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Injection anaesthesia with fentanyl-midazolam-medetomidine in adult female mice: importance of antagonization and perioperative care

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Abstract: Injection anaesthesia is commonly used in laboratory mice; however, a disadvantage is that post-anaesthesia recovery phases are long. Here, we investigated the potential for shortening the recovery phase after injection anaesthesia with fentanyl-midazolam-medetomidine by antagonization with naloxone-flumazenil-atipamezole. In order to monitor side-effects, the depth of anaesthesia, heart rate (HR), core body temperature (BT) and concentration of blood gases, as well as reflex responses, were assessed during a 50 min anaesthesia. Mice were allowed to recover from the anaesthesia in their home cages either with or without antagonization, while HR, core BT and spontaneous home cage behaviours were recorded for 24 h. Mice lost righting reflex at 330 ± 47 s after intraperitoneal injection of fentanyl-midazolam-medetomidine. During anaesthesia, HR averaged 225 ± 23 beats/min, respiratory rate and core BT reached steady state at 131 ± 15 breaths/min and $34.3 \pm 0.25^\circ\text{C}$, respectively. Positive pedal withdrawal reflex, movement triggered by tail pinch and by toe pinch, still occurred in 25%, 31.2% and 100% of animals, respectively. Arterial blood gas analysis revealed acidosis, hypoxia, hypercapnia and a marked increase in glucose concentration. After anaesthesia reversal by injection with naloxone-flumazenil-atipamezole, animals regained consciousness after 110 ± 18 s and swiftly returned to physiological baseline values, yet they displayed diminished levels of locomotion and disrupted circadian rhythm. Without antagonization, mice showed marked hypothermia ($22 \pm 1.9^\circ\text{C}$) and bradycardia (119 ± 69 beats/min) for several hours. Fentanyl-midazolam-medetomidine provided reliable anaesthesia in mice with reasonable intra-anaesthetic side-effects. Post-anaesthetic period and related adverse effects were both reduced substantially by antagonization with naloxone-flumazenil-atipamezole.

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Injection anaesthesia with fentanyl-midazolam-medetomidine in adult female mice: importance of antagonisation and peri-operative care

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Abstract

Injection anaesthesia is used commonly in laboratory mice; however, long-lasting post-anaesthesia recovery phases are disadvantageous. Here, we investigated the potential of shortening the recovery phase after injection anaesthesia with fentanyl-midazolam-medetomidine by antagonisation with naloxone-flumazenil-atipamezole.

To monitor the side-effects, depth of anaesthesia, heart rate (HR), core body temperature (BT) and concentration of blood gases, as well as reflex response were determined during a 50-min anaesthesia. Mice were allowed to recover from anaesthesia in their home cage either with or without antagonisation while HR, core BT and spontaneous home cage behaviours were recorded for 24 hours.

Mice lost righting reflex at 330 \pm 47 sec after i.p. injection of fentanyl-midazolam-medetomidine. During anaesthesia, HR averaged 225 \pm 23 beats/min, respiratory rate and core BT reached steady state at 131 \pm 15 breaths/min and 34.3 \pm 0.25 °C, respectively. Positive pedal withdrawal reflex, movement upon tail pinch and movement upon toe pinch, still occurred in 25%, 31.2% and 100% of animals, respectively. Arterial blood gas analysis revealed acidosis, hypoxia, hypercapnia and a marked increase in glucose concentration. After anaesthesia reversal by injection of naloxone-flumazenil-atipamezole, animals regained consciousness after 110 \pm 18 s and swiftly assumed physiological baseline values, yet they displayed diminished levels of locomotion and disrupted circadian rhythm. Without antagonisation, mice showed marked hypothermia (22 \pm 1.9 °C) and bradycardia (119 \pm 69 beats/min) for several hours.

Fentanyl-midazolam-medetomidine provided reliable anaesthesia in mice with reasonable intra-anaesthetic side-effects. Both post-anaesthetic period and related adverse effects were reduced substantially by antagonisation with naloxone-flumazenil-atipamezole.

Anaesthesia in laboratory mice is often induced by intraperitoneal injection of a mixture of two or three drugs, which usually belong to different substance classes and thus exhibit different mechanisms of action with possible synergistic or additive effects, e.g., ⁽¹⁻⁵⁾. Several substance combinations have been published over the years, with criteria for selecting and combining anaesthetic agents depending on the purpose for which anaesthesia is needed (e.g., surgery, imaging, terminal perfusion). In addition, the potential for drugs used for anaesthesia to interfere with experimental procedures and results can play a role when choosing an appropriate anaesthetic regime ^(4, 5). Additionally, problems of availability, particularly in drugs regulated under narcotic laws in many countries, can determine whether specific substances are applied or not ⁽¹⁻³⁾.

A well-known drawback of injection anaesthesia protocols for mice is the relatively long recovery phase, in which the animal is not in a deep stage of anaesthesia, but remains more or less immobilised while responding to stimuli. In this phase, hypothermia is the most prominent problem, but metabolic, respiratory and cardio-vascular functions are usually also impaired.

To circumvent these problems through abrogating anaesthesia, a mixture containing drugs that could be antagonised was suggested ⁽⁶⁾. A fully reversible three-component anaesthesia was proposed ⁽⁷⁾ that has been shown to achieve safe and reliable anaesthesia in chinchillas ⁽⁶⁾, ferrets ⁽⁸⁾, and rats ⁽⁹⁾. This anaesthesia protocol has since been used in mice: Thal and Plesnila ⁽⁴⁾ administered the anaesthetic mixture in the suggested species-specific dosages (fentanyl 0.05 mg/kg, midazolam 5 mg/kg, medetomidine 0.5 mg/kg) in female C57BL/6 mice. As the latter authors aimed to obtain long-term (approx. 3 hours) blood pressure and paCO₂ measurements under standardized conditions, animals were ventilated mechanically and injection of anaesthetics had to be repeated. Remarkably, at 1.5–2 hours after reversal of anaesthesia, a marked drop in body temperature (BT) to 32°C was found with punctual measurements using a rectal probe ⁽⁴⁾.

