Refinement: Evaluating stress and accuracy of different intraperitoneal injection techniques in mice

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1. Abstract

The intraperitoneal (i.p.) injection in mice is a common, efficient and easy way to administer an animal a substance. Several restraining methods for this injection are in use. Various studies mention a frequency of misplacing an i.p. injection using a certain restraining method, however the frequency of misplacing an i.p. injection has never been compared between methods.
To safely administer a substance through an i.p. injection, some form of restraint is necessary. The restraining itself causes a degree of stress in mice. However, the level of stress has never been determined when comparing different restraining methods. In the context of animal welfare and the 3R's principle, it is relevant to determine which restraining method is most accurate and causes the least amount of stress. In this study frequency of misplacing the i.p. injection and the degree of stress, was determined comparing three restraining methods. Restraining the mouse head down (HD), restraining the mouse head up (HU) and using a method described by Baek et al.(2015)[1] in which minimal restraint is required (BK). Accuracy was determined by administering a methylene solution intraperitoneally after which macroscopic pathology was conducted to determine the location. The acute stress reaction was measured by determining the level of blood plasma corticosterone (pCORT) and measuring eye, ear and tail temperature through infrared thermography (IRT).
These parameters were measured after mice were restrained either HD, HU or BK (within group comparison, n=). Temperature of thermal images and level of corticosterone were compared between different restraining methods.
Accuracy of the i.p. injection after all restraining methods was not significantly different, considering this all three restraining methods are equally valid to be used in practice. Corticosterone levels after HU restraint were significantly highest (HU n=18, HD n=19, BK n=18). However, this was not supported by the IRT data; temperature change patterns did not differ between procedures. When different restraints were combined with i.p. injection corticosterone results regarding HU restraint were not confirmed (group comparison, HU n=14, HD n=13, BK n=20 per group). Thermal changes were present in the eye-, tail- and in some extent ear area during an acute stress reaction. These changes could not be correlated to pCORT, further study is necessary to validate IRT as a reliable stress parameter.

2. Introduction

The intraperitoneal injection

The intraperitoneal (i.p.) injection is a common way to administer injection liquid substances to rats and mice. This injection provides a number of advantages such as the rapid absorption, the possibility of the administration of large amounts. It is a relatively non- invasive way and considered an easy injection[2]. However, there are consequences if the substance is accidentally injected outside the peritoneal cavity. Misplaced i.p. injections can cause damage to abdominal organs and peritonitis. Apart from the fact that the health and well-being of the animal is at risk, damage to the animal's health or injection of a certain substance in, for example, an intestine can lead to undesirable variation in the test results. [3-5]

There are several restraining methods which can be used to administer an i.p. injection in mice. At the Utrecht University the administration of an i.p. injection is carried out as described in the Handbook of Laboratory Animal Science.[5] The authors describe the following technique in mice: "*The needle should not be inserted horizontally(moves between abdominal wall and skin), nor vertical (puncturing the kidney). The puncture must be made in the left caudal quadrant of the abdomen next to the midline, due to the location of the bladder. The intestine is rarely punctured, because it's easily movable in the peritoneal cavity. It is important to use a short needle and in mice the head must be tilted back, so that the liver falls within the last rib. This reduces the chance of damage*. "

No specific references are given in this description; it seems to be based on the experience of the authors. To our experience this restraining method causes somewhat difficulty when the technique is new to the experimenter, due to the awkward position of the hand that is restraining the mouse. In addition, mice seem show more resistance when tilting the head backwards rather than just restraining the mouse in a horizontal position.

In the literature there are a few articles available on the technique of administering an intraperitoneal injection in rats and mice, most of these articles were published quite some time ago.[1,3,6-9] Several studies with rodents describe that the i.p. injection is relatively frequently administered outside the intraperitoneal cavity, between 1.2 and 24%[6-9]. However, restraining methods differ between studies and are different from the method described above. Causes of misplacing the injection are therefore difficult to determine. Due to the different restraining methods, these results are not useful to compare with the method described by Van Zutphen et al.

Davis et al.(2014)[2] have looked at the macroscopic lesions on the organs of mice after 30 days while administering an i.p. injection with saline once a day. Macroscopically they found no lesions or abnormal weight to the different organs. The restraining method in this study is performed as described by van Zutphen et al.(2009)[5]. The result may be an indication that by using this method, i.p. injections do not cause long-term complications very frequently. However, it must be taken into account that this study did not specifically evaluate the location of the administered i.p. injections directly after administration.

Scientific research on the frequency of a misplaced i.p. injection by evaluating the location directly after administering an i.p. injection according to the method described by Van Zutphen et al. and used at the University of Utrecht is not available. Without scientific research it remains unknown how accurate this method actually is.

The acute stress reaction

Following the principles of the 3R's[10] animal experimental techniques, such as the i.p. injection, should be refined as much as possible. Apart from the fact that the technique has to be accurate, the restraining method used should be substantiated to cause the least amount of stress to the animal.
A stressor can be defined as a factor which takes an animal out of a homeostatic status and to which an animal has to adapt (causing a physiological stress response) to reach homeostasis again. Stressors can cause pain, fear or excitement, which are all adaptive responses of the body to eventually reach homeostasis again.[11,12] For example, when an animal comes in contact with its predator it may experience fear, which can lead to fight or flight behavior that increases survival chances of the animal. When the behavioral response is successful, homeostasis is established again. A degree of stress can variate, the more an animal can predict or control the situation, the lower the degree of stress will be.[12] A stress response can be evaluated by various parameters. As illustrated in figure 1. in an acute stress reaction, the autonomic nervous system (as part of the central nervous system) is immediately activated to regulate the adreno-medulla to release catecholamines such as epinephrine and norepinephrine. The heartrate and arterial pressure change to prepare the animal for a 'fight or flight' reaction. In addition, a neuroendocrine reaction is initiated. The hypothalamic-pituitary axis is activated, causing the hypothalamus to release corticotropin-releasing hormone, to which the anterior pituitary releases adrenocorticotropic hormone. This initiates the adrenal cortex to a release hormonal glucocorticoids, which facilitate behavior adaption to a stress response. The glucocorticoids reach a maximum concentration in the blood minutes after the initial stress response and can be used as a reliable parameter to measure an acute stress response. As shown in figure 1. The released catecholamines and corticoids have a stimulating effect on the immune system as well. To reach homeostasis again, hormonal feedback is sent to the central nervous system and the hypothalamus.[13-15]
Core body temperature is known to increase as part of an acute stress response, this effect is known as stress induced hyperthermia(SIH) and has been shown in a variety of species.[16] In rodents, psychological stressors including placing the animal in a novel environment, restricting an animal's activity, noise and handling in a variety of ways induce hyperthermia.[17]
Groenink et al.(1993)[18] showed that this rise in temperature measured rectally can be correlated to the increase in blood plasma corticosterone. Although Meijer et al.(2006)[19] could not confirm the correlation between increasing levels of corticosterone and core temperature after exposing mice to a stressor and measuring temperature through radio telemetry. The body temperature seemed to rise slower than levels of corticosterone. Other factors, produced during an acute stress response, for example noradrenaline, various interleukinen or serotonine can contribute to hyperthermia as well.[20]

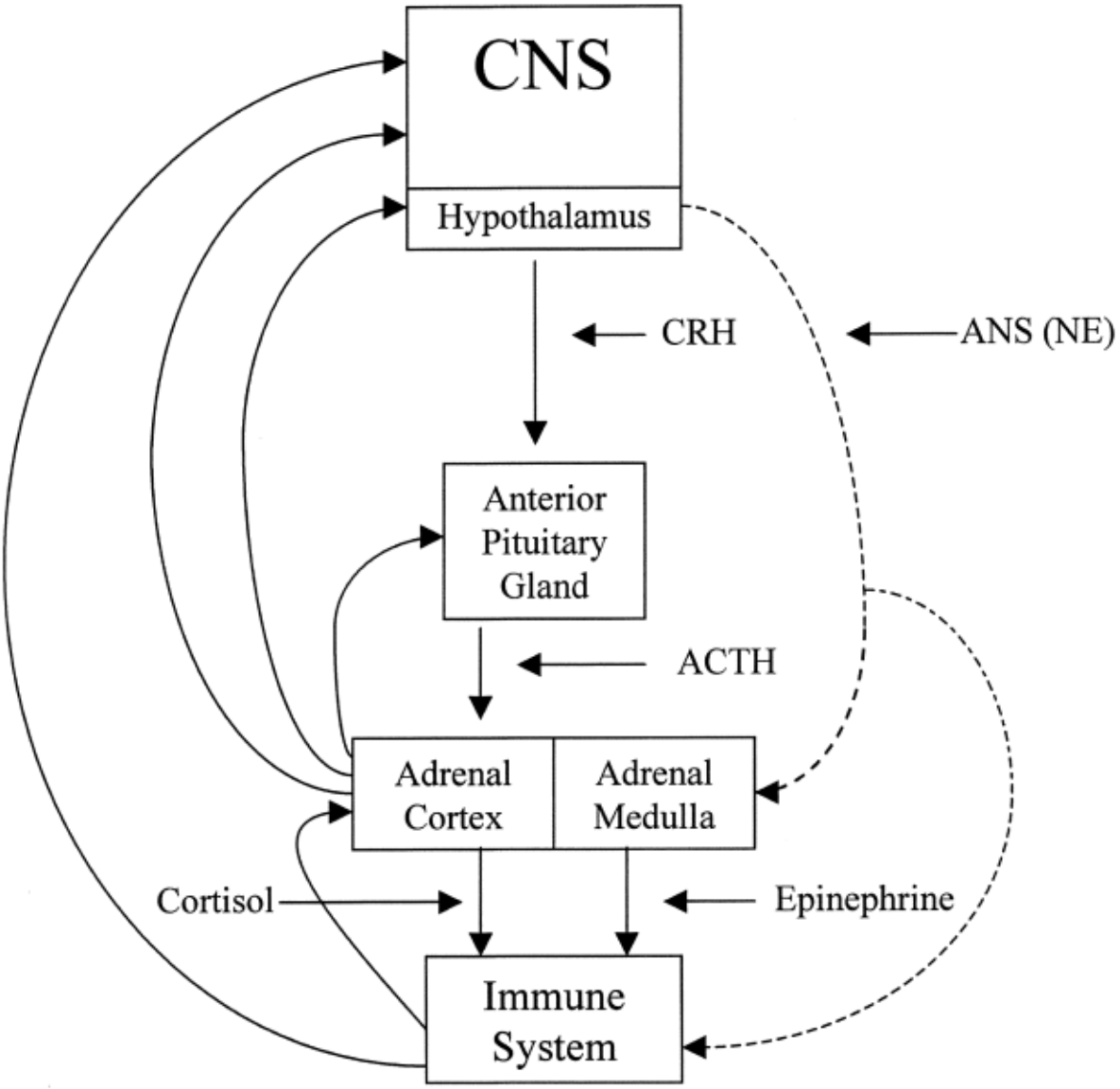

Figure 1. Illustration of an acute stress response. The central nervous system (CNS), the hypothalamic-pituitary-adrenal axis, the autonomic nervous system (ANS), and the immune system. Dashed lines indicate ANS neural pathways, solid lines indicate hormonal pathways.
ACTH adrenocorticotropic hormone; CRH corticotropin-releasing hormone; NE norepinephrine.[15]

