Chapter 10

Using Rodent Models to Simulate Stress of Physiologically Relevant Severity: When, Why and How

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1. Introduction

Given the demands of modern life, it is no wonder that the concept of stress has become a household topic for discussion. Also in the academic realm the phenomenon which is stress, is topping the charts in terms of research interest. The short term costs as well as the long term maladaptive effects of stress have been a popular topic of research in especially physiology and psychology for the past few decades, ever since Hans Selye defined the term "stress" in 1956 (Selye, 1956). Stress-related chronic disease, such as cardiovascular disease, diabetes and depression, places an ever-increasing burden on society – medically, socially and financially. Therefore, if we are to limit the spread and impact of this "pandemic", it is imperative to properly manage the effects of stress on our bodies. This of course, is only possible if we have a complete understanding of the body's response to stress.

The response to stress is almost never localised and contained. Rather, a stress response is initiated in response to a local physical (e.g. contusion to skeletal muscle) or mental (e.g. the loss of a loved one) stressor, but always culminates in a wide-spread, systemic response process that affects many organs and systems. Consider for a moment a less complex research model in a different discipline. Metabolic pathways (e.g. the Krebs cycle or glycolysis) can easily be manipulated in cell culture assays using one single cell type at a time, since these pathways (including substrate supply and waste removal systems) are contained in its entirety within each cell. In contrast, with the stress response pathways this is clearly not the case.

The stress response is a complex network of events, which is directed via two interlinked pathways, one endocrine (the hypothalamic pituitary adrenal (HPA)-axis) and one neural

(the locus coeruleus norepinephrine (LC-NE) or sympatho-adrenal medullary (SAM)-system). While the neural pathway is mainly activated neurally in response to stress perception, leading to the well-known "fight-or-flight" response, the endocrine pathway has many more triggers. Apart from neural activation, the HPA-axis is also activated by a large number of hormones and even chemical messengers, such as interleukin-6, a cytokine and mediator of inflammation, which is known to increase cortisol secretion. A contributing factor to the complexity of the HPA-axis is the fact that cortisol, the main end product of this stress response, has both endocrine and metabolic functions. Although cortisol is commonly known as the "stress hormone" in the context of psychological stress, its main function is actually metabolic – to maintain glucose supply to the brain. Therefore, the HPA-axis is structured not only for activation in response to perceived stress, but also to react to metabolic stimuli. Furthermore, while the stress response should be powerful and fast in an acute stress situation, the response should be controlled and relatively more limited in a situation of chronic stress, to prevent detrimental effects to the organism in the long term. One can appreciate therefore the need for relatively complex signalling networks in this regard, which serves to activate, limit or inhibit the stress response. To achieve this, numerous molecular mechanisms are in place, and react and interact in response to various stress signals. To give just one example, the glucocorticoid receptor, which is present on most cells to enable cortisol's effect on these cells, is up-regulated in response to acute stress, but down-regulated after a period of chronic stress.

Such complexities make the choice of a suitable stress research model both a difficult, and vital one. While some mechanisms, e.g. activation agents of specific adrenal or pituitary cell types, may be elucidated in cell culture, a whole-system model is required in order to assess the net effect of any stressor to these systems. This does not imply that there is no place for *ex vivo* or *in vitro* studies in the discipline of stress research, far from it! A large number of cell-based – and more recently organotypic culture-based – studies have contributed substantially to our understanding of specific mechanisms and/or partial pathways relevant to stress. The important point here is that ideally, *in vitro* work should at some point be followed up by *in vivo* investigations, in order to test the applicability of results obtained *in vitro*, to a whole system.

The importance of *in vivo* assessments, and the need for conducting them in a model specifically suitable to answer the question at hand, is clear when one considers the huge number of described animal models in the scientific literature. Apart from more conventional models using genetically "intact" rodents, recent advances in biotechnology have made possible research using non-physiological models such as gene-knock out animals. These animals may be genetically modified to erase the gene coding for a particular protein, so that the researcher may elect to produce animals completely lacking a particular protein of interest (e.g. IL-6 knockout mice), or in some cases lacking it in only one organ or system (e.g. STAT-3 knocked out or "switched off" in skeletal muscle only). These models may be used to shed light on various *in vivo* mechanisms which could previously not be properly elucidated using the conventional methods. However, these models have their

limitations. For example, when doing research on inflammation, an animal in which a pro-inflammatory cytokine was knocked out, may display increased or decreased basal levels of other pro-inflammatory cytokines, or an altered anti-inflammatory cytokine profile, or even up- or down-regulated cytokine responses on activation, as a spontaneous compensatory mechanism. The resultant net effect of the genetic manipulation therefore may result in a model that is not physiologically accurate, and responses measured may not accurately reflect normal *in vivo* responses. Furthermore, these compensatory mechanisms and/or the mere absence of an important protein may also result in other – sometimes unanticipated – side-effects (such as severe constipation in IL-6 knockout mice). Apart from being a confounding factor in the intended study, in some cases these undesired outcomes may result in poor health or even shortened life expectancy of the experimental animal, so that it limits the application of such a model even further. Of course, chain-reaction compensatory responses will also limit the extent to which results obtained in such models, may be extrapolated to a (at least genetically) normal situation.

Relatively "old-fashioned", or more conventional methods, when applied optimally, therefore still have an important place in research, both in applied areas such as pharmacology and in areas of basic research. Only when a situation that is physiologically relevant is recreated or simulated, can one realistically assess either the response to a challenge, or the outcome of a remedial intervention.

Therefore, in this chapter, I would like to reflect on methods used to simulate a variety of stressors to the body, starting with a variety of models used to simulate psychological stress, ranging in severity from non-extreme (mild) psychological stress to extreme mental trauma. I will also discuss general considerations in picking the appropriate animal model to use, which may determine the difference between success and failure in your research. Details on the various models will be provided, including issues such as repeatability and standardisation. Models will also be discussed in terms of their suitability for different research approaches or objectives, as well as in terms of their limitations. Arguments for and against the use of any particular model will also be illustrated using actual research data.

2. General considerations when choosing a rodent stress model

Small rodents are an obvious choice for research models in need of a whole body system, since they are relatively small and prolifically reproducing mammals, making them relatively economical to breed and house. Although rats and mice are physiologically very similar to humans in terms of organs and systems implicated in their response to stress, there are some fundamental differences between rodents and humans that may greatly influence results obtained using such models. It is necessary to understand these differences and the impact that it may have on any particular study employing rodents, and to adapt protocols to accommodate these differences in order to maximise the validity of results obtained. Let us consider just a few general factors that have huge impact on study outcome, but which may often be ignored or overlooked.