The protocol used by Thal and Plesnila ⁽⁴⁾ and Henke and colleagues ⁽⁷⁾ consisted of full reversal, i.e. all three components for inducing anaesthesia were antagonised at a predetermined time point.

Consequently the analgesic effect of fentanyl was equally completely abolished with naloxone, which could be a disadvantage if post-operative pain remains persistent after anaesthesia. With regards to post-procedural pain, it could be beneficial to omit naloxone and instead inject buprenorphine, which might antagonise some effects of fentanyl while providing pain alleviation for some hours. However, to

date the protocol of Henke and colleagues is often applied in experiments which do not induce long-term substantial pain, but require anaesthesia for immobilizing the animal for a certain time, e.g., for marking, probe sampling, intratracheal instillation, measurements of physiological values⁽⁹⁾, and the many kinds of imaging procedures (e.g., fMRI, PET). For such painless investigations, mice are often subjected to repeated anaesthesia for which a fully reversible anaesthesia protocol might be useful, as (side-) effects of fentanyl are antagonised and prompt recovery from anaesthesia could be provided at a predefined time point.

We aimed at using the fully reversible mixture as a protocol for routine laboratory settings (e.g., 30–50 min anaesthesia), where post-anaesthetic or post-operative care has to be considered. Therefore we analysed the potential of the protocol not only to provide reliable anaesthesia but also to ease termination of anaesthesia to promote smooth and fast recovery. Thus, antagonisation ought to mitigate against persistent hypothermia, reduced general condition, inactivity and lack of food and water intake as well as other spontaneous behaviours and signs of hampered recovery, impaired health and reduced wellbeing.

This study aimed to characterize intraperitoneal injection anaesthesia with fentanyl-midazolam-medetomidine in terms of safety, reliability and post-anaesthetic health and wellbeing. Since fast and smooth recovery after anaesthesia is desired in many experimental settings, we focused on the impact of reversal of anaesthesia by investigating the beneficial, as well as any possible adverse, effects of antagonisation by recording continuous telemetry and video data for a full 24-h period after anaesthesia.

Methods

Ethics statement

The animal housing and all procedures and protocols were approved by the Cantonal Veterinary Office (Zurich, Switzerland) under the license number 86/2011. Housing and experimental procedures were in accordance with Swiss animal protection law and also conform to Directive 2010/63 EU of the European Parliament and of the Council of 22 September 2010 on the *Protection of Vertebrate Animals*

used for Experimental and other Scientific Purposes and to the Guide for the Care and Use of Laboratory Animals⁽¹⁰⁾.

Animals and standard housing conditions

Thirty-two 4-week-old female C57BL/6J mice were obtained from a commercial supplier (Harlan, Horst, The Netherlands). Mice were housed in groups of two to four animals prior to and after the experiments. All mice were housed individually during the experiments as single housing is prerequisite for data acquisition and analyses (e.g., obtaining telemetric recordings, filming of behavior and automated analysis). The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations, except for *Helicobacter* species. The animals' health status was monitored by a sentinel program according to FELASA guidelines throughout the experiments⁽¹¹⁾.

Animals were kept in Eurostandard Type III open-top plastic cages (425 mm × 266 mm × 155 mm, floor area 820 cm²; Techniplast, Indulab, Gams, Switzerland) with autoclaved dust-free sawdust bedding (80–90 g per cage; LTE E-001 Abedd, Indulab). A standard cardboard house (Ketchum Manufacturing, Brockville, Canada) served as a shelter, except during the experiments. Animals were fed a pelleted and extruded mouse diet (Kliba No. 3431, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to sterilized drinking water. Autoclaved hay (8–12 g/cage) and 1 Nestlet™ per cage (about 5 × 5 cm, consisting of pressed cotton fibres; Indulab, Gams, Switzerland) were provided as nesting material.

The light/dark cycle in the animal room consisted of a 12-h/12-h cycle (lights on at 15:00, lights off at 03:00, according to experimental set-up and recording sequences) with artificial light of approximately 40 Lux in the cage. The mean room temperature was 21±1°C, with a relative humidity of 50±5% and 15 complete changes of filtered air per hour (HEPA H 14 filter); the air pressure was controlled at 50 Pa. The animal room was insulated to prevent electronic or other noise.

Experimental design

Treatment groups

Mice were assigned randomly to four groups of eight animals. All animals underwent anaesthesia lasting 50 min. Sixteen mice were implanted with telemetric transmitters prior to the experiments in order to obtain intra- and post-anaesthetic measurements of heart rate (HR) and core body temperature (BT), either with or without reversal of anaesthesia. Another 16 animals were used for behavioral

analysis (video-recordings) during the post-anaesthetic recovery period, either with or without reversal of anaesthesia. At 4 weeks after the initial anaesthesia experiment, eight of these animals underwent a terminal experiment to obtain arterial blood with which to determine intra-anaesthetic acid-base balance and blood gas concentration during a second, 50-min- duration anaesthesia.