An intraperitoneal injection will cause a degree of stress in mice. Restraining the animal may lead to an experience of fear. The puncturing of the skin, muscular tissue and peritoneum leads to damage of the body, to which the animal will experience pain. An acute stress reaction is initiated by these experiences and stress response systems like the autonomic- and HPA system will be activated to eventually reach homeostasis again.[19]
There are several articles available in literature about the degree of stress and consequences to the well-being of an animal caused by an i.p. injection. Davis et al.(2014)[2] have evaluated parameters to measure the degree of stress and pain, as well as the effects on health an i.p. injection can have. They found no significant effect on behavior, physical condition, fecal cortisol (parameter for chronic stress) and hematology after the 30-day administration of an i.p. injection once a day. Following these results, they conclude that mice injected under this scheme experienced no increase in pain or chronic stress and stay in relatively good health. This conclusion, however, is very premature, because behavior is a difficult parameter to evaluate in prey animals. If the mice actually experienced no increase in pain during the study is difficult to say. In addition, these results do not give information

about the degree of acute stress during an i.p. injection when using this method of restraint.
Meijer et al.(2006)[19] have also evaluated the acute stress response, to an i.p. injection in comparison to a subcutaneous and intramuscular injection using heart rate and body temperature as stress parameters. Although the reaction to an i.p. injection seemed slightly different when administering sham injections. This effect was not seen when administering real injections. The type and volume of an injection may contribute to the degree of stress experienced by mice. No difference was found in change of temperature between groups administered sham or real injections.
The restraint itself may be a significant part of the stress response which the mouse exhibits.
Baek et al.(2015)[1] have evaluated the degree of stress which a conventional i.p. injection restraining method can cause in comparison with an alternative i.p. injection method in which minimal restraint is required. Although the sample size is small, the researchers found that the blood plasma level of corticosterone is significantly lower in the alternative i.p. injection method. However, there are several mouse strains available in scientific research. More research is necessary to further validate the method and determine whether this effect applies to all strains as well as if the technique is accurate and suited for all strains.

Infrared thermography as a stress parameter

Different methods to determine an acute stress reaction, such as blood collection or determining rectal temperature, can often cause an animal extra stress in their application. In the search of a non-invasive way to measure a degree of stress and the knowledge that body temperature can change as part of a stress reaction, infrared thermography (IRT) has been used in a variety of species to evaluate an acute stress response.[21,22]
The theory behind the idea of measuring stress with IRT is based on the indication that activation of the autonomic nervous system can cause peripheral vasoconstriction, which can lead to a decrease in temperature on IRT images.[23-25] This effect is suggested to have two functions; redistribution of the blood to important organs such as the brain and prevent of blood loss in case of when the body is damaged.[26]
It has been indicated by Blessing et al.(2003)[27] that vasoconstriction in the rabbit ear and in the tail of the rat can be mediated by the sympathetic system. A study with sheep indicated that the temperature in the ear-pinna decreases in lambs during exposure to a stressor.[25]
In addition several studies indicate that there is a change in eye temperature during and after exposure to a stressor in several animal species. [28-33]
There have been only two studies with rodents where an acute stress response was assessed through IRT images. Lecorps et al.(2016)[29] found a significant decrease in temperature of the tail and an increase of temperature of the eye with correlations to anxiety-related behaviors by exposing mice to an open field and a plus maze test. Vianna et al.(2005)[28] have evaluated heartrate, arterial pressure and body(i.p.) temperature with radiotelemetry in addition to evaluating anxiety-related behavior and IRT images. When a group of fear and sham conditioned rats were exposed to a stressor, the arterial pressure of the fear conditioned rats was significantly higher and the temperature in the tail and paws of the fear conditioned rats showed a decrease in comparison to the sham conditioned rats.

In the context of animal welfare and the 3R's (refinement, reduction and replacement) principle, it is important that the injection techniques in experimental animals used are scientifically substantiated. Only if this condition is met, more sophisticated techniques can be evaluated and put into use. In addition, methods of measuring a degree of stress should be refined to improve the well-being of the animal.
In the present study, the degree of stress and the frequency of outside the peritoneal cavity injected i.p. injections is determined of three different restraining methods. The technique described by van Zutphen et al.[5](HD) is compared with an alternative technique, were the head is held up instead of down(HU) and the technique described by Baek et al.[1](BK). The methods are compared to determine which is most accurate and which method causes the least amount of stress. In each of these restraining methods, the degree of acute stress will be measured by the blood plasma level of corticosterone(pCORT). In addition, body temperature will be assessed by assessing IRT images. Blood plasma corticosterone levels are compared with IRT images to determine whether or not the measurement of body temperature with IRT is a reliable way to measure an acute stress response in

mice. The accuracy of the i.p. injection technique will be determined by assessing the location of the injection after injecting with a methylene solution[6,8] and macroscopic pathology after euthanasia. Expected is that the HD and BK technique will be equally more accurate than the HU technique, as in both restraining methods the mouse is held in a way that the organs are moved cranially.
The acute stress reaction is expected to be higher using the HD technique and the lowest using the BK technique. The images obtained with the infrared thermal camera are expected to show an increase in eye-temperature, a decrease in tail temperature and ear temperature, which can be correlated to blood plasma cortisol. By determining the degree of stress and accuracy, restraining methods of i.p. injection can be refined. Determining accuracy can lead to reduction as well, since misplacement of the injection causes variation in results. With these results the IRT technique can be validated to be used as a reliable, non-invasive way to measure the acute stress response in mice.

3. Material and methods

Animals and Housing

All of the described procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of Utrecht University (DEC number 2014.III.07.057. approved working protocol 104893-9). A total of 114 mice, 36 C3H/HeNCrl (C3H) female, 20 C3H male, 58 BALB/cAnNCrl (Balb/c) female, 42 weeks of age, which have been primarily used for educational purposes were used in this study.
20 Balb/c female mice were used for determination of stress levels after different restraining methods, measured by blood plasma level of corticosterone and IRT images in four subsequent experiments. In the last experiment location of i.p. injection was determined.
20 C3H male- and 29 Balb/c female mice were only used for determination of stress levels by measuring blood plasma level of corticosterone and determining location after i.p. injection. 36 C3H female and 9 Balb/c female mice were only used to determine location after i.p. injection.
Mice were held in conventional animal room. Temperature 18–24.1 ° C; 12/12 h light/dark cycle with lights on at 07:00 h, light intensity at shelf level about 100 lux; radio on during light hours. The mice were socially housed (10 mice per cage) in Makrolon type 3 cages (36x20 cm) with a bedding of sawdust. Cage enrichment in the form of a cardboard shelter, tissues and a plastic tube was present. Cages were cleaned once a week. Food pellets (CRM(E) 801730, Special Diet Services) and tap water were provided ad libitum. Mice were primarily used for educational purposes, behavior, injection and handling practicals were performed once to twice a week for students of the university in the weeks prior to the experiment and in the first week of the experiment. However, mice were not handled for at least three days- and at least one day after the first test.

Procedures

Restraining methods

Mice were restrained for the i.p. injection by in three different methods illustrated in figure 2. Basal values were obtained in a control experiment.
Control(C): Mice were only lifted up by the tail to move them from the home cage to the test cage
Head Down(HD): The injection technique described in the introduction, the mouse is fixated in one hand with the back of the animal in the palm of the hand, the head is tilted downwards to move the organs cranially. i.p. injection is administered in the left caudal quadrant, parallel to the vertebrae.[4]
Head Up(HU): The method is quite similar to the HD method, with the important difference that the head of the mouse is not tilted downwards but upwards.
Baek(BK): This method was first described by Baek et al.(2015)[1]. The mouse can grip a surface with the front legs, while the experimenter with the left tail and right hind leg of the mouse slightly fixes and with the other hand administering the i.p. injection in the left caudal quadrant, parallel to the vertebrae. Although this is not an actual restraint, the method will be referred to as BK restraint.
The time the mouse is held in a certain restraint is equal to the time necessary to administer an i.p. injection. Determined by an employee at the Department of Animals, Science and Society at around 15 seconds. At the second and third time point an actual i.p. injection was not administered (figure 4.). The abdomen of the mouse was gently palpated, as was performed in Meijer et al.(2006)[19].