2.1. When to stress: lights on or off?

The timing of stress exposure, interventions to relieve stress and sampling of blood or tissue for analysis is a vital consideration, with many confounders complicating the issue. Firstly, the rat is nocturnally active, while humans obviously are not naturally nocturnal. Therefore, the question arises of whether to stress the rats during their active time, at night, or during the day, when they are asleep – which would most accurately mimic the physiological responses of humans? One could argue that it would be more applicable to expose an animal to a psychological stressor while it is awake, i.e. in the darkness – after all, how can one stress a rat when it is half asleep during the day anyway? However, this seemingly logical argument is not correct, for a very simple reason. Whether the rodent is asleep or wide awake when exposed to an experimental stressor is not the determining factor – rather, the normal rhythmic changes of hormones over the course of a day hugely affects the ability to respond to stress.

A typical circadian rhythm graph for corticosterone is presented in Figure 1. The circadian rhythm illustrated is expected in experiments employing a normal light-dark cycle – convention would be a 12 hour light-dark cycle, with lights switched on at 7am, and off at 7pm. Reversal of the light cycle has significant effects on the circadian rhythms, the "pattern" of which follows the delay in timing from the conventional one. This effect of light and darkness may be partially explained by the fact that sympathetic input to the adrenal gland is photo-sensitive: in periods of darkness, a dramatic increase in basal norepinephrine secretions from sympathetic nerves occurs (Hashimoto et al., 1999), so that basal corticosterone secretion is up-regulated in periods of darkness. However, one can also see from the curve that corticosterone secretion starts to increase after the nadir at a time of day when there is still much light – this further illustrates the complexity of this regulation, pointing to the existence of additional important causative factors.

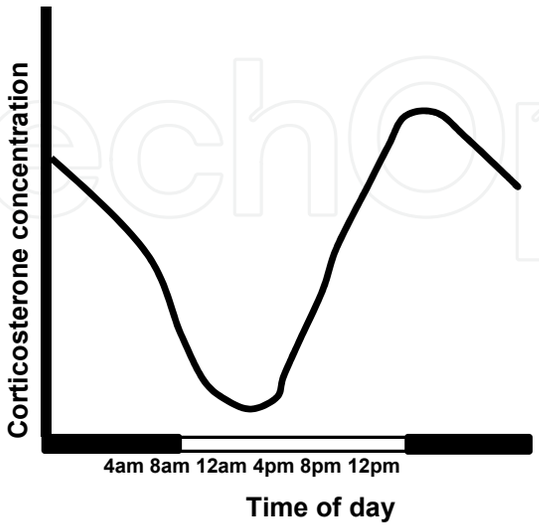

Figure 1. Expected circadian rhythm graph for corticosterone in rats. Dark bars at the bottom indicate "lights off" periods and open bar indicates the "lights on" period.

It is of importance to note that adaptation to changes in lighting conditions is not synchronised, and so does not occur within similar time frames, for all hormones. For example, while the corticosterone rhythm was shown to adapt to a 12-hour delay (phase reversal) and become constant after about 6 days, the rhythm for adrenaline only adapted after 10 days, indicating that the pituitary adrenocortical system adapts more readily to light-dark cycle shifts, while the sympatho-adrenal medullary system requires relatively more time (Miki and Sudo, 1996).

Researchers working on models using juvenile animals should also take note that the circadian rhythms for these hormone fluctuations are not fully developed at birth. The diurnal rhythm for corticosterone for example is only regular from about day 30-32 in rats (Allen and Kendall, 1967). Also, circadian rhythms are affected by many stress-related disorders – in this context, a chronic mild stress model of depression has been shown to cause fluctuations in corticosterone rhythm which only normalised after 8 weeks of chronic mild stress, and which was dependent on resilience of animals exposed to stress (Christiansen et al., 2012).

Therefore, in planning an experiment, it is most important to decide whether the stress exposure and sample collection should take place during the rising or falling phase of a hormone pulse. In the context of stress for example, one would time stress exposure and sample collection to coincide with the natural decrease of hormones expected to increase in response to stress, such as corticosterone and the catecholamines. Otherwise, if done at a time when hormone levels were increasing naturally, the circadian rhythm may effectively mask the acute response to stress. Even though these experiments should always include control samples taken from unstressed animals at the same time of day, unsynchronised sampling may still increase the variability of data, and thus decrease statistical power. This consideration is especially important in models employing physiologically relevant levels of stress, since the response seen is usually not enormous, and any potential confounders should be excluded as far as possible. Therefore, a suggestion is that all stress exposure interventions should be performed in the early morning hours, so that subsequent sample collection may to completed before noon, when the nadir for corticosterone occurs.

2.2. Metabolic rate

Another very important way in which rodents differ from humans is their much faster basal metabolic rate. Rats have a metabolic rate roughly 10 times and mice 30 times that of humans. This would obviously have huge implications for any study design with a pharmacological component. For example, when testing the potential of a stress relief medication, one would have to either increase the dose recommended by the manufacturer for human consumption, or decrease the dosage interval in rodents to ensure the maintenance of a therapeutic concentration of the drug at the level of the target tissue. Both these approaches have their drawbacks though. On the one hand, administration of a mega dose may result in intolerance reactions to the drug, most often including side effects such as gastroenteritis, with obvious confounding results given the interaction between

inflammation and the glucocorticoid response. When choosing this method, parameters to monitor gut integrity, such as prostaglandin E2 levels or serum lipopolysaccharide levels, should ideally be included in the testing profile. On the other hand, decreasing the dosage interval requires more frequent handling of the experimental animals, which increases the possibility of an undesired stress response to the constant handling. This last obstacle can be partially overcome with the use of osmotic mini-pumps – these tiny pumps are implanted subcutaneously behind the neck of the rodent where it cannot reach, and releases the drug constantly at a pre-selected rate and over a pre-selected number of days. It is debatable however whether or not this method accurately reflects *in vivo* conditions for and effects of a drug that is, for example, intended to be administered orally once or twice a day, rather than continuously. A further limitation of this method is that labile substances can't be tested in this way, since the drug can only be maintained at body temperature (i.e. not cooled) for the duration of the infusion.