Transmitter implantation

Prior to the experiments, at about age 8–9 weeks, 16 mice were instrumented with telemetric transmitters. TA10ETA-F20 transmitters (Data Sciences International, St. Paul, MN, USA)—which measure HR and core BT in freely moving mice—were implanted as described previously in detail ⁽¹²⁾. Briefly, under anaesthesia with sevoflurane (Sevorane[®], Abbott, Baar, Switzerland), the transmitter body was implanted in the abdomen under aseptic conditions. One wired loop electrode was fixed with silk sutures (Perma-Handseide 6–0, Ethicon, Norderstedt, Germany) between the muscles located to the right of the trachea, whereas the other wired loop lead was sutured to the xiphoid process. Muscle layers and skin were closed with resorbable sutures (VICRYL 6–0, Ethicon, Norderstedt, Germany). Together with pain relief (twice daily: buprenorphine, 0.1 mg/kg and meloxicam 5 mg/kg), supportive therapy, consisting of 300 µL glucose (5%) and 300 µL saline (0.9%) warmed to body temperature, was applied subcutaneously twice daily for 4 days ⁽¹²⁾. After transmitter implantation, mice had a period of 5–6 weeks convalescence before the first experiment.

Experimental setting

Data were acquired in the regular housing room from telemetric and behavioural recordings in undisturbed animals in their home cage during the 24 hours following anaesthesia. To avoid interference, all necessary husbandry and management procedures were completed in the animal room at least 1 day before starting an experiment or data recording, and disturbances (e.g. unrelated experimental procedures) were not allowed.

Prior to the experiment mice were placed individually in a new cage to habituate for 3 days. The cage was equipped with clean sawdust bedding and one Nestlet[™]. The cardboard house and hay were omitted to allow a better view of the animals during the experiments and for video recordings. Throughout the experiments, the mice lived in the same cage; this cage was not cleaned and the bedding was not changed during the experiments. All experiments were conducted when the mice were aged 16–32 weeks with body weights ranging from 25 to 30 g. To avoid any influence of circadian rhythm, anaesthetic procedures were carried out between 1:00 and 3:00 p.m. (=at the end of the dark phase).

Anaesthesia was performed in a separated experimental area within the animal room to avoid transportation and thus allow continuous telemetric data recording of animals in their familiar environment.

Anaesthesia experiments

Agents and dosages for anaesthesia and antagonisation as well as administration route were taken from the literature ⁽⁷⁾, and from a study employing the same strain, sex and approximate body weight range of mice as in our study ⁽⁴⁾.

After weighing the animals, an intraperitoneal injection of fentanyl (0.05 mg/kg; Fentanyl, Kantonsapotheke Zurich, CH), midazolam (5 mg/kg; Dormicum[®], Roche Pharma AG, Reinach, CH) and medetomidine (0.5 mg/kg; Dorbene[®], Graeub AG, Bern, CH) mixed in NaCl (0.9%) to a volume of 3 µl/g body weight was applied. Animals were then placed back in their home cage and observed for 7 min to allow time for the mice to calm down in a familiar environment and for the drugs to be absorbed. Time to immobility was established by observing the animals' spontaneous behaviour and posture. Righting reflex was tested every 10 to 15 sec after the animal became motionless by carefully turning the mouse on its back; any attempt of the animal to right itself from dorsal to sternal recumbence was judged as a positive reflex response. At 7 min after injection of anaesthetics, the mice were transferred to the experimental area of the animal room. Mice were placed in dorsal recumbence on a water-filled warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 38°C±1°C, and protective eye-ointment (Vitamin A Blache, Interdelta SA, Givisiez, CH) was applied. Anaesthesia was continued for 50 min while the mice breathed room air spontaneously.

For reflex testing during anaesthesia, noxious stimuli in the form of a tail pinch, and cervical as well as abdominal skin pinch were applied at 10 min intervals. Furthermore, the pedal withdrawal reflex (grasping the skin between the toes of one hind leg and pulling the leg away from the animal), toe pinch (forceps placed on the first phalangeal bone of one hind leg and slight pressure applied) and whisker movements (gently brushing whiskers with forceps) were tested at the same time intervals. All reflex tests were induced by the same investigator using blunt forceps with a spacer between its arms. The reflex tests were registered as positive or negative, i.e. whether any motor response or reactive movement was observable or not.

Respiratory rate (RR) was assessed by counting the movements of the thorax wall and recorded at 10-min intervals. During anaesthesia, mice were observed for abnormalities of respiratory rhythm (i.e.

irregularities, pauses etc.). In addition, they were monitored for alterations in heart rhythm by use of a real-time telemetry electrocardiogram read-out.

In half of the animals, anaesthesia was stopped after 50 min by intraperitoneal injection of naloxone (1.2 mg/kg; NaloxonOrPha, OrPha Swiss GmbH, Kusnacht, CH), flumazenil (0.5 mg/kg; Anexate®, Roche Pharma AG, Reinach, CH) and atipamezole (2.5 mg/kg; Alzane®, Graeub AG, Bern, CH) mixed in 0.5 ml NaCl (0.9%) to a volume of 9 µl/g body weight. In the remaining animals, 9 µl/g body weight of saline was injected, i.e., anaesthesia was not antagonised. All animals were returned immediately to their respective home-cage where either telemetric or video recordings were performed continuously for the next 24 hours. The time elapsed to regaining of righting reflex and spontaneous locomotion was determined from the video recordings.

Telemetric data acquisition and analysis

Telemetric data were recorded with the Dataquest LabPRO program (Data Sciences International, St. Paul, MN, USA). Data acquisition started 3 days before anaesthesia and continued for 24 h following the 50-min period of anaesthesia. To establish normal values (in the 3 days before anaesthesia) and to investigate the post-anaesthetic effects (during the 24 h following anaesthesia), HR and core BT were measured in the free-ranging mouse in its home cage every 5 min for 30 s and 10 s, respectively.

To determine the intra-anaesthetic effects of anaesthesia, bi-polar ECG and core BT were monitored continuously. ECG tracings were used both to monitor heart rhythm for alterations and to calculate HR (one value every 10 s). From these data, the mean values of HR and core BT were calculated for each minute for each individual.