Figure 2. Illustration of the different restraining methods described

Test cage

Mice from the same home cage were habituated in the test cage per pair, a week before the first experiment (ten minutes a day for two days) and were habituated two days after each experiment for 20 minutes to prevent an anticipation on blood collection.
After restraining the mice were put in pairs in a test cage for 20 minutes. The test cage (21x15 cm) had transparent sides of plastic and sawdust bedding. The cage was divided into two areas of equal size with a septum of transparent Perspex, so that the mice could see, but not reach each other. This was required to be able to keep the mice apart on infrared images. After a pair of mice had been in the test cages the bedding was replaced.

Infrared Thermography

Mice were recorded via infrared thermography in the test cage during 4 separate experiments. A FLIR T430 sc camera (resolution 640x480, setting ultramax during recording, sensitivity <0,03 K.) on a tripod was used to record the mice. The emissivity was automatically set at 0,95. FLIR ResearchIR analysis software was used to collect the data. During the time in the test cage, the mice were recorded every 4 minutes for 60 seconds. Resulting in 5 measurements per mouse. Before selecting a frame, the view was set at ‘manual’ and ‘linear scale’. Frames of the videos were selected every 4 minutes when the area of interest was most visible. The eyes, ears and tail temperature were assessed. The eye temperature was assessed by selecting the eye-area (the orbital region of the whole eye) on one of the eyes with a cursor of a set surface (9 pixels) from the software program. The ear temperature was assessed by drawing a line of three pixels on the distal site of the pinna medially from the midline in one ear. The tail temperature was assessed by drawing a line of ten pixels from the base of the tail. The mean temperature of these areas, shown in a corresponding table was used for analysis (Figure 3). The temperature in the room was determined after each experiment by a logger in the room.

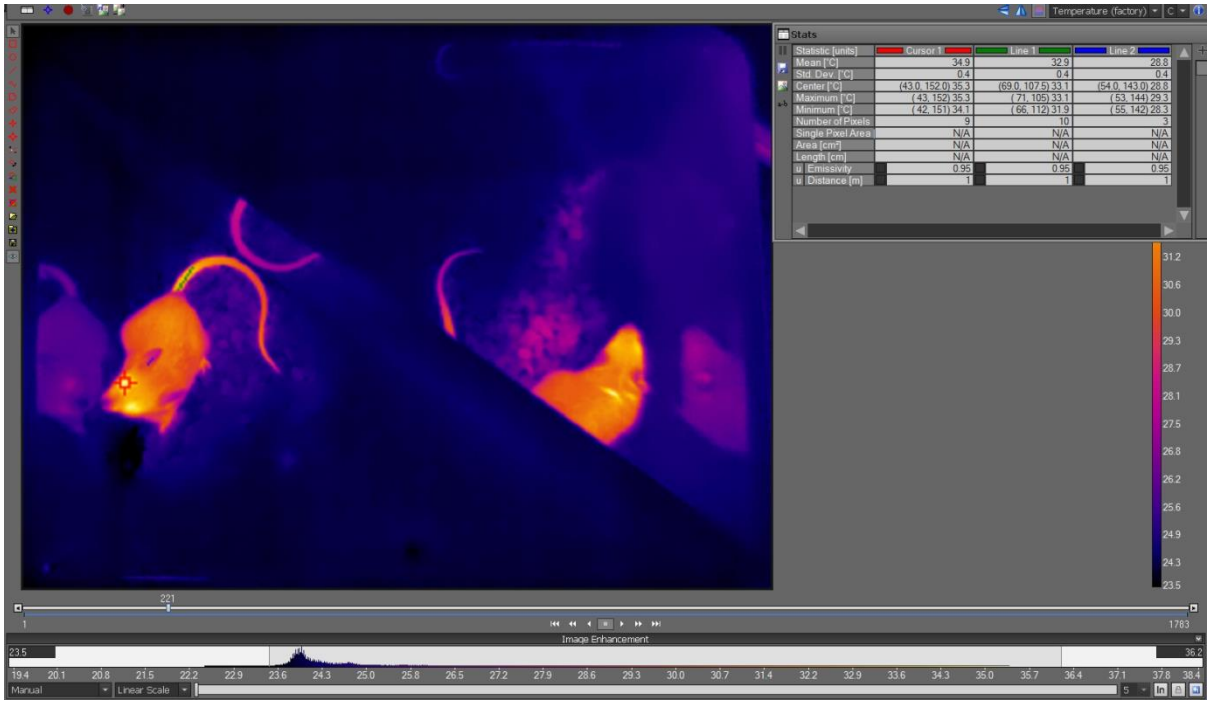

Figure 3. Data was collected by measuring eye-, ear- and tail temperature. Mean temperature of these areas, shown in a corresponding table was used for analysis.

Endocrinology

Collecting blood from the mice was performed 20 minutes after restraining by tail vein incision, collecting blood from the lateral vein of the tail. The procedure lasted less than 2 minutes, to prevent measuring corticosterone due to blood collecting.[13,34] All blood samples were collected in a separate room to prevent possible communication (sounds, odors etc.) between animals. When blood was collected by tail incision, the mouse was taken out of the test cage, its head and body covered with a tissue. With an extra sharp razor blade (GEMs Scientific, American Safety Razor Co, Staunton, VA, USA) perpendicular incision was made at the ventral side of the tail, initially about 3 cm away from the base, subsequent times more proximal. Drops of blood were collected in a Microvettes CB 300 capillary collectiontube coated with EDTA. When finished, a gauze was placed on the incision and gentle pressure was applied for a moment before the mouse was returned to the home cage. After the last experiment the mouse was taken out of the test cage, restrained and decapitated with decapitation scissors within 2 minutes. Trunk blood was collected in a Minicollect Tube, 1ml coated with EDTA. The blood samples were centrifuged at 3000 rpm for 30 min at a temperature of 4 °C. Plasma was stored in Plastibrand Micro Tubes, 1,5 mL of polyprolene at -26 °C. Plasma corticosteron levels were measured by RIA with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MP Biochemicals, Orangeburg, New York.

I.p. injection

i.p. injection was administered by one of the three described techniques. A solution of saline 0,9% and methylene was administered using a Terumo Neolus 26Gx ½ inch needle and a BD plastibak 1ml sterile syringe.
In the group of mice used only to determine the location of the injection (n=45) a solution of saline 0,9% and methylene was injected, after which the animals were euthanized by cervical dislocation.

Pathology

Immediately after euthanasia the abdomen of the mouse was incised in the midline to expose the abdominal cavity. The location of the i.p. injection was assessed macroscopically. Location was determined as intraperitoneal or outside of the peritoneum. When the solution was visibly injected in another organ, retroperitoneal or subcutaneously. A photograph was made of the abdomen cavity; the image was evaluated by the two experimenters, blind to the used method.

4. Experiment

The mice, home cages, restraining method and performing experimenter were randomized before conducting the experiment. One mouse pair a day was measured to prevent test sequence effects that could contribute to the results on the IRT images or the blood plasma level of corticosterone during the experiment.

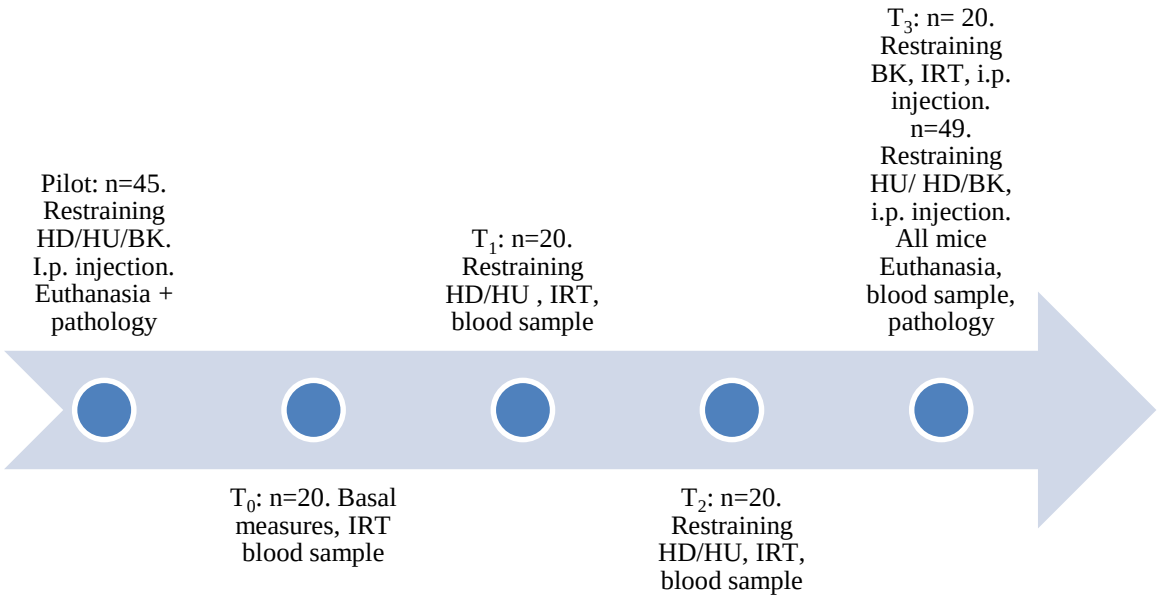

Figure 4. Overview of the experiment with a 7 day interval in between T0,1,2,3.

Pilot

To assess the visibility of the exact location after peritoneal injection with the methylene solution, 45 mice were used only to determine a location after i.p. injection (Figure 4.). The i.p. injection was administered, directly after the mouse had deceased the location was macroscopically evaluated. This data was included in the analysis.

T0

The basal values of corticosterone and the body temperature of the eyes, ears and tail measured by IRT of mice (n=20) were determined (Figure 4.).
2 mice from a cage were put in the test cage for 20 minutes. The infrared camera recorded the mice in the cage and data was collected as described above. After 20 minutes, blood was collected, the mice were marked and put back in their home-cage. The measurements were spread over 2 days, one pair of mice from the same home cage were measured on the first day. A second pair was measured on the second day to prevent testing order effects.