2.3. Social issues

A factor that should be of particular interest to researchers investigating effects of psychological stress, is the social hierarchy that exists within experimental rodent colonies. Rats in particular are a very social species, and individual housing of rats actually causes a degree of psychological stress. Therefore, standard practise is to house rats in groups of four to five, when using standard sized cages. This in itself is a limiting factor, since it is logistically not really possible to monitor appetite or food and water consumption of individual rats (which are usually done using metabolic cages in which rats are individually housed) without causing a stress response to housing conditions. Logistic factors aside, it is interesting to see that within these small groups, a social hierarchy quickly emerges, with some rats being submissive, while others are clearly dominant. Dominant rats have been shown to grow faster and to be relatively more resistant to stress interventions that submissive rats. This is both good and bad for the researcher. On the one hand, having this social hierarchy in a way simulates human situations, making the model more representative of the human population as a whole. On the other hand, the variation in the response to stress resulting from social hierarchy results in great variations in data obtained within the same experimental group, which could hide differences between experimental groups. This lowers the statistical power of any experiment, necessitating the use of larger experimental groups, which of course is more time and resource consuming. In our experience, experimental groups for the purpose of research into the psychological stress response should consist of at least 10-15 rats, on condition that all rats have been properly accustomed to the environment, handlers and protocols.

2.4. Practical tips

Apart from the factors discussed above, there are a few more general considerations to keep in mind when setting up an animal model of stress. I will touch on these just briefly. Research has shown that the mood (emotional state) of the animal handler(s) also affect the

basal anxiety level of animals. Therefore some personality types may be more suited to work using animal models than others. For example, in our group we had two students conducting stress studies on sibling rats from the same litters. One student was completely at ease with the rats and handled them with natural ease, while the other student was very nervous around the rats and anxious about handling them. When assessing corticosterone levels in the control rats from the first student's study, serum concentrations were all lower than 10ng/ml. However, those from the more nervous student all had values in excess of 40ng/ml. (All samples were collected at the same time of day, so that diurnal variation did not play a role.) Of course, the fact that even unstressed animals had clearly elevated corticosterone levels, severely limits the conclusions that may be drawn from this specific experiment.

Different strains of animals have also been shown to vary substantially in their natural sensitivity to stress. This has been comprehensively reviewed elsewhere, in the context of neurobiology (Ellenbroek et al., 2005). Perhaps of specific interest for the stress researcher is the fact that these differences in stress sensitivity seems to be the effect of differences at adrenocortical level, rather than a central effect, since restraint were reported to elicit similar hippocampal and hypothalamic responses across five rat strains, although differences were quite clearly present at adrenal level (Gomez et al., 1996). An interesting fact is that some of these supposedly strain-dependent differences are more the result of nurture than nature: for example, if a spontaneously hypertensive rat (SHR) is reared by a Wistar-Kyoto rat (WKY), its hypertension is significantly less pronounced. One should therefore exercise caution in the selection of a strain to breed for the purpose of stress research. Furthermore, even within an established strain, differences occur. For example, first-time rat mothers have been shown to yield pups with relatively less resistance to stress, so that litters from first-time mothers should be avoided by the stress researcher. Also, a vital point to remember is that the experimental animal does not speak human! When conducting research in humans, it is possible – and ethically required – to explain to any volunteer the intervention that he or she will be subjected to, including expected risks. Therefore, when a human is stressed experimentally (e.g. by participating in a maths test or public speaking), although they will mount a psychological stress response, they also know that the test, or stressor, won't be permanently detrimental. A rodent on the other hand, has no way of knowing whether an acutely applied stressor will be lethal or not, so that even mild stressors are perceived as quite severe the first time. Therefore, if the requirement for research purposes is to simulate stress of a physiologically relevant severity in rodents, the stress intervention may actually seem relatively mild in comparison to what one might expect to be necessary.

From just these few considerations it is clear that the ideal *in vivo* model for psychological stress may simply not exist. However, if one is aware of potential confounders, the protocol may be optimised, and interpretation of results approached with the necessary caution, making in vivo models very valuable and realistic tools. So, how does one go about setting up the optimum model?

3. Design and setup of an animal model

Moving on to the actual setting up of a model, there are several precautions to include in the protocol, that are unique to studies on stress, especially psychological stress. For this section, I will limit myself to a discussion of rat models for stress, since this is the species of choice for this discipline, and also the species that I have most experience working with.

Putting first things first, one has to decide what situation of stress should be simulated. This is directly dependent on the research question. For example, if the question is related to the effect of a calming tablet administered to someone who has been exposed to a sudden trauma (e.g. car hi-jacking), a model where rats are subjected to a severe acute stressor is obviously the best choice. When a daily supplement is tested for stress relieving properties, or the effect of long-term occupational/stress on a specific organ is investigated, a model with multiple exposures to a relatively milder stressor would be more ideal. Sometimes, it may even be useful to combine protocols to achieve a mix of acute and chronic stress, in order to most accurately simulate actual human situations. Rats have been reported to be able to adjust to any mild stressor within a period of about 3 days (Garcia et al., 2000). Therefore, a study requiring mild stress to continue for a relatively long time, may require combination of a number of stressors in order to maintain a stressful environment.

3.1. What does a rat find stressful?

The decision of the type of stressor again depends on the situation being simulated. Stressors in real life vary from "mild irritation" to traumatic. Similar variety is therefore required in models for stress. Arguably the most popular simulation of prolonged trauma is a model known as maternal separation. Normally, pups remain with their mother throughout the first few weeks of their life until they are weaned at the age of 21-30 days, dependent on laboratory standard operating procedure. In the maternal separation model, rat pups are removed from their mother during a critical time in their development, usually during the first two or three weeks after birth, for a period of three hours per day. This traumatic separation is characterised by changes in both behavioural responses (such as anxious-like behaviour and hyperactivity in the open field test) and HPA-axis responses (such as decreased expression of glucocorticoid receptor in the hippocampus and ≈15% higher basal blood corticosterone concentrations) to stress, that persists into adulthood. These changes suggest an increased natural anxiety in response to chronic severe stress during early development. This technique is uniquely suited for and commonly used for investigating the development of psychiatric disorders such as anxiety and depression. Note that the endocrine responses seen in this model is relatively small in comparison to for example restraint stress models, even though it represents trauma, i.e. the most severe type of stress. One has to keep in mind though that these changes are assessed in the "rested" state, and reflects chronic changes, which are always smaller in magnitude than acute responses assessed directly after application of an acute stressor.

A somewhat milder form of stress may be simulated using restraint (sometimes called immobilisation). This technique is highly variable due to research group-specific differences in the execution of this technique. On the extreme end, animals are literally taped down on a flat board, immobilising them completely, for a period between one and two hours. Rats are fairly vocal in response to this particular protocol, so that it is advisable to conduct this particular protocol in soundproof facilities, to prevent negative effects on the rest of the animals housed in the same unit. A much milder form of restraint is to place rats in small cages that limit their movement. An example of a Perspex restraint cage (restraining up to 6 rats simultaneously) as used by our group is presented in Figure 2.