Acid-base balance and blood gas concentration

Arterial blood was sampled at 50 min of anaesthesia to investigate the intra-anaesthetic side effects of anaesthesia on the partial pressure of oxygen (paO₂, mmHg) and carbon dioxide (paCO₂, mmHg) as well as on acid-base balance (pH), hematocrit and blood glucose concentration.

Blood sampling and analyses were carried out as described previously⁽¹³⁾. Briefly, following incision of the anterior neck, dissection of the right common carotid artery, and cutting a small hole in the artery using a fine-bladed pair of scissors, arterial blood was collected in a heparinized syringe. Acid-base balance, paCO₂, paO₂, hematocrit and concentration of blood glucose were determined immediately

using a blood gas analyzer (EPOC, Alere Inc, Ottawa ON, Canada). These animals died immediately from the subsequent rapid loss of blood under anaesthesia.

Video recordings and behavioural analysis

In order to establish baseline values as well as to analyse behaviour in mice with or without reversal of anaesthesia, animals were recorded digitally in the absence of a human observer, 24 h before and after the anaesthesia experiment, with infrared-sensitive cameras suspended 1.5 m above the cages. The recorded material (24 h of continuous footage) was subsequently analysed using EthovisionXT 7 software (Noldus, Wageningen, Netherlands) measuring the distance moved (in cm) of the animals' centre point.

In order to determine the temporal distribution of changes in general activity, the 24-hour period was divided into four consecutive 6-h periods according to the light-dark cycle in the animal room. Data were summed and analysed for the following time frames: 0000–0600 hours (light phase), 0600–1200 hours (light phase), 1200–1800 hours (dark phase) and 1800–2400 hours (dark phase).

Latency to drink, eat and nest building was also determined, using ObserverXT™ software (Noldus, Wageningen, Netherlands). These time points were defined as the first purposeful contact with water nipple, food-pellet or Nestlet™.

Statistical analysis

Data are presented as mean \pm standard deviation, except the post-anaesthetic telemetry recordings, which are presented as boxplots for every hour during the 24-hour observation period.

Statistical analyses were performed with SPSS 22 software (IBM, Armonk, NY, USA). All data were tested for normal distribution and homogeneity of variance (Shapiro–Wilks, Levene's test).

One way ANOVA was performed to compare group means of latency times and activity levels defined as distance moved. Post hoc analysis with Bonferroni tests was carried out to identify significant differences between groups; p-values ≤ 0.05 were considered significant.

Results

Anaesthesia period

Following intraperitoneal injection of anaesthetics, animals were motionless at 210 \pm 42 sec and lost their righting reflex at 330 \pm 47 sec.

Seven minutes after injection (i.e. at the beginning of the 50 min anaesthesia), reflex testing (Table 1) was able to provoke motor responses in 100% of animals for toe pinch, and in 31.2% and 25% of animals for tail pinch and pedal withdrawal reflex, respectively. During the course of anaesthesia, responses subsequently diminished; however, toe and tail pinch responsiveness was not excluded reliably at any time during anaesthesia.

During anaesthesia HR averaged 225 \pm 23 beats/minute and core BT reached steady state at 34.3 \pm 0.25°C (Figure 1). RR ranged from 96 to 180 breaths/minute with a mean of 131 \pm 15 breaths/minute in all animals. Analysis of arterial blood from 8 mice at 50 min of anaesthesia showed the following values: paO₂ 83.6 \pm 8.1 mmHg, paCO₂ 43.9 \pm 6.1 mmHg, pH 7.16 \pm 0.04, concentration of blood glucose 22.1 \pm 2.5 mmol/L, hematocrit 46.3 \pm 3.9 %.

Post-anaesthetic period

Upon completion of the 50 min anaesthesia period, animals received an injection of either antagonists or saline and were placed back into their respective home cages. After reversal of anaesthesia, HR and core BT returned to baseline values within 2 hours, whereas mice who received no reversal went through a 16–24h period of acute hypothermia and bradycardia, reaching average values of 22 \pm 1.9 °C and 119 \pm 69 beats/min, respectively (Figure 2A). After antagonisation, animals regained physiological posture after 1.9 \pm 0.3 min and locomotor activity returned after 4.3 \pm 0.5 min. However in mice without antagonisation, a further 726 \pm 36 min and 792 \pm 42 min passed before those animals were able to resume physiological posture and be ambulant (Figure 3); all animals survived.

Activity levels were measured (Figure 2B) and analysed during four consecutive 6-h periods (0000–0600 hours and 0600–1200 hours during light phase; 1200–1800 hours and 1800–2400 hours during dark phase) (Figure 4). In the first 6 h after anaesthesia, all mice displayed a significant reduction in locomotor activity (measured as distance moved) when compared with baseline values ($p= 0.014$ compared to animals with antagonisation and $p= 0.001$ compared to animals without antagonisation). During the next 6-h period (6–12 h after treatment, light period), the group without antagonisation displayed the lowest levels of activity, whereas the group that received antagonisation was even more active than the corresponding baseline, i.e. disruption of circadian locomotor activity rhythmicity occurred after

antagonisation ($p= 0.0001$ compared to animals with antagonisation and $p= 0.007$ compared to animals without antagonisation). During the first 6 h of the dark period (12–18 h after anaesthesia), the activity levels of both experimental groups were significantly lower than the corresponding baseline ($p = 0.0001$). This trend continued to the end of the dark phase (18–24 h after anaesthesia), with activity levels of experimental groups below baseline levels ($p= 0.015$ compared to animals with antagonisation and $p= 0.138$ compared to animals without antagonisation).