T1 and T2

T0, T1 and T2 were performed with a 7 day interval. Mice were restrained by the HU or HD method, corticosterone was measured, IRT images were collected (Figure 4.).
T1: The same mice used on T0 (n=20) were restrained by the HU or the HD method. Mice did not actually receive an i.p. injection, but the abdomen was gently palpated and mice were held for about 15 seconds. 2 mice out of a cage, randomly chosen out of the 4 marked mice used on T0 were restrained. The 2 mice were restrained at the same time, by two experimenters. After restraining the mice were put in the test cage at the same time for 20 minutes. The infrared camera recorded the mice in the cage. After 20 minutes, blood was collected and the mice were put back in their home-cage. The measurements were spread over 2 days. On the first day one pair mice out of 4 marked mice from

one cage was measured. On the second day the other pair of mice was measured.
T2: is quite similar to T1 except that the mice restrained HU were now restrained HD and vice versa.

T3

The mice were restrained either by HU, HD or BK method of a total of 69 mice. An i.p. injection was administered. Corticosterone was measured. IRT images were collected of mice used on T0, T1 and T2. Mice were euthanatized (Figure 4.).
Mice and cages were randomly chosen. A pair of mice were restrained at the same time by two experimenters. The mice that were measured on T0, T1 and T2 were restrained according to the BK method (n=20) and recorded with the infrared camera. The other mice (n=49) were restrained according to the HD, HU or BK method.
An i.p. injection was administered. After restraining, the mice were put in the test cage at the same time for 20 minutes. After 20 minutes the mice were euthanatized by decapitation (within 2 minutes) and blood was immediately collected. One pair per cage was measured a day. When two pairs of mice were left in the cages, the two pairs were restrained and injected immediately after one another and two test cages were used to prevent extra stress.

5. Statistics

The data was analyzed using SPSS software of IBM corporations, version 24. Charts were edited in Windows Microsoft Office Excel 2010 or in the chart editor function of SPSS.

I.p. injection data

The i.p. injection data was divided into two categories(restraining method and location) with subcategories (restraint: HD/HU/BK and location: i.p./not i.p.). A Pearson's Chi Square test was conducted to check the data for significance.

Endocrinology

The pCORT data was divided in two categories. Mice that had been restrained on T0, T1, T2 and T3 (Repeated restrain category, RR) and mice that had been restrained and injected on T3 (Single restrain category, SR). The RR category consisted of female Balb/c mice was sorted in 4 treatments: control, HD restraint, HU restraint and BK (and IP injection) restraint. Data was checked for normality using a Kolmogorov-Smirnov test. A linear mixed model was conducted to determine if there was a significant difference between restraining methods. To control for repeated measures, mouse ID was included as a random factor in all models. Restraining method was included as fixed predictor. Residuals of the fitted models were tested for normality, and in addition visually assessed using histograms, Q-Q plots and Box plots. When an interaction between time of measurement and restraining method showed significance (<0,05) a post-hoc test with Bonferroni correction was conducted.
The SR category consisted of the group of all mice that were restrained and injected on T3. This group consisted of both Balb/c female and C3H male mice. The animals were sorted in HD-, HU- and BK restraint. A linear mixed model was conducted to determine if there was a difference between these different strains/sexes, with strain as a between subject factor The same standards as described above were set, gender was added as a covariate. Data was checked for normality using a Kolmogorov-Smirnov test. A one-way ANOVA test was performed to determine if there was a significant difference between the restraining methods.

Temperature data

Data was missing at certain measurement points due to the occurrence that a tail or an eye was not visible in the test cage during the recorded 60 seconds. Data was first checked for normality using a Kolmogorov-Smirnov test. To compare the course of the five temperature measurements after the different restraining methods, a linear mixed model was conducted for ear-, eye- and tail measurements. To control for repeated measures, mouse ID was included as a random factor in all models. Measurement and restraining method were included as fixed predictors. Residuals of the fitted models were tested for normality, and in addition visually assessed using histograms, Q-Q plots and Box plots. When an interaction between measurement and restraining method showed significance (<0,05) a post-hoc test with Bonferroni correction was conducted. For the ear-, eye- and tail measurements significance of a change in temperature during 5 subsequent measurements was checked to confirm accordance with the results of Vianna et al.(2005)[26] and Lecorps et al.(2016)[27]. The delta of 5 subsequent measurements of the eye- and tail measurements of all groups was determined. A Pearson correlation was applied to evaluate correlation between change in eye- and tail temperature.

pCORT and body temperature correlation

To determine a correlation between temperature change and stress levels of the mice, the area under the curve(AUC) of the mean temperatures of the RR category was calculated for body areas separately. A Pearson correlation was applied between AUC of temperature and pCORT per timepoint.

6. Results

I.p. injection data

Results are illustrated in figure 5. and table 1. No significant difference was found between the three restraining methods regarding the location of the injection (X^2_2 = 2,393, p=0,302).

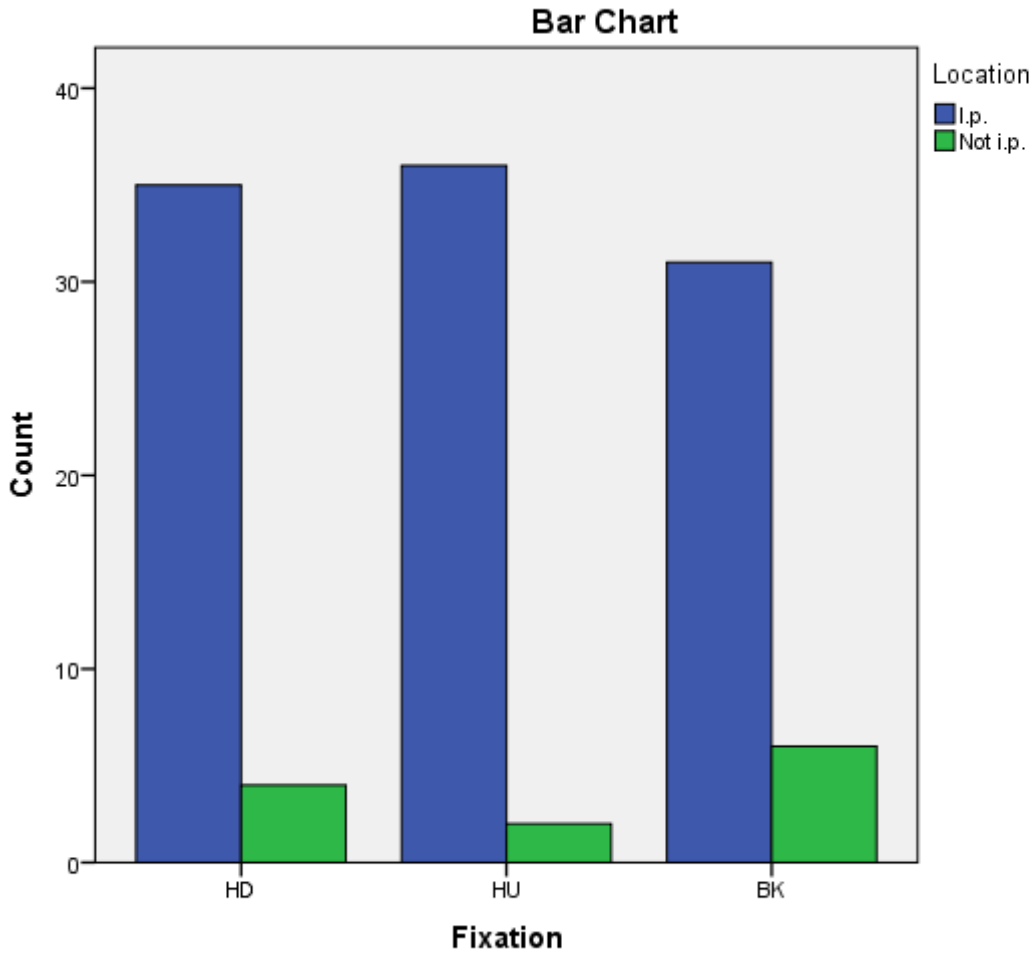

Figure 5. Bar chart of the location of the administered i.p. injection to a total of 114 mice (HD n=35, HU n=36, BK n=31). Y-axis shows number of times administered in or out of the peritoneum. X-axis shows the restraining method in which the animals were injected.

Table 1. Overview of injection locations in percentages.

Location	HD	HU	BK
I.p.	89.7%	94.7%	83.8%
Not i.p.	10.3%	5.3%	16.2%
Intestine	7.7%	2.6%	5.4%
Bladder	-	-	2.7%
Retroperitoneal	-	-	2.7%
Fat	-	2.6%	-
Subcutane	2.6%	-	5.4%

Blood plasma corticosterone data

Results and standard deviations of the RR category are shown in figure 6. In some cases, pCORT was missing due to failure of collection of blood or an error during processing of the blood. Therefore, group sizes were: On T0 n=19, T1 n=19, T2 n=18 and T3 n=18. Data was normally distributed. A significant interaction between measurement and restraint was found between restraining methods (F_{52}=5,516 p=0,002). The HU restraint resulted in significantly higher corticosterone plasma levels compared to the control and the BK (and injection) restraint.
Results and standard deviations of SR category are shown in figure 7. For the same reasons as mentioned above data is missing in some cases. A significant difference in pCORT was found between male (C3H)- and female (Balb/c) mice (F_{29}=33,854 p=0,000). Only the data of female mice was used for statistics. Due to this number of mice in per groups were: HD restraint n=13, HU restraint n=14 and BK restraint n=20. Data of the HU group was normally distributed. No significant difference between restraining methods was found after performing a one-way ANOVA (F_2=0,373, p= 0,691).