This particular cage has compartments 6cm wide x 7cm high x 18 cm long, and works best for restraint of mature Wistar rats, weighing around 300-350 g (this type of restraint is only successful when rats fit tightly into restraint compartments). Note the use of Perspex as material for the cages: this prevents the rats from having a stress response to being isolated from their group because they can still see their "neighbours". Also, body heat from peers warms the sides of the cages, creating a similar effect to when rats sleep clumped together, as they habitually do. When rats are put into these compartments, they typically turn around once or twice (invariably getting stuck halfway through the turn), and then stop trying to move. Grooming behaviour – a known self-pacifying behaviour in rats – indicates that rats are feeling claustrophobic, i.e. a psychological stress response can be expected. Rats are usually restrained for a period of 30 minutes to 2 hours once per day. During this time, they do not have access to food or water, but sufficient ventilation holes at both ends allow for normal ventilation. Keep in mind that when restraining nocturnal animals during light hours, they won't have a huge requirement to feed or drink, so that the absence of food and water is not perceived as stressful and does not impact significantly on their normal metabolism.

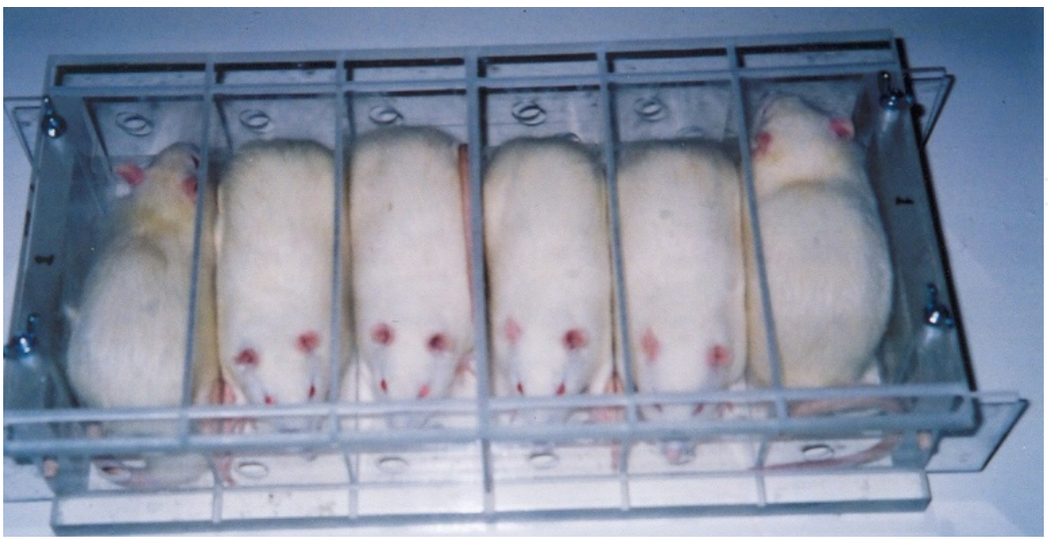

Figure 2. Restraint stress by confinement in purpose-designed Perspex cages elicits a mild form of psychological stress in rats. Adult male Wistar rats weighing more than 300g were used in this particular instance. Note the two rats on each side that were able to turn around in the cage once, but prefer not to attempt it again.

The response obtained using this model is of mild severity, and is ideal for studying normal adaptation to both acute and chronic stress. Given the wide relevance of this severity of stress to the human population, it is also a valuable model to use in the pharmacological, psychological and physiological testing of therapies, drugs and daily supplements intended to decrease stress levels or counter the side-effects of stress. This particular model is relatively easy to standardise in terms of diet, stress duration, light-dark cycle, etc. and is highly repeatable within a research group, as long as particular care is taken in selection of animal handlers and other factors already discussed. However, inter-research group differences do exist, so that care should be taken to consider changes in stress intervention protocols when comparing results reported by different laboratories. Commonly expected values with the restraint model used as an acute stress intervention lasting one hour, in our hands, are presented below (Figure 3) for changes in body mass, corticosterone, testosterone and the pro-inflammatory cytokine interleukin (IL)-1β.