Latency to engage in spontaneous home cage behaviours such as eating, drinking and nest building were not altered significantly when anaesthesia was reversed (Figure 5). Animals that received no reversal of anaesthesia displayed significant latency prolongation in eating ($p= 0.0001$ and $p= 0.0001$ compared to baseline and animals with antagonisation, respectively, while there was no significant difference between baseline and animals with antagonisation $p = 0.51$). The same effect was seen in drinking ($p= 0.0001$ and $p= 0.0001$ compared to baseline and animals with antagonisation, respectively, while there was no significant difference between baseline and animals with antagonisation $p = 0.051$), and nest building ($p= 0.0001$ and $p= 0.0001$ compared to baseline and animals with antagonisation, respectively, while there was no significant difference between baseline and animals with antagonisation $p = 0.081$).

Discussion

Intraperitoneal injection of fentanyl-midazolam-medetomidine induced anaesthesia for up to 50 min in adult female C57BL/6 mice. Induction of anaesthesia was rapid, however a decrease in response to reflex testing was delayed in a few individuals as in the pedal withdrawal reflex (often considered to be a sign of deep anaesthesia). Whereas in most animals the pedal withdrawal reflex was negative at 10 minutes, it was still positive at 20 minutes in two out of 32 mice, which probably results from individual variability of pharmacodynamics and sensitivity to the agents⁽¹³⁾. In general, during anaesthesia no response could be provoked upon most of the reflexes tested, with the exception of pedal withdrawal reflex, toe pinch and tail pinch. The reason for the latter movements could be that anaesthesia was not deep enough due to low dosage. The text book dosages used⁽⁷⁾ were identical to those in another study using the same strain, sex and approximate body weight of mice⁽⁴⁾. Because both anecdotal evidence and clinical experience support the suggested dosages, no preliminary dose-testing was deemed necessary to adjust the commonly proposed dosages to the conditions of our laboratory setting (e.g.,

strain, sex, age, etc.) as generally recommended for injection anaesthesia in mice ⁽¹³⁾. Moreover, clinical experience with widespread use of this kind of anaesthesia (sometimes called “neuroleptanalgesia”) suggests that, due to the properties of the drugs, a specific reflex might not in fact be indicative of the depth of anaesthesia. However, no further focused investigations were carried out to ascertain the exact stage of anaesthesia reached in our study. Thus, given our findings, we conclude that, due to the inconsistent loss of reflexes and abolishment of response upon noxious stimulation, the dosages might need to be adapted, particularly if the protocol is used for invasive or painful interventions.

During anaesthesia, stable, albeit depressed, HR and RR were observed. Arterial blood gas analysis detected hypoxia, hypercapnia, acidosis and hyperglycemia. These characteristics should be considered when measurements for cardio-vascular studies or of glucose metabolism are performed under anaesthesia. In mice, such typical intra-anaesthetic alterations of physiological equilibria can hardly be prevented except hypoxia, which could be treated by providing additional oxygen in breathing air.

Intraperitoneal injection of the antagonists naloxone-flumazenil-atipamezole resulted in a clear reversal of anaesthesia within a few minutes. Animals regained all reflexes, turned in sternal recumbence and showed normal posture, movement and locomotion. Spontaneous home cage behaviours such as food and water consumption as well as nest building were not altered significantly after reversal of anaesthesia compared to unanaesthetized mice. While locomotion appeared within a few minutes of antagonisation, monitoring of locomotor activity with continuous 24-hour infrared-sensitive video recordings revealed an overall decrease in locomotor activity, i.e. a shorter distance was covered in the day following anaesthesia with antagonisation. In addition, the circadian rhythmicity of locomotor activity was clearly disturbed, as shown by the abnormal distribution of activity bouts in contrast to the regular distribution of activity bouts relative to day-time (i.e. high activity during light phase, low activity in the dark). The aberrant locomotion periods during day time hint at reduced sleep times and possible restlessness. After antagonisation, HR as well as core BT quickly regained normal values. A slight decline in core BT to values of 33–34°C was found at 1 hour after antagonisation. Such a decrease in BT after reversal of anaesthesia was also reported previously by Thal and Plesnila ⁽⁴⁾ from measurements with a rectal probe. With telemetric measurements we could confirm that this decrease in core BT occurred for less than an hour and thus was transient and probably of minor influence on animal wellbeing.

In contrast, if antagonisation was omitted, severe hypothermia and bradycardia occurred for about 16 hours or more after the 50-min period of anaesthesia. Locomotor activity was almost absent for

about 12 hours. Locomotor activity bouts appeared from 12 hours onwards but overall activity was reduced and did not reach normal levels even by 24 hours. In addition, the time until animals resumed spontaneous home cage behaviors such as food and water consumption as well as nest building was markedly prolonged to 15 and 20 hours, respectively. Such extension and aggravation of anaesthesia-related side-effects was probably in part due to the methodology used, i.e. the experimental set-up, which aimed to treat both groups identically with continuous sampling of telemetry and video recording data for 1 day. Therefore, animals were transferred back to their familiar home cage environment without further heat support, which was of negligible relevance if anaesthesia was reversed but was clearly disadvantageous for those that did not receive antagonisation. This underpins the well-known aspects and importance of peri-operative care in mice, particularly providing warmth during recovery from injection anaesthesia, which is applied routinely in laboratory work.

In summary, reversal of anaesthesia was advantageous and recommended as the recovery phase was shortened markedly by antagonisation and the adverse effects of anaesthesia were acceptable. In this study, all substances administered in the mixture for anaesthesia were antagonised hence the analgesic properties of these agents were completely abolished when anaesthesia was reversed. Since no invasive or painful intervention was conducted in this experiment, the antagonisation of analgesic effects was justified from animal welfare and methodological considerations. However, if surgery or otherwise persistent pain-inducing interventions are performed under anaesthesia, post-operative pain must be considered. Consequently, we suggest that either (i) the opioid fentanyl is not antagonized with naloxone, or (ii) buprenorphine is administered at antagonisation to provide post-operative pain alleviation for several hours⁽¹⁴⁾. Since the presented protocol (fentanyl-midazolam-medetomidine, antagonised with naloxone-flumazenil-atipamezole) comprises reversal of all anaesthetic and analgesic components, it might be useful in situations where no post-anaesthetic pain is present and a rapid recovery is aimed for. Therefore, for minor interventions, less invasive measurements, and investigations needing immobilization as imaging procedures this protocol might be preferable to other injection anaesthesia protocols (e.g., well-known ketamine-based mixtures with alpha-two-agonists).