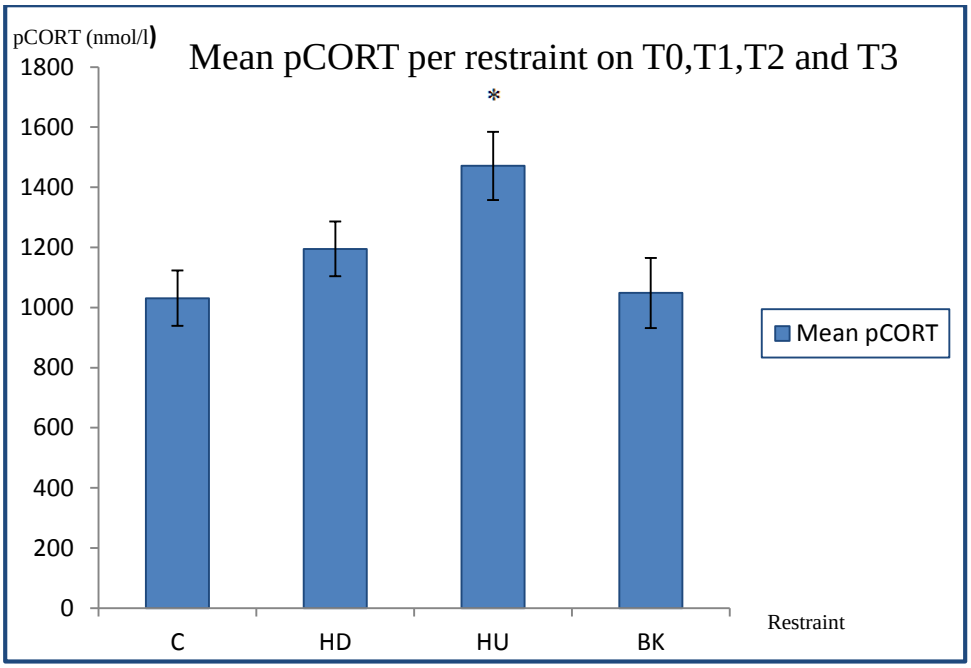

Figure 6. RR category. Mean pCORT of female Balb/ c mice after the control(C), HD-, HU- and BK restraint on T0 (n=19), T1 (n=19), T2 (n=18) and T3 (n=18). pCORT is plotted in nanomol per liter. Error bars depict standard deviations of the mean pCORT, * p<0,05.

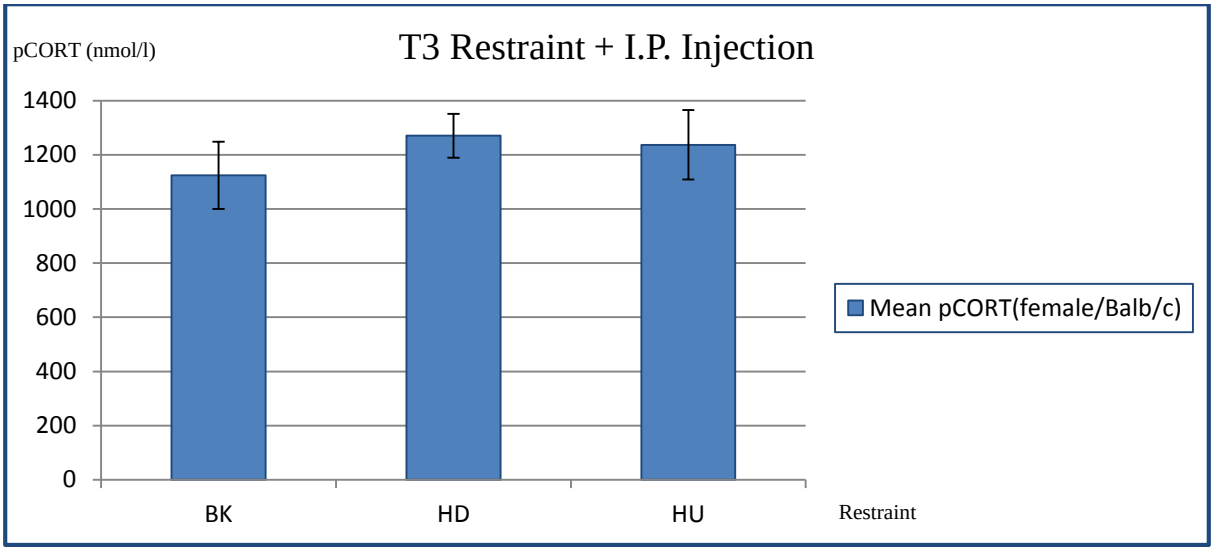

Figure 7. SR category. Mean pCORT after BK (n=20), HD (n=13) and HU (n=14) restraint in female balb/c mice. pCORT is plotted in nanomol per liter. Error bars depict standard deviation of mean pCORT.

Temperature data

Figure 8. illustrates a representative example of the collected IRT images of 5 subsequent measurements after BK restraint and i.p. injection.

Ear temperature

Results and standard deviations shown in figure 9a and table 4a. Data was normally distributed. A significant pattern during 5 subsequent measurements after all restraining methods was found ($F_{4,356}$=4,183, p=0,003). After conducting a Bonferroni test, temperature significantly decreased between measurement 1 and 2. The temperature significantly increased between measurement 2 and 4. No significant interaction between measurement and restraining method was found ($F_{12,356}$=0,659, p=0,791). There was a significance in restraining method, after conducting a Bonferroni test. This was due to a significant difference between the ear temperature in mice after BK restraint (with IP injection) and the other restraining methods. Ear temperatures were significantly higher during all measurement after BK restraint (with i.p. injection).

Tail Temperature

Results and standard deviations shown in figure 9b. and table 4b. When the residuals of not normal distributed data was visualized in a Q-Q plot, a normal distribution was approximated.
A decrease in tail temperature during 5 subsequent measurement was confirmed after all restraining methods ($F_{4,340}$=160,3, p=0,000). A significant interaction between measurement and restraining method was found ($F_{12,340}$= 4,787, p=0,000).
After conducting a Bonferroni test the first measurement after BK restraint and injection was significantly higher compared with first measurements of other restraining methods. Temperatures of the subsequent measurements after BK restraint (with i.p. injection) were not significantly different from other restraining methods. Temperatures after the control-, HD- and HU restraint did not differ.

Eye Temperature

Results and standard deviations shown in figure 9c. and table 4c. With the exception of one measurement, data was normally distributed. A rise in eye temperature during 5 subsequent measurements was confirmed after all restraining methods ($F_{4,355}$=214,8, p=0,000). No significant interaction between measurement and restraining method was found ($F_{12,355}$=0,743, p=0,709).

Eye-Tail correlation

A correlation between the increase in eye temperature and decrease in tail temperature was not found. However a trend was found after a Pearson correlation during the control measurements (r= 0,398, p=0.082).

pCORT and body temperature correlation

There was no correlation present between the calculated areas under the curve of the means of eye-, tail- and ear temperature measurements and pCORT of mice in the RR category (p>0,05 for temperature of all measured body areas and pCORT).

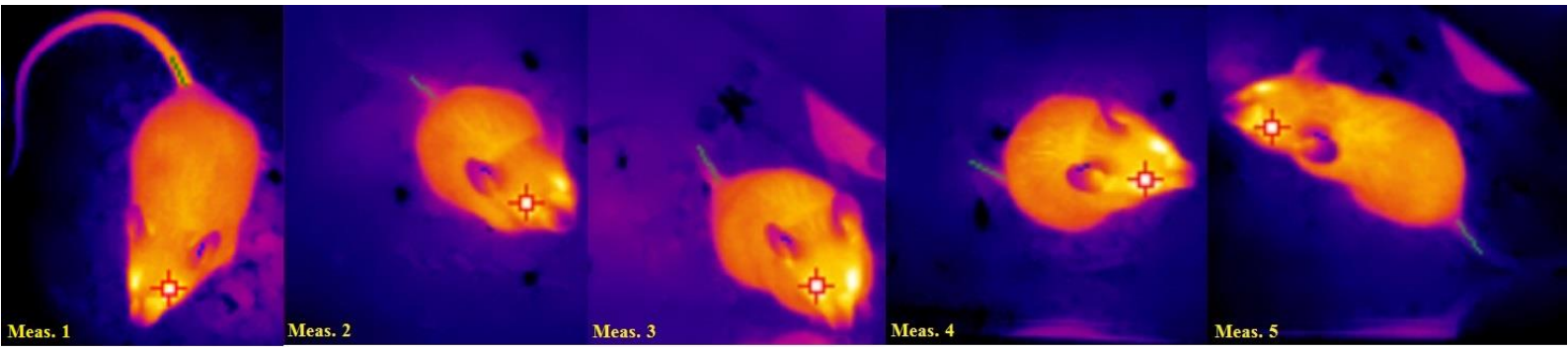

Figure 8. IRT images of the 5 subsequent measurements after BK restraint and i.p. injection.

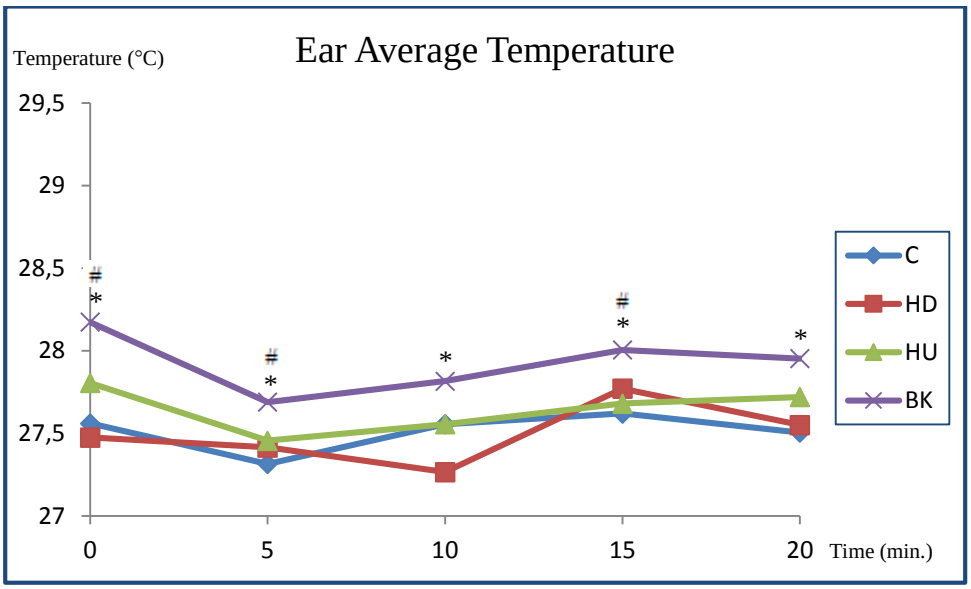

Figure 9a. Average Ear Temperature at 5 subsequent measurements after control (C), HD, HU and BK+injection restraint (n=20). Temperature is plotted in degrees Celsius, time in minutes. # p <0,05* p <0,05.