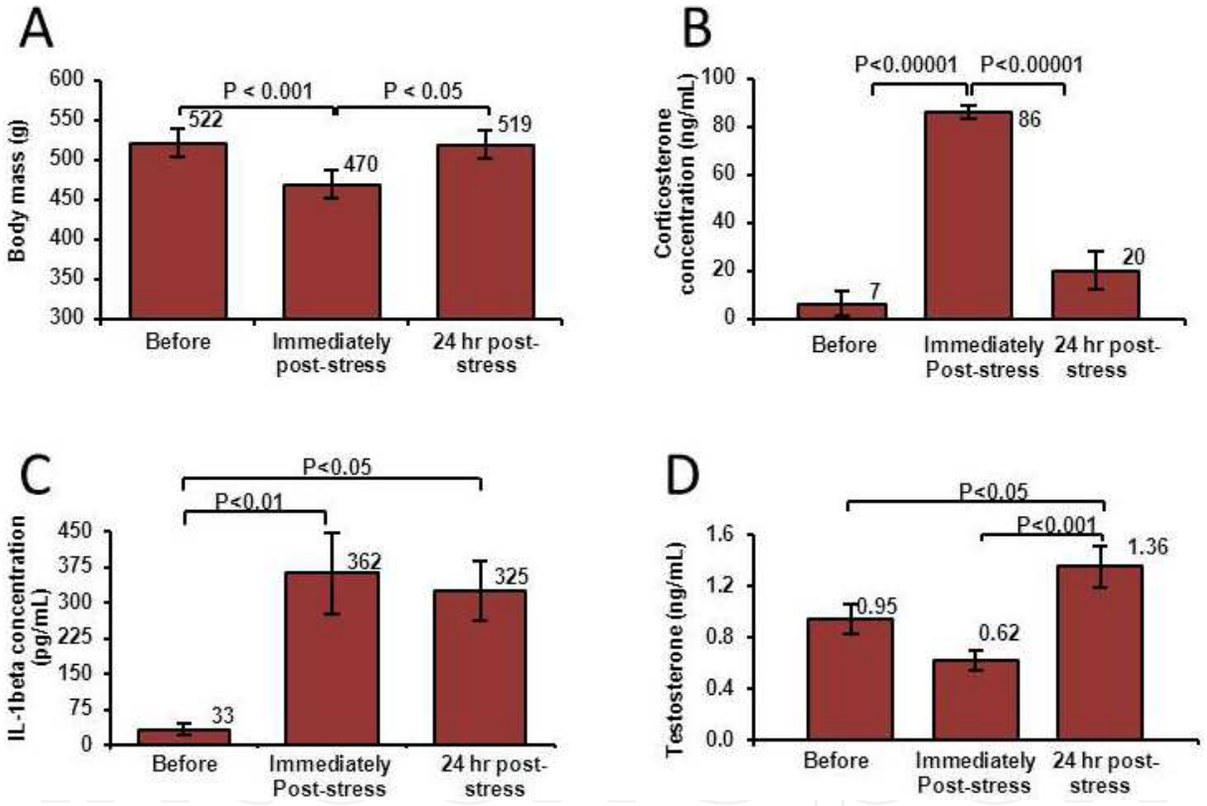

Figure 3. Effects of acute short-term restraint stress (1 hour) and recovery from stress on mean a) body mass, b) serum corticosterone, c) serum interleukin-1β and d) serum testosterone concentrations. Bars on graphs illustrate mean values, while error bars indicate standard deviations.

Body mass decreases significantly, but only transiently, in response to acute stress as applied by our group. This is mainly the result of increased defecation and urination. In terms of corticosterone, an acute increase of between 8-12-fold is seen. This response plateaus after one hour, and rats are able to recover from one exposure to restraint within one day. Testosterone concentrations are not acutely affected by acute stress, but it may increase during the recovery period. This effect is similar to that seen in athletes after a

stressful bout of exercise, and may suggest an ability to cope and resist the stressful effects of the particular stressor.

In the chronic model, since the rat is not able to fully recover between stress exposure sessions when done daily for an extended time, testosterone levels do decrease with this model, resulting in a more catabolic state, and even up-regulation of the proteolytic pathways. In other words, although only mild in severity, this model is severe enough to result in undesirable side-effects in the longer term, making it an excellent simulation for chronic stress such as occupation-related stress in humans. From the cytokine data, restraint stress clearly has a pro-inflammatory effect as well, which makes this a particularly suitable model for investigations into the efficacy of e.g. anti-inflammatory interventions. Note that the IL-1β levels are still significantly elevated even after the recovery period – this is most likely due to the relatively long half-life of the cytokine. Again, in the long term, a shift toward a pro-inflammatory status is achieved.

Some groups have used involuntary swimming (forced swimming in a $1m^3$ swimming pool warmed to 24°C) as stressor. Although acute forced swimming is a recognised test to assess depressive-like behaviour (although this is being disputed), rats are natural swimmers, so it is doubtful whether this method – when applied chronically - is really a significant stressor. In fact, in the discipline of exercise science, researchers train rats to swim in order to study hypertrophy and metabolic adaptation to exercise training. These anabolic responses are the direct opposite of the catabolic response that is the stress response, further placing doubt on the use of this technique to realistically simulate chronic stress. Furthermore, in our experience, females are more willing swimmers than males. Males were found to simply climb onto the most submissive animal, which would then literally be drowned without investigator intervention. Alternatively, they might hold their breath and sit at the bottom of the pool for as long as they can before jumping/swimming up for a breath of air, rather than exercising the whole time. Although females tend to actually swim a lot better without the constant prodding required with males, their voluntary exercise capacity/willingness to exercise also varies dramatically. Therefore, as with voluntary running models (using purpose-designed running wheels), the "natural athletes" have to be selected from a larger cohort prior to the study. This then has the disadvantage of possible genetic pre-selection, which may yield data that is not widely applicable across the whole population. It is clear therefore, that this model has many limitations and should not be a first choice for simulation of psychological stress.

A number of other stressors may be employed, and some of these are not very labour-intensive, so that they are commonly used in combination with the stressors discussed above, to prevent adaptation, as mentioned earlier. These include soiled bedding, tail flick, and inversion or cage tilt. Bedding is soiled with water by simply pouring 300ml of water onto cage bedding and leaving rats to endure this discomfort for an hour before changing the bedding again. For the tail flick protocol, a rat is manually restrained and its tail placed in a water bath kept at 49 °C until the rat voluntarily flicks it out. For the cage tilt, the restraint cage is turned upside down for the duration of a restraint session which usually

lasts from 30 minutes to an hour when used in combination with cage tilt. These are all examples of mild severity stressors. Extreme heat or cold are also referred to as stress models, but these stressors are more metabolic than psychological in nature.

3.2. Keeping experimental animal stress free

Although left for last in this section, the following point is perhaps the most important. When conducting any experiment investigating the response to stress, it is of major importance to keep all animals "otherwise" stress free. In other words, one has to ensure that rats are only exposed to the standardised stressors used as interventions in the study. Several precautions may be needed to prevent other stressors from confounding data. For example, vibration has recently been identified as a stressor in terms of the immune system. Animals exposed to constant low grade vibration may deplete their lymphocytes in as little as two to three weeks. Considering that lymphocytes make up the bulk of rat white blood cells (about 75%), it is clear that the end result is an immune-compromised animal with very abnormal cytokine profile. Therefore, while it may seem like a good idea to have a generator handy, the constant vibration it causes, may in fact be detrimental to your study.

Furthermore, new male rats should never be introduced to existing housing groups (e.g. if one rat dies, it should not be replaced with another adult rat), and adult male rats should not redistributed between cages after they have established their hierarchy. They have a social hierarchy and such changes will result in social stress, the result of which is difficult to determine before it is too late. Lastly, rodents in particular have to be handled to accustom them to their handlers. During this time, they also become used to the sounds and smells associated with their housing environment. Introduction of a new sound or smell may result in an uncontrolled, unstandardized stress response. In our laboratory, the simple guideline during acclimation of rats after arrival from the breeding unit, is to expose them to all sounds, smells (e.g. disinfectants used both during every day maintenance and during sample collection procedures), actions (e.g. weighing, sham injections, oral gavage with tap water only) and people required for the intervention study, with the exception of the intervention itself. During sacrifice, a meticulous procedure has to be followed: Firstly, all surfaces should be disinfected with a disinfectant the animal has been habituated to, in order to disguise any body odour from the previously sacrificed animal. Then, the rat is taken from its cage and euthanasia applied as soon as possible. Rats still in line for sacrifice have to be protected from any sound or smell that could alert them to what is happening; otherwise they will have a severe acute stress response. Sprague-Dawley rats for example has been shown to have increased heart rate and mean arterial blood pressure when present in the same room where other rats were being exposed to a variety of interventions, which included routine actions such as cage changes, but also experimental interventions such as decapitation (Sharp et al., 2002).