Conclusion

Intraperitoneal injection of fentanyl-midazolam-medetomidine provides reliable anaesthesia for up to 50 min in laboratory mice, with stable although depressed cardio-vascular parameters together with slight hypoxia, hypercapnia, acidosis and hyperglycemia. Dosages of all components of anaesthetic

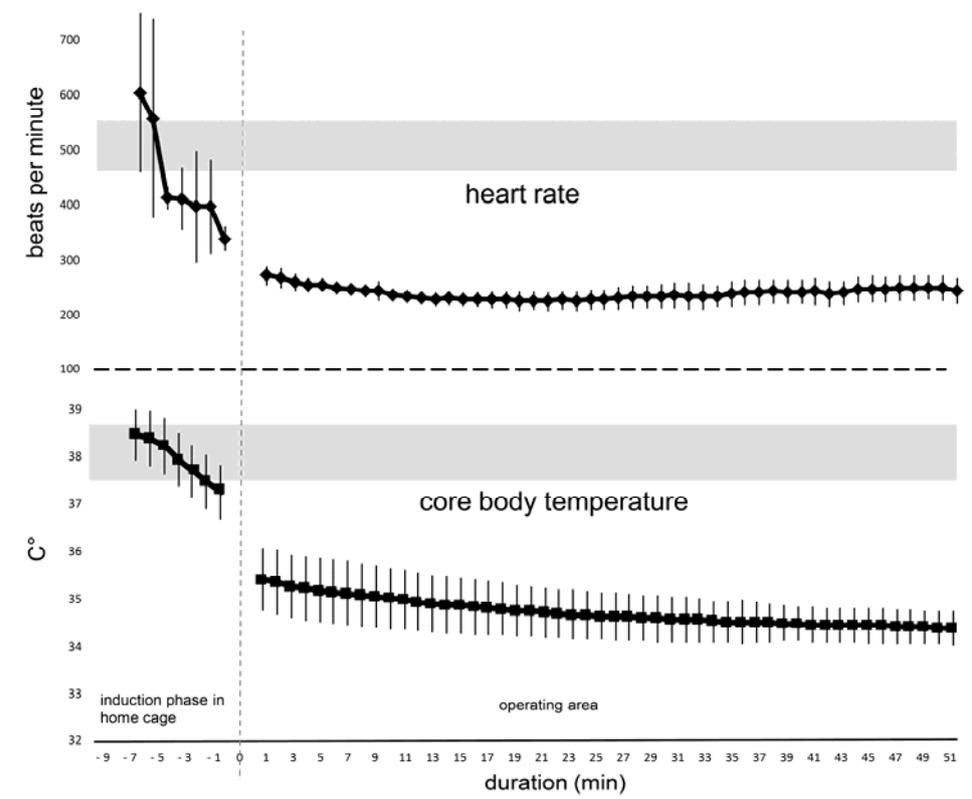


Figure 1: Heart rate (HR) and core body temperature (BT) during induction and anaesthesia. Mean \pm SD ($n = 16$ mice) for HR and core BT after injection of fentanyl-midazolam-medetomidine in the home cage and during 50-min anaesthesia while mice breathed spontaneously and lay in dorsal recumbency on the warming mat (38°C). Grey areas indicate mean baseline values (measured for 3 days before anaesthesia) at the corresponding time of day in conscious mice.