Table 4a. Standard deviations from the ear average temperature (means depicted in fig. 9a) of 5 subsequent measurements after the control, HD, HU and BK+injection restraint.

Time(min.)	0	5	10	15	20
Std. C	0,15	0,16	0,14	0,14	0,15
Std. HD	0,09	0,14	0,15	0,09	0,11
Std. HU	0,23	0,12	0,11	0,12	0,13
Std. BK	1,13	0,15	0,13	0,14	0,10

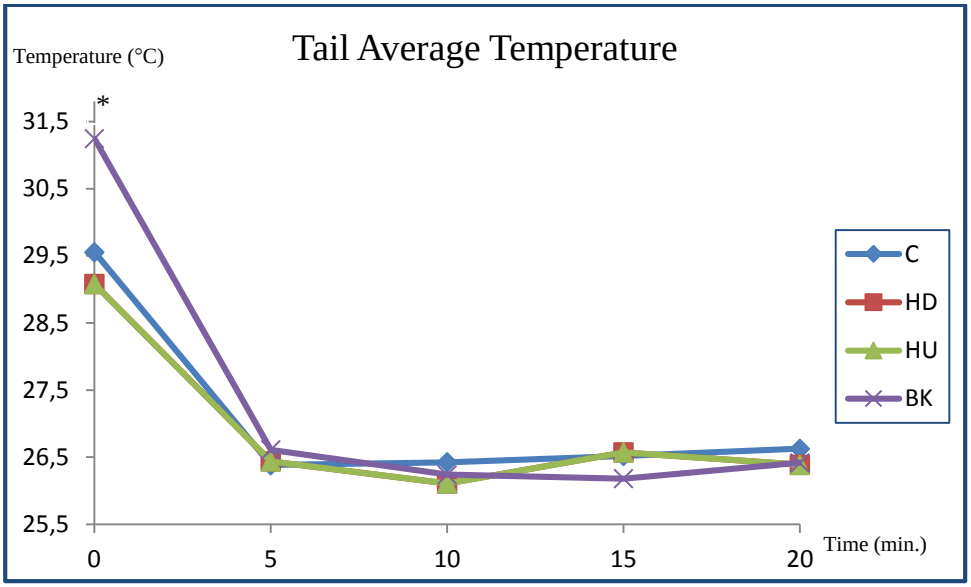

Figure 9b. Average Tail Temperature at 5 subsequent measurements after the control (C), HD, HU and BK+injection restraint (n=20). * p <0,05.

Table 4b. Standard deviations from the tail average temperature (means depicted in fig. 9b) of 5 subsequent measurements after the control, HD, HU and BK+injection restraint.

Time(min.)	0	5	10	15	20
Std. C	0,22	0,11	0,32	0,38	0,45
Std. HD	0,23	0,18	0,13	0,14	0,15
Std. HU	0,30	0,13	0,39	0,37	0,38
Std. BK	0,26	0,15	0,08	0,11	0,08

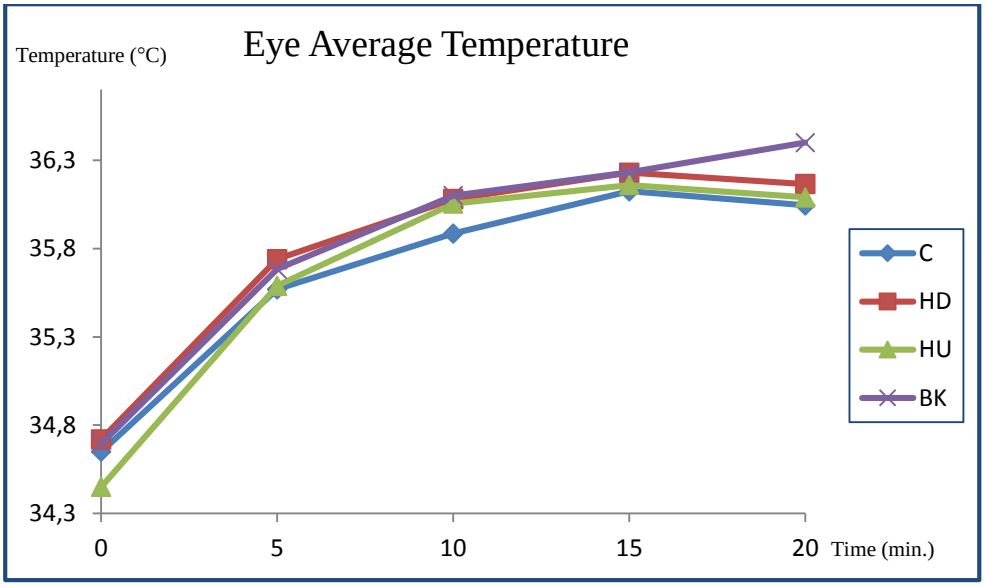

Figure 9c. Average Eye Temperature at 5 subsequent measurements after the control (C), HD, HU and BK restraint(n=20).

Table 4c. Standard deviations from the eye average temperature (means depicted in fig. 9c) of 5 subsequent measurements after the control, HD, HU and BK+injection restraint.

Time(min.)	0	5	10	15	20
Std. C	0,10	0,08	0,07	0,07	0,08
Std. HD	0,11	0,08	0,06	0,04	0,08
Std. HU	0,14	0,12	0,07	0,06	0,07
Std. BK	0,14	0,12	0,07	0,06	0,07

7. Discussion

Considering the accuracy of the i.p. injection, HD, HU and BK restraints are equally valid to be used in practice. In contrast to our expectations application of an i.p. injection during HU restraint is equally accurate to the HD and BK technique. Although no statistical difference was found the BK technique does seem to show more variation in location when it is misplaced.
Mice had highest stress levels after the HU restraint in the RR category, during subsequent measurements. pCORT was significantly higher in comparison to control and BK restraint. However, no difference could be found between restraining methods in the SR category, when mice received an i.p. injection on T3. This could be due to several factors such as the fact that all mice in this category received an i.p. injection, the fact that these were not naive mice or the fact that most of the mice in this category were not repeatedly used.
The IRT images showed an increase in eye-temperature and a decrease in tail temperature confirming part of our hypotheses. However, this pattern was not seen in ear temperature which was considered a peripheral area as well. The patterns of temperature changes after the control-, HD-and HU restraint were not significantly different. This may indicate that an acute stress reaction in the control group is present as well. After BK restraint and i.p. injection, change in temperature seems to differ from other conditions.
No correlation could be made between pCORT levels and the AUC of mean temperature change in body areas separately. Which may indicate that the HPA-axis does not fully mediate change in body temperature in these areas.

Accuracy

For the wellbeing and health of a laboratory animal and for the results in an experiment it is relevant to know the accuracy of a restraining method used to administer an i.p. injection. Reduction and refinement can be achieved when this is substantiated.
Manual restraint to administer the i.p. injection is necessary to prevent the mouse from moving. The method of Baek requires minimal restraint, which may lead to a lower degree of stress in the animal. Guarnieri (2016)[35] describes in a comment on the article of Baek et al.(2015)[1] that the BK restraint introduces several uncontrollable variables; Reduced visibility on the injection site, the difference in behavior of different sexes and mouse strains. The investigator's experience which could have greater influence on the process using this method in comparison to other methods and a limited amount of time to administer the injection.
These arguments seem justly, our experimenters did experience the limited amount of time and reduced visibility of the injection location. However, these variables seem not to have led to a significant difference in misplacing the injection. The injection does show the most variability in locations when it was administered outside the peritoneal cavity. The ability of mice to change posture during restraint or the reduced visibility on the injection site may have contributed to this result. The BK restraint was relatively new to the assisting experimenters, causing the procedure to show some difficulty in the first cases. When it was done several times the procedure became more natural to the experimenters. A certain injection method always takes time and repeated practice to learn, if the BK method is practiced more often, frequency of misplacing may even decrease. The BK method was suited for the two strains of mice used by us. The fact that these were not naïve mice could also have been of influence. As described earlier it needs further investigation if the method can be applied in other strains and in naïve mice.
No significant difference was found between the three restraining methods regarding the location of the injection, restraining methods are equally valid to be used in practice. It may be that this result is due to the size of the needle used (26G ½ inch). However Miner et al.(1969)[8] have used two different needle sizes(26G ½ inch, 22G 1 inch) and concluded that needle size was not a factor in frequency of misplacement. In this experiment only one restraining method was used, therefore this factor cannot be excluded in our experiment. Another factor which could have had effect on these results was the experience of the experimenters, four different experimenters performed the i.p. injections on different time points (one experimenter was present at all-time points). Matter of experience could lead to a higher or lower frequency of misplacing the injection.
Van Zutphen et al.(2009)[5] state that the HD restraint may be more accurate, suggesting the organs

would be moved cranially when the head is tilted downwards, although no literature confirms this. From the results in this study we conclude that restraining method does not have a significant effect on the frequency of misplacing an i.p. injection when using a 26G ½ inch needle.
The i.p. injection technique can be refined by determining what degree of stress the restraining methods cause.