Interestingly in this study, witness rats that were individually housed, showed a greater stress response than rats group housed, further illustrating the additive effect of different stressors. The magnitude of this acute stress response can indeed be enormous. In a study

by our group, corticosterone responses were determined in rats that could smell and hear experimental procedures for sacrifice. When sorted according to the order of sacrifice, it is clear that the rats waiting their turn were experiencing acute stress that accumulated with time (Figure 4).

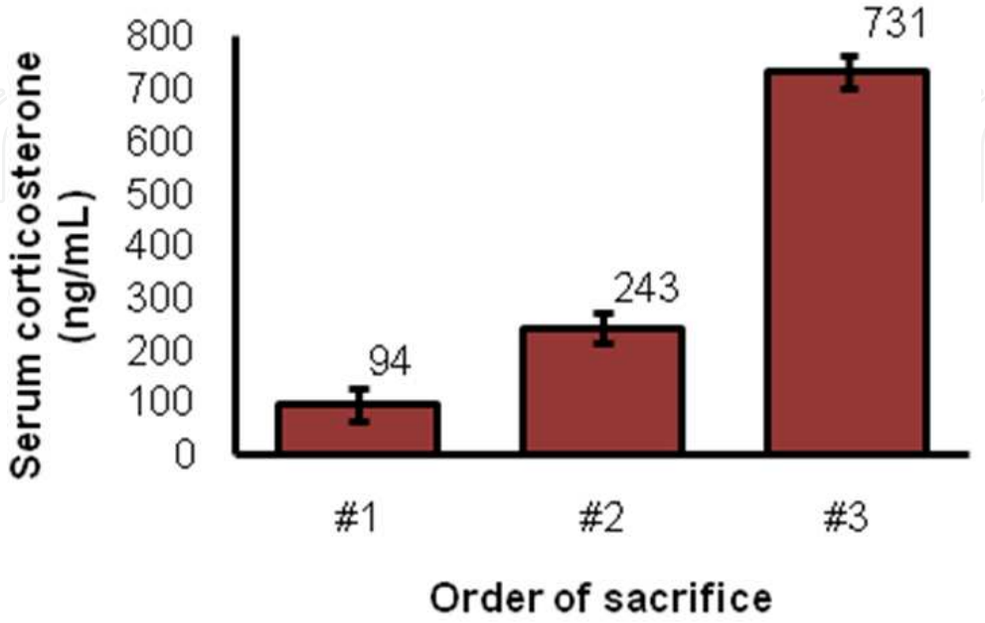

Figure 4. Cumulative corticosterone levels in an acute stress response that was elicited by witnessing the experimental killing of littermates in male Wistar rats killed approximately 15-20 minutes apart.

4. Quantifying stress responses

In terms of psychological stress, an obvious and very popular assessment technique in humans are the use of validated, standardised questionnaires designed to assess levels of perceived stress, anxiety, depression, hardiness, job satisfaction, etc. Quite clearly this method is not of use in animal models. Instead, tests to analyse and quantify stressed behaviour have been developed. The most common techniques in this context are the open field and elevated plus maze tests, as well as the forced swimming test mentioned earlier. To increase the accuracy of interpretations made from behavioural tests, it is advised to combine at least two behavioural tests, rather than to rely on the results from only one technique.

For the open field test, the researcher relies on the fact that rats naturally fear large open spaces, since this would expose them to predators. For this test, an "open field" of $1m^2$ with gridlines, with high walls around all sides, are used (Figure 5). The rat to be assessed is simply placed in the centre of the open field, and its exploratory behaviour assessed by quantification of movement frequency and distance. A variation of this test is to have a second open field test on a separate day, which involves placing a novel object in the centre of the open field – the number of approaches made to this object is recorded. The interpretation of the results is not without complexity though. While a greater degree of

locomotor activity and more time spent in the inner zone is usually seen as indicative of a relaxed emotional state, this same result is obtained in young rats after the traumatic experience of maternal separation. The latter condition is seen as an anxious, hyperreactivity or hyperarousal state. The "novel object" open field test can distinguish between these two explanations for the same behavioural test result: while an emotionally relaxed rat would approach the novel object often to investigate, the hyperaroused rat would be much less keen to explore the novelty.

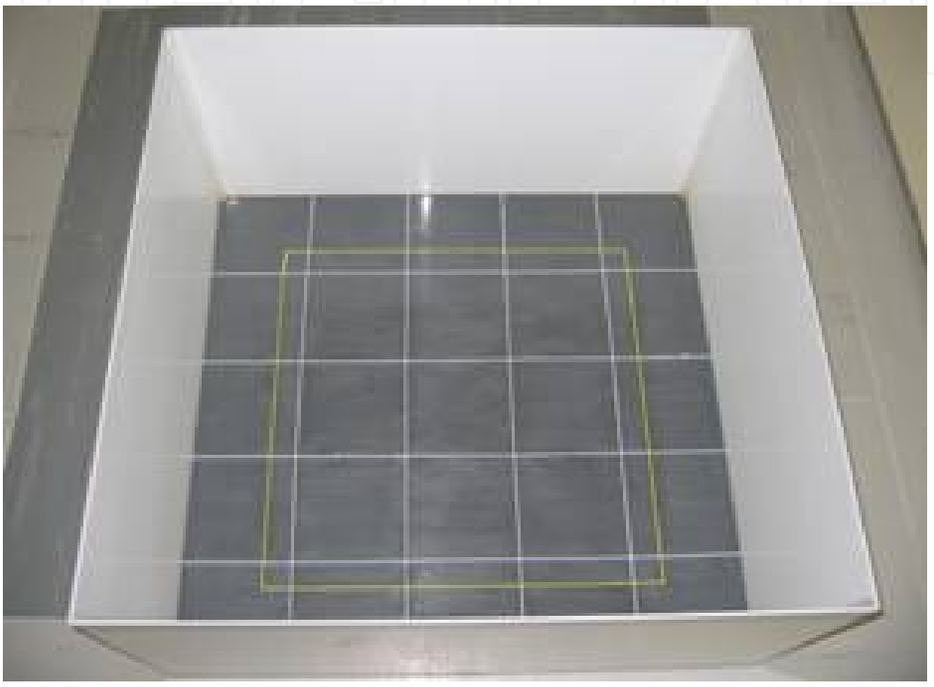

Figure 5. The open field test platform

The elevated plus maze test uses this same basic principle. The maze consist of a platform in the shape of a plus sign (+), with two opposite arms open (i.e. looking a bit like a diving platform) and the other arms closed along the sides. This platform is placed at a height of 0.5 m off the floor (Figure 6). Similar to the open field test, the rat is placed in the centre of the plus, and its courage to enter the open arms, *versus* the relatively safer closed arms (at least as perceived by a rodent), is assessed in terms of not only the number of times an open or closed arm is entered, but also the time spent in the respective arms, either moving about or sitting in one position, as well as the rat's aggressive (rearing) or self-soothing (grooming) behaviour while in the arms. In this way, a lot of data on behavioural changes may be generated, to use on its own, or to correlate with physiological data such as hormone levels. However, as with the open field test, the data is not easy to interpret. Therefore, again, no one measure should be considered as a stand-alone result.