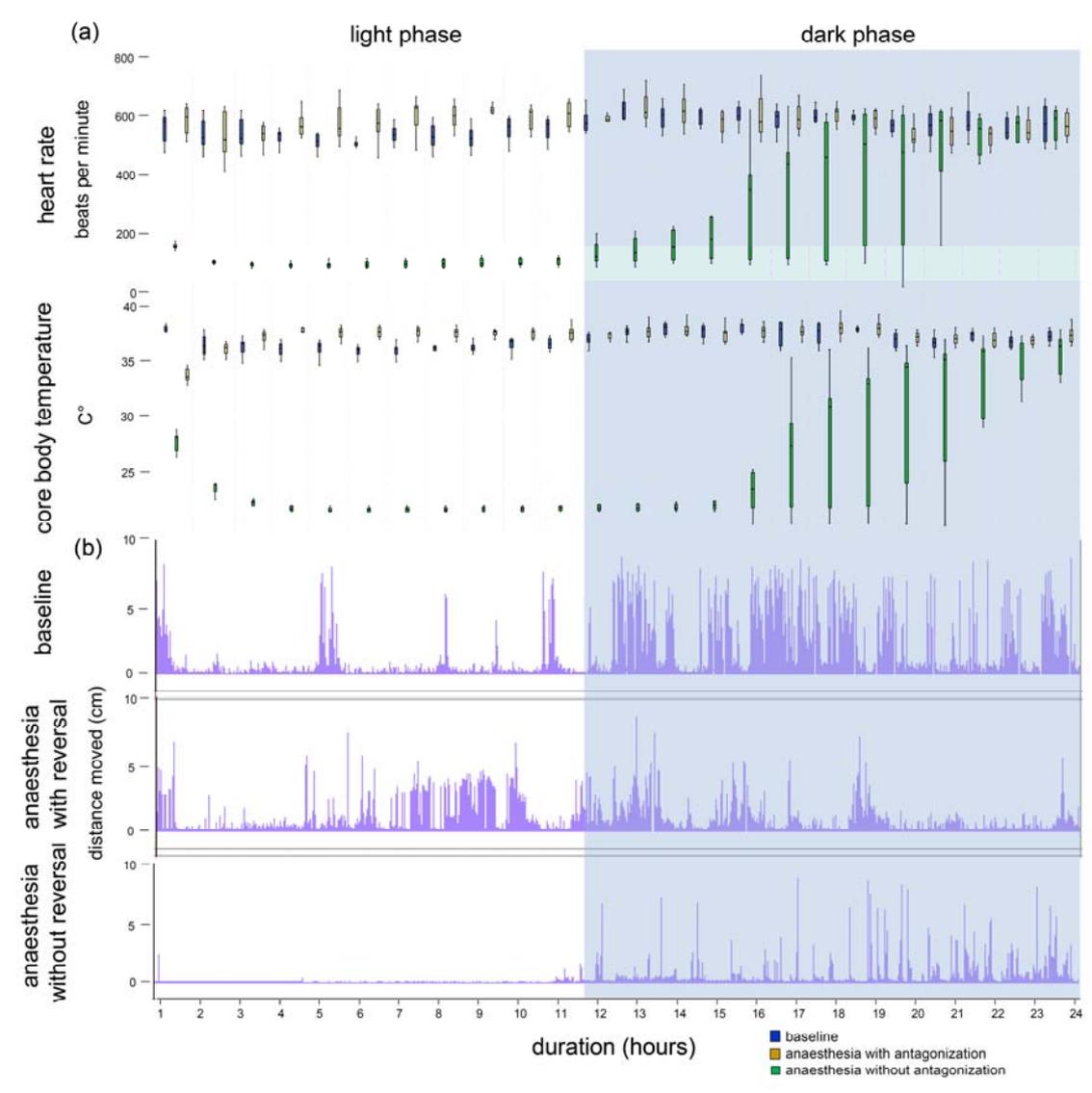


Figure 2: Post-anaesthetic period (24 h). (A) Telemetric recordings of HR and core BT for 24 h during baseline and after anaesthesia with and without reversal are presented as box-plots for each hour ($n = 8$ mice). (B) Activity shown as distance moved during 24h. A single representative data-set of locomotor activity levels (represented as distance of animals' centre point moved in cm) during baseline as well as after anaesthesia with or without reversal as analyzed by Ethovision® software is presented.

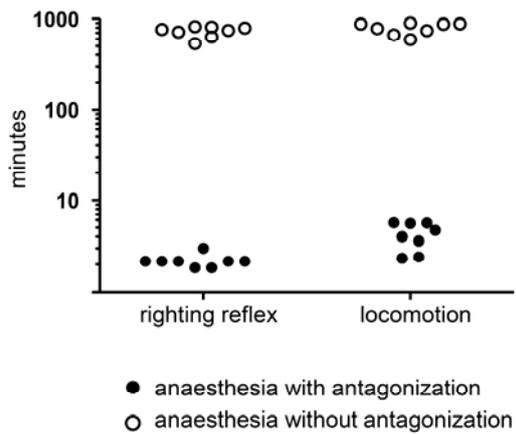


Figure 3: Duration until recovery of physiological posture (righting reflex) and spontaneous locomotion. Time elapsed after anaesthesia is depicted in minutes per individual (n = 8 mice per group).

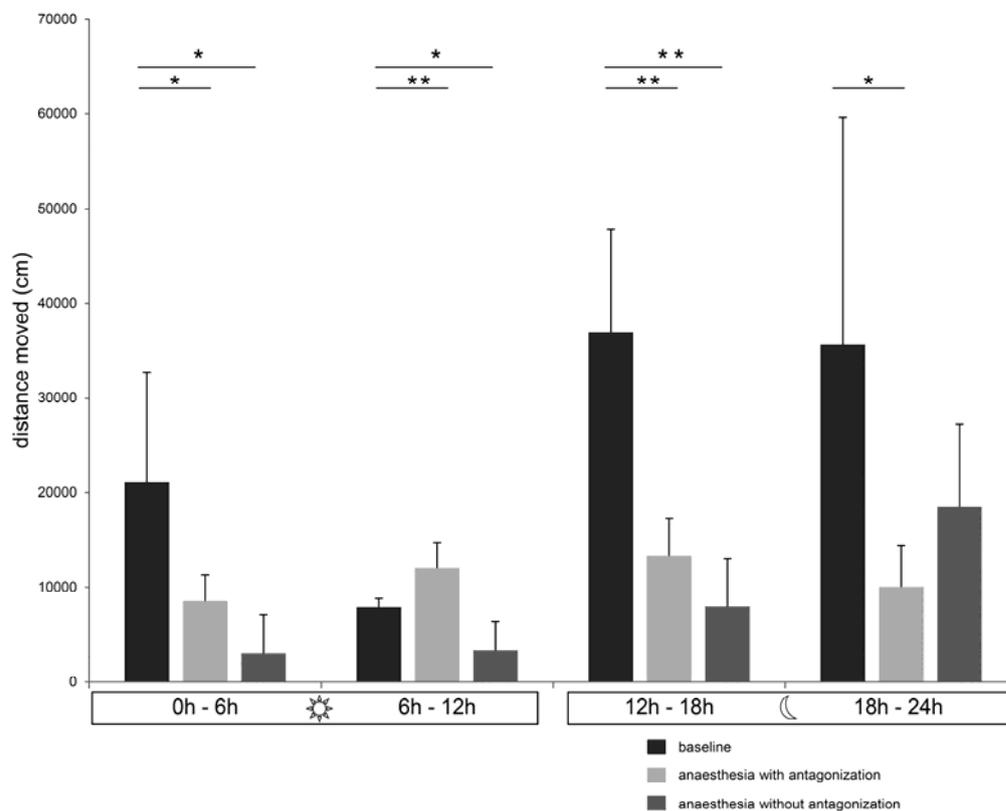


Figure 4: Home cage activity represented as distance moved in cm for 0-6h, 6-12h, 12-18h and 18 - 24h. Mean \pm SD (n = 8 mice). Activity was in general decreased on the first day after anaesthesia, except at 6-12 h after antagonisation where significant hyperactivity occurred. P-values are shown for the comparison with baseline; * $p \leq 0.05$; ** $p = 0.0001$.