Blood plasma level of corticosterone

Mice in the RR category had highest stress levels after the HU restraint. pCORT was significantly higher in comparison to control and BK restraint. In contrast to our expectations HD restraint did not cause the highest stress levels and BK restraint did not lead to a significantly lower stress level. These results indicate that HU restraint caused the highest degree of stress. This might be explained by the fact that these mice were used for educational purposes. The HD restraint is taught to students at the Utrecht University. Which means that the mice have been held in this restraint regularly. They might have habituated to this restraining method causing a lower degree of stress. The HU restraint on the other hand was a restraining method which they had never experienced. This could have led to a higher degree of stress, explaining the high corticosterone blood plasma level.
When this group of mice was restrained according to the BK restraining method, the animals received an i.p. injection. Curiously this did not lead to a different degree of stress compared with the control and HD restraint. This indicates that the experience of an injection (painful stimulus) does not lead to a significant rise in bloodplasma corticosterone in comparison with the control experiment (although it is probable that mice did show an acute stress reaction). Jensen&Toates (1997)[12] confirm this is a review about the psychological factor as part of stress reaction. The authors refer to an article of Weiss (1971) where the HPA activity in rats was different after giving the rat an electrical shock and comparing situations where the rat could and could not control the situation, concluding that the psychologic factor has a greater influence on the HPA-reaction that the actual sensation of the shock. The mice that were restrained by the BK method had more control over the situation, the minimal restraint enabled them to grab a surface with their front paws.
Mice had not experienced this form of restraint as well as the HU restraint, which did significantly differ from other conditions. This indicates that the BK method does lead to a lower degree of stress in the animals, in accordance with the results of Baek et al.(2015)[1].
The control group did not differ from the HD- and BK restraint. It would be expected that some form of restraint causes a degree of stress in comparison to a baseline. Explanations for result could be that although mice were habituated to the test cage, full habituation did not take place or that the transfer of a mouse to a different cage caused a degree of stress. In a study of Meijer et al.(2005)[34] pCORT in mice was significantly higher after being lifted by the tail in comparison to a control group, in our study we lifted the mice by the tail to transfer them to the test cage. Results of IRT seem to confirm a probable stress response in the control group. This would mean that a stress reaction due to transfer or unsuccessful habituation is present during all our experiments. Measurements in the control group cannot be considered as basal value of pCORT and this effect is probable to have interfered with our other stress related results as well thus not obtaining reliable answers to our hypotheses.

Although mice were habituated to the test cage a week before the experiment started, apparently full habituation to the test cage did not take place. Natelson et al.(1988)[36] describe after reviewing several studies with rats that habituation to a stressor depends on intensity, duration and frequency of the stressor. A stress response can lessen over time but this is not always the case in several studies were the stressor was of short duration. Leussis et al.(2006)[37] confirm this, adding that studies with mutant mice indicate that there is an genetic component as well. Salomons et al.(2010)[38]_ used Balb/c mice to study habituation of certain strains(measuring blood plasma corticosterone among other parameters). The researchers describe that Balb/c mice were initially highly anxious (evaluating behavior), but habituated quickly to the new test environment. Mice were put in the novel environment for a shorter duration in comparison with our experiment, but the frequency of which they were put in the novel environment was higher (4 times a day for 5 days).
Considering this, habituation in this strain of mice is possible. Our habituation protocol differed from the study of Salomons et al.(2010)[38], this may have led to unsuccessful habituation.

When pCORT was compared in the SR category, no significant difference was found. It would be logical if the same differences were found as in the mice that were restrained repeatedly. We cannot exclude the possibility that this could be due to the i.p. injection given. The unsuccessful habituation or transfer of the mouse to the test cage could also have been of influence here.
On T3, not only female Balb/c, were used, but C3H male mice as well. Statistics revealed that the C3H male mice had significantly lower pCORT levels, which indicates that the stress reaction of different sexes or breeds of mice is not the same.
In our experiment different experimenters worked with the mice. Experience of the experimenter could lead to difference in time needed to restrain the mouse (even though we attempted to keep this equal for all mice), which could lead to a higher degree of stress. In addition two of the experimenters had been working with the mice before this experiment, mice may have recognized these experimenters and experienced a different degree of stress.
If further study should be performed, it is advisable to use naïve mice of the same gender and breed which can be measured in the home-cage or can be fully habituated to a test cage. Experimenters, unknown to the mice, with the same experience should perform the tests to obtain reliable results. Recommendations for further study are described in box 1 and 2.

Infrared Thermography

Considering that mice held according to the HU restraint had the highest pCORT blood plasma levels it would be logical to see a different temperature change compared with the restraining methods and the control group.
However, tail- eye- and ear temperatures of the mice after the control-, HD- and HU restraint did not differ. Explanations for this effect are that temperature change does differ, but this difference is not significant enough to be detectable by the infrared camera. It may also be that that the pCORT reaction, which is mediated through the HPA-axis, differs from the body temperature reaction during an acute stress response, because temperature is mediated by another stress response system. Supporting this theory is the fact that significant differences between temperature change after BK(+injection) restraint and after other restraints are not seen in the pCORT level of the mice. In addition, the fact that correlation between eye-, tail- or ear temperature and pCORT was not possible. The temperature change pattern of the control group does not differ from the other groups where mice were restrained. Considering pCORT blood plasma level mentioned earlier in the control group, this result is likely due to the unsuccessful habituation or transfer of the mice to the test cage, causing an acute stress reaction visible in temperature change during all the experiments. Due to this we did not obtain reliable baseline values of temperature in the different body areas.

Peripheral areas

The IRT images significant decrease in tail temperature during a stress reaction, confirming part our hypotheses and the results of Lecorps et al(2016)[29] and Vianna et al.(2005)[28]. However, this pattern is not seen in ear temperature which was considered a peripheral area as well.

The cause of temperature change during an acute stress reaction in different areas of the body has been discussed in literature. Several studies have determined changes in blood flow in the mouse tail[39], however the function of this has been studied in rats more than in mice. In rats various studies have indicated that tail temperature plays an important role in thermoregulation of the rat. Arteriovenous anastomoses (AVA's) near the base of the tail have an important contribution to this effect.[40] AVA's are normal vascular channels between the arterial and venous side of the circulation.[41] They play an important role in cutaneous blood flow regulation and are innervated by the sympathetic nervous system.[42] The sympathetic system can cause vasoconstriction in the rat's tail after aversive stimuli. As mentioned earlier the functions of this effect are suggested to be the redistribution of the blood to important organs and prevention of blood loss[26]. Busnardo et al.(2010)[26] suggest that measuring the temperature in the rats tail is an indirect way to assess the activity of the sympathetic nervous system. If mice have arterial anastomoses in their tail remains unclear in literature, but is likely considering the similar thermoregulative potency in comparison to rats.[39] The observed decrease of temperature in this study is therefore likely to be mediated by the sympathetic

system.
We found that tail temperature after the BK restraint and i.p. injection decreases to similar levels as after the HD and HU restraint, but from a significantly higher starting point (about 2 degrees). This indicates a lesser vasoconstriction or even a vasodilatation (baseline reference remains unknown) of the tail vessels immediately after restraint and i.p. injection.
The BK restraint + i.p. injection caused a significant different temperature change. Without baseline values it remains unknown what this significant difference means in terms of stress, but it is obvious that different degrees of stress can be measured through IRT in the tail.

Other studies have observed vasoconstriction in the ear, mediated by the sympathetic nervous system as part of peripheral vasoconstriction in several species.[25,27] Vianna et al.(2005)[28] could not confirm this in rats, even though a decrease in temperature in other peripheral body parts was measured. The authors suggest that ear vasoconstriction may be species-specific. For example, guinea-pig's and rabbits have arteriovenous anastomoses (AVAs) in the ear[39], while the rat's ear is small and does not have arteriovenous anastomoses.[43] A study of Barker et al.(1989)[44] indicated that mice don't have AVA's in the ear. However the ear does also have capillaries which can vasodilate as well[43] and may therefore have an effect on ear temperature.
In our study even though no significant interaction between measurement and restraining method was found, considering the differences in restraining methods, all 5 subsequent measurements after the BK+injection restraint were significantly higher than after other restraining methods. This effect can also be seen in the average ear temperature in figure 9a. Apparently even though the mouse ear does not contain AVA's, after BK restraint and i.p. injection the ear capillaries can vasodilate to a degree that a significant temperature change can be measured through IRT.
A significant pattern during 5 subsequent measurements after all restraining methods was found, but in contrast to our expectations, temperature did not decrease during the 5 measurements. Temperature between measure 1 and 2 decreased, indicating vasoconstriction immediately after restraint with and without i.p. injection. An increasing temperature between measurement 2 and 4 was found indicating vasodilatation between 5 and 15 minutes in the test cage. Without more knowledge about how this area is innervated it is difficult to say to what extend the stress response systems of the body were of influence on this effect.
From these results it is difficult to conclude that an acute stress reaction can be measured in the ear, a pattern was seen, it was not the pattern that we expected from an area that we considered peripheral. From the significant differences that were found after BK (+ i.p. injection) restraint, we can conclude that different degrees of stress can be seen in ear temperature to some extent.

Central area
The IRT images showed an significant increase in eye-temperature during an acute stress reaction, confirming part of our hypotheses. No significant difference was found when comparing eye temperature changes after different restraining methods.

Eye temperature change during stress is observed in several species through IRT. How this change in temperature is mediated is difficult to say. In larger animals such as horses, dogs and cattle a change in eye temperature is found after a pain- or fear experience.[30,31,33]
Eye temperature changes after restraint or handling have been measured before. For example in a study where chickens were handled, the animals showed an initial decrease in eye temperature which after a few minutes increased above baseline Edgar et al.(2013).[45] There is not literature available on restraint and eye temperature in small laboratory animals. However Vianna et al.(2005)[28] and Lecorps et al.(2016)[29] indicate that eye temperature increases in mice and rats after exposing them to fearful or anxious situations. The present study confirms this although we cannot differentiate between treatments.