It is of importance to note that the intervention protocol, or stress model used, may also dictate or limit the assessment techniques that are possible. Firstly, the behavioural tests are performed over the space of a few minutes. Therefore, if the investigation was related to the upstream stress responses to acute stress on the level of the brain, the physiological aspects

of these responses need to be assessed immediately after exposure to the stressor. Doing a behavioural test first will result in central effects being missed, because the tissue sample will be collected too late. A suggestion to get around this is to perform the behavioural tests one day prior to the collection of tissue and blood samples for physiological and/or biochemical analyses. The use of appropriate control animals will prevent the behavioural tests from confounding results in such cases.

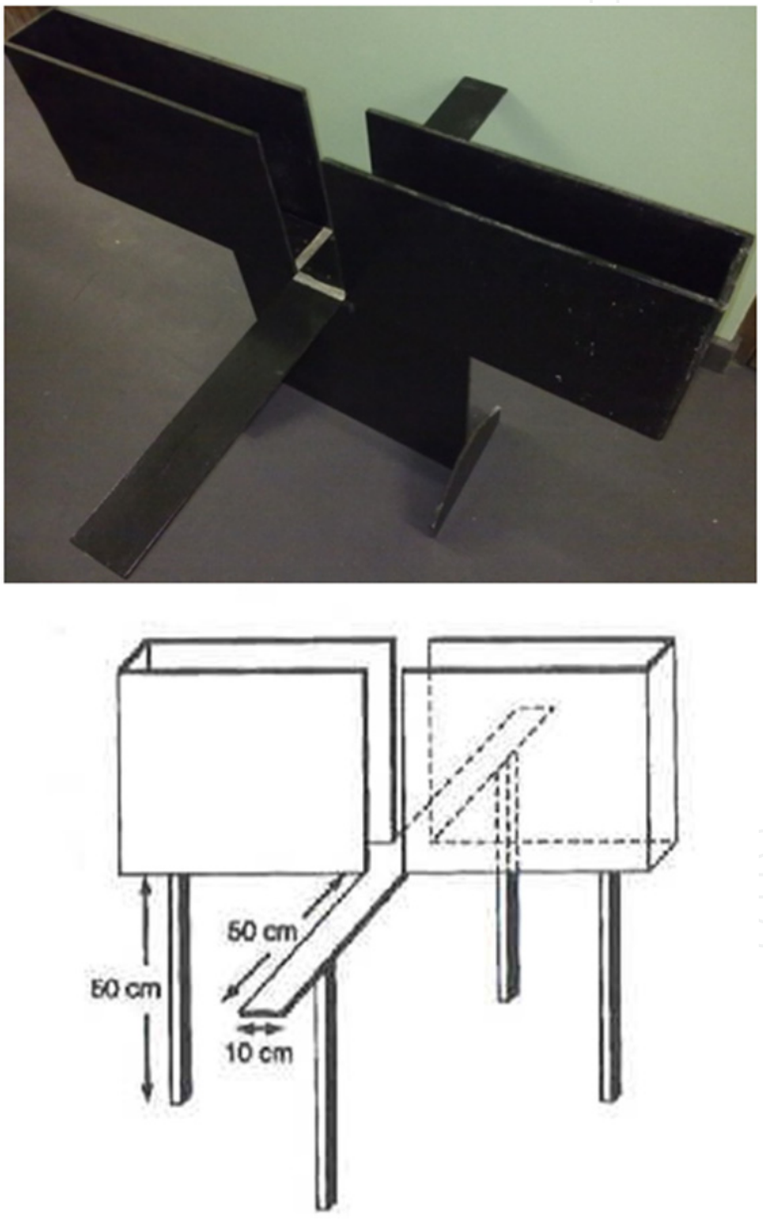

Figure 6. The elevated plus maze, with a technical drawing below to indicate dimensions.

Secondly, the type of stress intervention chosen may influence behaviour quite dramatically. For example, when considering the elevated plus maze, an anxious or stressed rat does not move about freely and would prefer the closed arms of the elevated plus maze, while an emotionally relaxed animal will exhibit more exploratory behaviour, and be more willing to enter and explore the open arms. However, when testing stressed or anxious behaviour in a rat that has just been restrained for an hour, the opposite effect is seen: an example of behavioural data illustrating this phenomenon in an elevated plus maze test is provided in Table 1. Data show that stressed rats chose to enter open arms more frequently than controls, which in this case may be interpreted as a counter reaction to having been confined to a small space during restraint. The latter explanation is very feasible, since the restraint stressed rats entered the closed arms less frequently than the controls. Although this decision would normally indicate a relaxed state, one has to keep in mind that the normally comforting closed arms would now resemble the restraint cage unit the rat had just "escaped" from, so that the rat, even though stressed, decided that the open arms are the safer option. The third parameter illustrated in Table 1, grooming, which is a self-soothing behaviour as stated earlier, clearly shows that despite the atypical result just described, the restrained rats were indeed stressed, since they spent more than four times as long trying to calm themselves than the control animals.

	Number of entries into open arms	Number of entries into closed arms	Time spent grooming (in seconds)
Control	4.3 ± 0.5	8.3 ± 0.7	11.3 ± 2.3
Stressed	5.9 ± 0.6*	6.1± 0.5*	49.8 ± 8.5**

Table 1. Selected parameters indicating behavioural responses to repeated restraint stress in male Wistar rats. Asterisks indicate values significantly different from controls (ANOVA with Bonferroni *post hoc* tests: *P<0.05; **P<0.001).