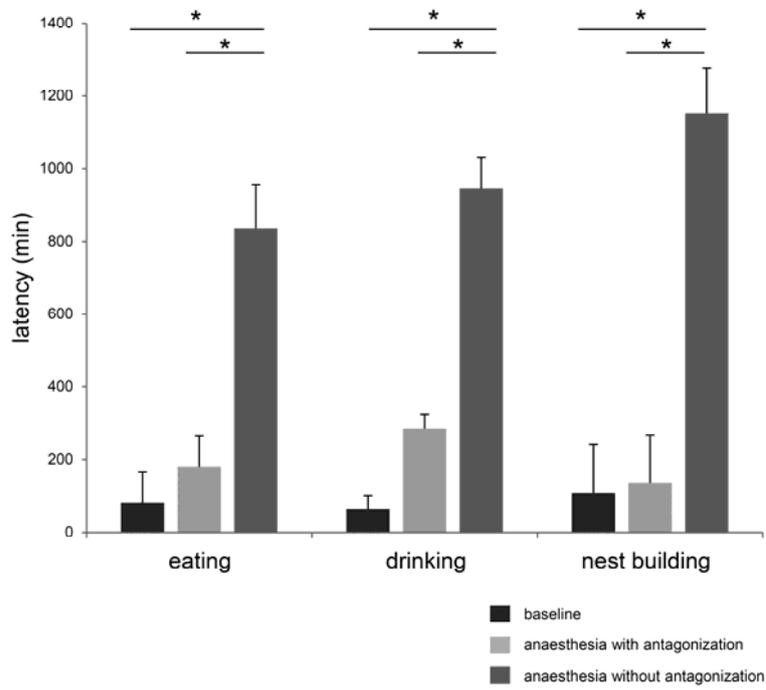


Figure 5: Latency to eat, drink and building nests during the post-anaesthetic period. Mean \pm SD for baseline and time elapsed after the 50-min anaesthesia period until animals started eating, drinking and building nests. ($n=8$ mice); * $p \leq 0.05$.

References

1. Kawai S, Takagi Y, Kaneko S, Kurosawa T. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp Anim.* 2011;60(5):481-7.
2. Kirihara Y, Takechi M, Kurosaki K, Kobayashi Y, Kurosawa T. Anesthetic effects of a mixture of medetomidine, midazolam and butorphanol in two strains of mice. *Exp Anim.* 2013;62(3):173-80.
3. Kirihara Y, Takechi M, Kurosaki K, Kobayashi Y, Saito Y, Takeuchi T. Anesthetic effects of a three-drugs mixture--comparison of administrative routes and antagonistic effects of atipamezole in mice. *Exp Anim.* 2015;64(1):39-47.
4. Thal SC, Plesnila N. Non-invasive intraoperative monitoring of blood pressure and arterial pCO₂ during surgical anesthesia in mice. *J Neurosci Methods.* 2007;159(2):261-7.
5. Zuurbier CJ, Koeman A, Houten SM, Hollmann MW, Florijn WJ. Optimizing anesthetic regimen for surgery in mice through minimization of hemodynamic, metabolic, and inflammatory perturbations. *Exp Biol Med (Maywood).* 2014;239(6):737-46.
6. Henke J, Baumgartner C, Roltgen I, Eberspacher E, Erhardt W. Anaesthesia with midazolam/medetomidine/fentanyl in chinchillas (*Chinchilla lanigera*) compared to anaesthesia with xylazine/ketamine and medetomidine/ketamine. *J Vet Med A Physiol Pathol Clin Med.* 2004;51(5):259-64.
7. Henke J, Erhardt W. Aästhesiemethoden bei der Maus. In: Erhardt W, Henke J, Haberstroh J, Baumgartner C, Tacke S, editors. *Anästhesie und Analgesie.* 2. Stuttgart: Schattauer; 2012.
8. Schernthaner A, Lendl CE, Hartmann K, Pragst I, Preissel AK, Henke J. Medetomidine/midazolam/ketamine anaesthesia in ferrets: effects on cardiorespiratory parameters and evaluation of plasma drug concentrations. *Vet Anaesth Analg.* 2011;38(5):439-50.
9. Albrecht M, Henke J, Tacke S, Markert M, Guth B. Effects of isoflurane, ketamine-xylazine and a combination of medetomidine, midazolam and fentanyl on physiological variables continuously measured by telemetry in Wistar rats. *BMC Vet Res.* 2014;10:198.
10. Worlein JM, Baker K, Bloomsmith M, Coleman K, Koban TL. The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (2011); Implications for Behavioral Management. *American Journal of Primatology.* 2011;73:98-.
11. Mahler Convenor M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, et al. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim.* 2014.
12. Cesarovic N, Jirkof P, Rettich A, Arras M. Implantation of radiotelemetry transmitters yielding data on ECG, heart rate, core body temperature and activity in free-moving laboratory mice. *J Vis Exp.* 2011(57).
13. Arras M, Autenried P, Rettich A, Spaeni D, Rulicke T. Optimization of intraperitoneal injection anesthesia in mice: drugs, dosages, adverse effects, and anesthesia depth. *Comp Med.* 2001;51(5):443-56.
14. Jirkof P, Tourvieille A, Cinelli P, Arras M. Buprenorphine for pain relief in mice: repeated injections vs sustained-release depot formulation. *Lab Anim.* 2015;49(3):177-87.