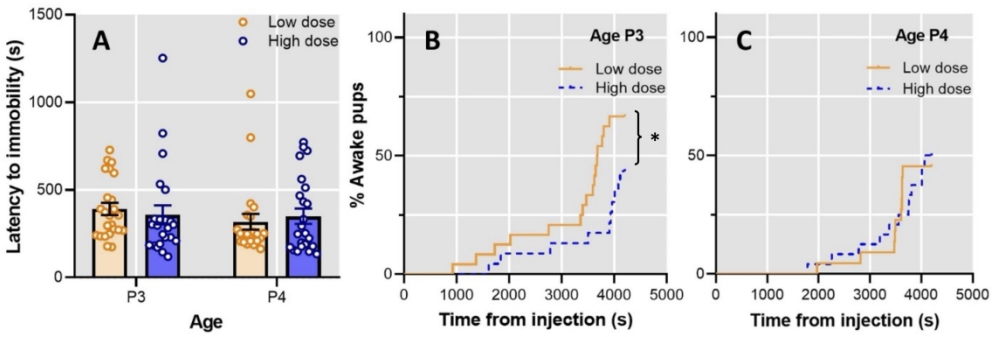

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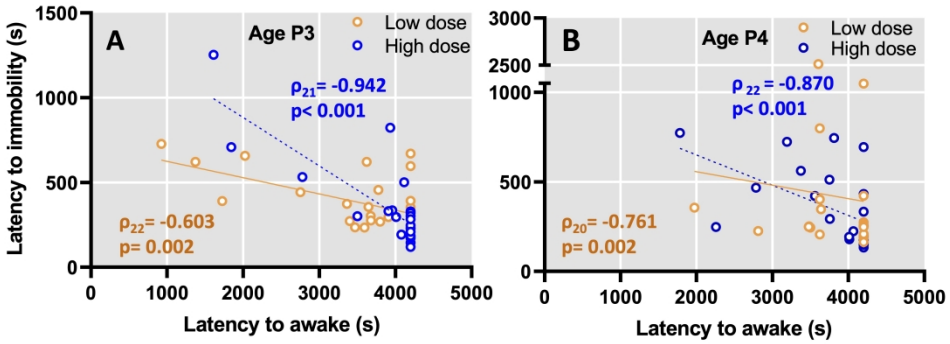

Figure 3. Correlation analysis between latencies to immobility and awakening after ketamine/xylazine anaesthesia. Biplots of the latency to immobility after anaesthetic injection (ordinate) and the latency to awake after anaesthesia (abscissa), in P3 (**A**) and P4 pups (**B**) during experiment 1. Data on animals under the low dose of the K/X mixture (37.5/3.75 mg/kg) are plotted as orange dots, whereas blue dots represent animals having received the high dose (50/5 mg/kg). Regression lines illustrate the trend in the correlations, and the results of the Spearman's correlation analysis (correlation coefficient, ρ; associated p value) are indicated for each age and dose, using the same colour code as for the dots and lines.

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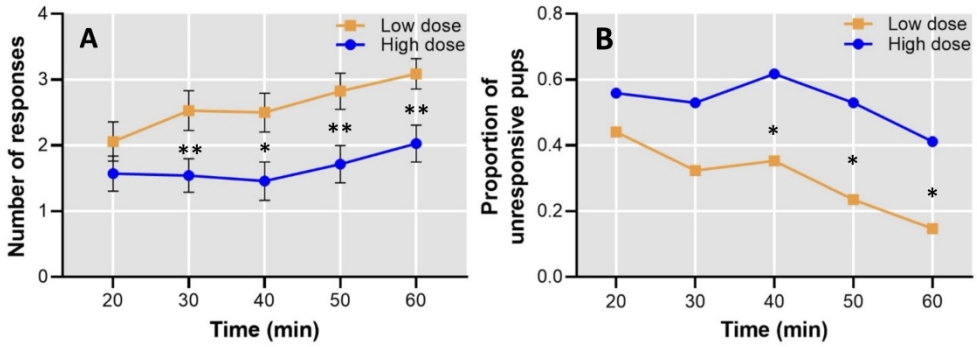

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1540x582mm (72 x 72 DPI)