In terms of physiological assessment, stress can be assessed in terms of neuronal and endocrine pathways, as well as signalling proteins such as cytokines. Factors which may impact significantly on the quality of data is the method and timing of sacrifice and of sample collection. Recent studies on rodents commonly use intraperitoneal injection of a sodium pentabarbitone overdose. This is relatively painless and the animal loses consciousness fairly quickly. This method is also useful in the context of stress, with the exception of studies with the aim of investigating central changes. The reason for this is that the rodent will perceive the "loss of control" when losing consciousness, resulting in a central stress effect. While this effect may not reach downstream tissues in time to affect the outcome of analyses significantly, definite changes will be seen in the brain itself. Therefore, when conducting *in vivo* studies in the field of neurophysiology, it may be advisable to rather use cervical dislocation or decapitation techniques. The timing of sample a sample is of course vital. Sample collection for hormones should take into account diurnal variation in glucocorticoid levels, as discussed earlier. (For rodents,

corticosterone is the glucocorticoid produced in highest quantities, whereas in humans it is cortisol.) For example, samples for determination of corticosterone levels should all be taken at the same time of day AND at the same period of recovery after the last stress exposure, so that the experiment may require quite a bit of logistical synchronisation. Also, the biological half-life of parameters of interest should be considered. For example, while corticosterone is a down-stream output of the stress pathways and has a relatively long half-life, ACTH is secreted fairly early in the stress response and has a half-life of less than 15 minutes, so that samples obtained at the end of a two-hour restraint protocol will probably not have detectable levels of ACTH, but sufficient corticosterone to be able to quantify the stress response. The design of stress protocols will therefore have different endpoints, depending on the aim of the investigation, for example a short restrain period may be more ideal for detection of upstream events in the stress pathways, while a longer one may be required for down-stream parameters to become available in circulation. Therefore, in order to time the sacrifice of an animal and collection of samples optimally, it is necessary to understand the basic biochemistry and/or pharmacology of parameters of interest.

In some instances it may be even more useful to determine down-stream effects related to earlier events, rather than trying to "catch" upstream parameters in circulation at an optimal time. This is also true when the parameter of interest can have its origin from more than one source. For example, when considering the inflammatory component of the response to stress – which has been linked to many chronic diseases recently – it is difficult to pinpoint the origin of cytokines when only assessed in blood, since most of them are released from a wide variety of cells. Also, since some cytokines, such as IL-6, have an autocrine-type action, its level in circulation is often not indicative of events at cellular level. In these instances, immunostaining of tissue levels of these parameters are very useful. Indirect measurements of e.g. inflammatory responses can also inform on the response to stress. For example, instead of measuring TNF-α levels in blood, activity of the proteolytic pathways in tissue may be employed as indirect indicator of TNF-α activity, which known to play an important role in muscle wasting, or cachexia. In this way, the timing of sampling become less critical, and the effect at the level of the target tissue, may be directly elucidated.

5. Characterisation and standardisation of stress models

The severity of the stressor will determine the extent of acute activation of the HPA-axis and/or SAM pathway, as well as the adaptability of the animal to the stressor, i.e. the chronic response to any particular stressor. This necessitates the standardisation and characterisation of any particular model by researchers prior to its application for research purposes. Our group have characterised our model of restraint stress in terms of a variety of parameters. One of these is the corticosterone response, which is presented for protocols of different durations in Figure 7.

This figure illustrates the significant difference in the response to a specific stressor acutely, and after chronic intermittent exposure, after which one may expect habituation to the

stressor. One can see from these data that the stressor employed was indeed mild; although there was a substantial increase in corticosterone concentration in serum immediately after the restraint (at the "acute" time point), the rat was able to completely recover its corticosterone levels to control levels one day after the single restraint session. The data further indicates the effectiveness of this model to induce chronic stress: the value labelled "4 days" indicates that 3 restraint stress sessions over 3 days resulted in a corticosterone response that the rats could not completely recover from overnight, resulting in a significantly elevated corticosterone level even after a period of recovery, albeit not as highly elevated as in the acute version of the model.

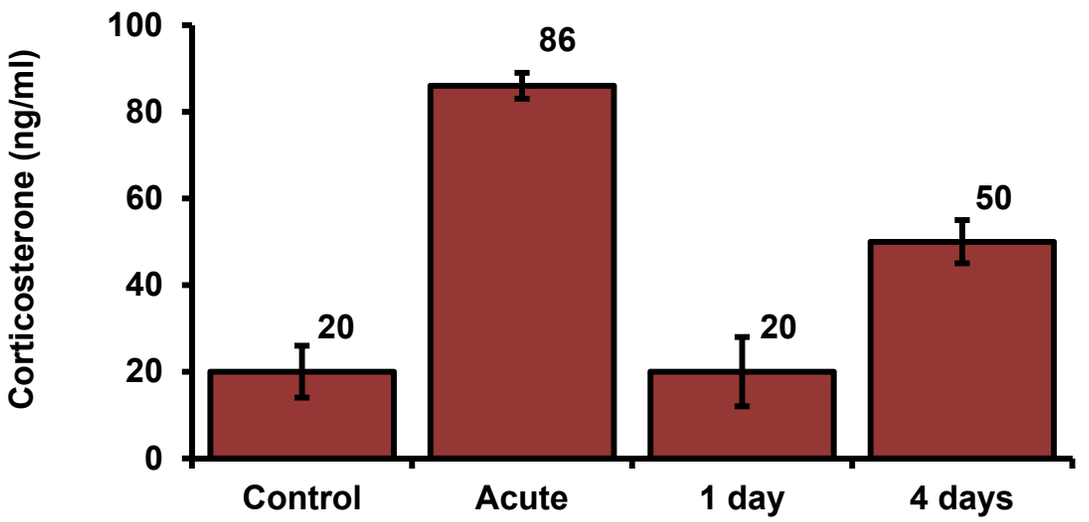

Figure 7. Corticosterone responses to one hour of restraint stress daily, for protocols of different durations. Values are means for at least n=10 rats per experimental group.

However, as discussed earlier, these results in its entirety will probably only be valid for the model as executed in our hands. Although the same trend should be seen – e.g. the increased corticosterone levels in stressed rats – the magnitude of this response as well as the animal's ability to habituate to it, is largely dependent on the execution of the model by various research groups, who each adapts the protocol to be best suited for their own particular research interests. Therefore, it is vital to include sufficient control groups for all interventions, in order to facilitate cross-group comparisons of results.

6. Conclusion

Conducting research using experimental animal models is a complex endeavour, with many considerations, adaptations to make and precautions to take. However, when applied by researchers with the ability to adapt a protocol to make the most of it, results achieved are very satisfactory in terms of quality, repeatability and direct applicability to actual physiological situations. Therefore, in conclusion, cell-based scientists and systems biologists should combine efforts to successfully counter the effects of stress.

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