No explanation is described in literature for the rise in eye temperature of small mammals.
A delayed increase of temperature during or after exposure to a stressor could be due to vasodilatation of the capillaries or larger blood vessels behind the eye as part of an increase in the core body

temperature after exposure to a stressor, earlier described as the phenomenon SIH. If this is the case corticosterone in the blood may be correlated to eye temperature.[18]. In our study it was not possible to correlate AUC of mean eye temperatures to the pCORT in the RR category. Furthermore in the study of Vianna et al.(2005)[28] the core body temperature (measured by radio telemetry) of rats seems to rise only after exposure to the stressor, while the eye temperature already rises during exposure, though they could not confirm this statistically.
Considering this, if there is an immediate increase in temperature during exposure to a stressor this cannot be part of SIH which occurs after several minutes, but may be the result of redistribution of blood to the brain mediated by the sympathetic nervous system discussed earlier.[26] If this is the case it would be interesting to correlate another parameter mediated by the sympathetic nervous system to the immediate change in eye temperature. From our results and literature, it is clear that an acute stress response can be measured through eye temperature, if different degrees of stress can be measured in this area remains unclear.

Correlations
A trend, but not a significant correlation between the increase in eye temperature and the decrease in tail temperature was found. When looking at other deviations in average temperatures, for example the significantly high tail temperature after BK restraint and injection, this is not obvious in the eye area of the mouse.
This indicates that the changes in temperature due to an acute stress reaction in the measured body areas is at least in some extend mediated by different stress response systems. If further study should be performed it would be advisable to measure more than one body area with IRT as they do not show the same pattern of temperature change.
In our study it was not possible to correlate AUC of mean eye-, tail- or ear temperatures to the pCORT after different restraining methods or in the control group. This would indicate that the HPA-axis does not fully mediate change in body temperature in these areas. However, we only calculated the AUC of mean temperature. It may be that correlation can be found when calculating the AUC of the three body areas of every mouse individually.

Concluding this discussion about the mechanisms that are most probably behind the temperature changes in different body areas measured by IRT. The temperature reaction during or immediately after the stressor-exposure is mediated by the autonomic nervous system. After several minutes the HPA-axis may contribute to the temperature reaction in different body areas, most likely in the more centrally located body areas reflecting core body temperature as part of SIH.[18] Magnitude of the thermogenic effect will likely depend on the innervation as well as the size of a certain innervated area or structure, for example AVA's will likely have more effect on the overall temperature than capillaries. Therefore the temperature change will not be the same in the eye- ear- and tail areas of the body.
An acute stress reaction can be measured through IRT in the eye-, tail- and in some extent ear area in mice. If IRT is a sensitive enough parameter to determine different degrees of stress in body areas and how this effect is mediated is yet to be defined. More research is necessary to determine if IRT is suitable to determine different degrees of stress in mice.

In this experiment we measured the body temperature change of female Balb/c mice. There are several factors that could have influenced the results and these should be considered when performing further study, described in the recommendations in box 1 and 2. It is unknown if the oestrus cycle of the female mice has an influence on body temperature during a stress reaction. Health status could also have an effect on temperature change. We did not determine in which part of the cycle mice were and performed tests to determine if mice were completely healthy.
We found a difference in pCORT between female Balb/c and male C3H mice which differ in sex as well as in breed indicating a difference in stress reaction. This difference may be present in temperature change as well, further study is necessary to confirm this.

8. Conclusion

In the context of the 3R principle we determined the accuracy and degree of stress using different i.p. injection restraining methods. With these results we have gained information to be able to further refine the i.p. injection technique, a broadly used technique in laboratory animal science.
The accuracy of the i.p. injection using either the HD, HU or BK restraining method was equal. Although the BK restraint seemed to show more variation in location when it is misplaced.
The acute stress reaction of mice that were repeatedly restrained during four subsequent experiments was significantly highest after HU restraint. However, this effect could not be seen in the following experiment where mice were restrained HU and received an i.p. injection. A follow up study with naïve mice that are habituated to their environment should be performed to confirm this.
An acute stress reaction initiates a thermogenic response which can be measured through infrared thermography in the area of the eyes- tail- and in some extent the ears. The thermal changes of the measured body areas could not be related to the pCORT levels in mice. To validate infrared thermography as a reliable stress parameter further study is necessary. A follow-up experiment can determine if body temperature change can be correlated to a validated stress parameter and if infrared thermography is a sensitive enough parameter to determine different degrees of stress in mice.

9. Acknowledgements

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10. Appendix

Box -1

Recommendations

I.p. injection technique in practice

From the results in this study considering accuracy and degree of stress, it is the authors opinion that the BK technique is a valid i.p. injection technique to be used in practice. Although there seems to be more variation in location when the injection is misplaced, the frequency of misplacement is not significantly different in comparison to other techniques. When an experimenter becomes more experienced with this technique, the method of injecting will become more natural and frequency of misplacement or variation of location may even decrease. Considering the results of Baek et al.(2015)[1], the fact that the BK restraint with i.p. injection did not lead to significantly higher pCORT levels and the fact that the psychological factors seems to have a greater influence on the degree of stress than the actual experience of an injection contribute to the probability that the BK technique is less stressful for the animals, due to the minimal restraint of the animal. Of course it needs to be tested if this method of injecting is suitable for different strains, ages and sexes of mice as well as naïve Balb/c mice.

Use naïve mice of the same strain and sex

The mice used in this study were of 42 weeks of age and had already been used for educational purposes, this may have had affected the results. The degree of stress in an animal is not only mediated by the stressor that the animal is exposed to. A variety of factors contribute to the degree of stress that is measured in a laboratory animal. In addition to the kind of stressor and genetic factors, previous experience and environmental conditions are relevant when comparing studies.[46]

The mice have been handled by students practicing restraining methods and i.p. injections. In addition the mice have been lifted up by employees once a week to clean the cage. The degree of stress measured in this study may not be representative for other mice, because the studied mice could have to some extend habituated to the handling. If further study should be done an experiment with naïve mice could be performed to exclude this possible interference.

Avoiding environmental differences and measuring mice in their home cage

The study of Garcia et al. (2001)[47], where an ultrasonic probe was placed around the tail artery, indicates that the blood flow in the tail of the rat is very sensitive to environmental events, tapping on the cage caused vasoconstriction. Vianna et al.(2005)[28] suggest that noises in the room could even have affected the variation in baseline measurements of tail temperature. To determine if the infrared camera is suitable to measure different degrees of stress, environmental changes, which can cause vasoconstriction and therefore temperature change, should be avoided in a follow up experiment.

To avoid a reaction due to disturbance of the cage, we restrained and measured a pair of mice at the same time and measured one pair of mice out of one cage per day. However our protocol on T3 may have caused extra stress. During the 4 subsequent days, each day the number of mice in a group became less. This may have caused social stress. In a follow up experiment, disturbance of the cage and changes in the group should be avoided.

As discussed, in our experiment the transfer of mice by lifting them by the tail and the fact that mice have probably not fully habituated to the test cage may have influenced all our stress related results. To obtain reliable pCORT results. It would be best to measure mice in their home cage. If this is not possible certainly a longer more frequent habituation protocol as used in Salomons et al.(2010)[38] is necessary.

Correlating pCORT and temperature change

To determine if there is a correlation between blood plasma corticosterone and body temperature change measured by IRT, an experiment in which cortisol is administered to mice after which the temperature change is measured through IRT could be performed.

Box-2

Recommendations

Measuring other stressparameters regulated by autonomic nervous system

Meijer et al.(2006)[19] could correlate the corticosterone plasma level tot the heartrate of mice. Nevertheless the authors advise to measure both parameters as they work through different stress response systems (neuroendocrine response vs. autonomic response).
An autonomic response parameter such as heartrate or arterial blood pressure may be more correlated to initial change seen in the body temperature measured by IRT. As the change in temperature during or immediately after exposure to a stressor is most likely only mediated by the autonomic nervous system. pCORT is shown to rise after several minutes. After several minutes body temperature may be correlated with the level of corticosterone as part of SIH.[18] This potential correlation however does not exclude a contribution of the autonomic nervous system.
In a follow up experiment it would be advisable to measure both pCORT and an parameter that is mediated by the autonomic nervous system.

Measuring temperature more frequently

In studies with other species eye temperature shows a decrease in the first moments of the acute stress reaction.[,33,45] Therefore measuring more frequently and if possible measuring during experience of the stressor would be advisable in a follow-up experiment to determine if a decrease in eye temperature is present in mice as well.

Measuring temperature change of more than one body area

As described earlier, during an acute stress reaction the temperature change in different body area's is different. In a follow-up experiment is would be advisable to measure more than one body area. An acute stress reaction can be measured in the eye- tail- and in some extend ear area in mice. Temperature change do not show the same patterns, therefore to further evaluate temperature change as a parameter of stress more than one body area should be measured.

Measuring temperature change for a longer period

It would have been interesting to measure the course of temperatures longer than 20 minutes because the eye temperature still seemed to be increasing after the BK restraint and i.p. injection.
A temperature baseline can be determined by measuring temperatures for a longer period. Zethof et al.(1993)[48] reported that temperature after handling measured rectally was back to baseline after 60 minutes. Clement et al.(1984)[49] describes that is can take several hours before temperature(measured by radio telemetry) will go back to baseline after handling mice. This can be taken into account when designing a follow-up experiment.

Attempting a set-up where eyes and tail are always visible

In our experiment certain data was missing due to the occurrence that a tail or an eye was not visible in the test cage during the recorded 60 seconds. In a follow up experiment it would be advisable to design a set-up in which the areas of interest are always visible to the camera.

Separately measure restraining method and i.p. injection

In our study we observed several differences in temperature change after BK restraint and i.p. injection. To know which factor is responsible the factors should be measured with the infrared camera separately. pCORT should also be measured with separate i.p. injections and restraining method to determine if an i.p. injections has an effect on pCORT in mice held in the same restraint.