TOOLS TO MEASURE AND IMPROVE WELFARE OF LABORATORY RATS: REWARD-RELATED BEHAVIOUR AND ENVIRONMENTAL ENRICHMENT

METHODES OM HET WELZIJN VAN LABORATORIUMRATTEN
TE METEN EN TE VERBETEREN:
ANTICIPATIEGEDRAG EN KOOIVERRIJKING

(met een samenvatting in het Nederlands)

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The question is not
Can they reason?
Can they talk?
but
Can they suffer?

Jeremy Bentham, 1789,
Principles of Morals and Legislation

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

The experiments described in this thesis aim to investigate tools to assess and improve welfare of laboratory rats. A concept of welfare based on reward-evaluating mechanisms in the brain plays a central role in the approach of this thesis. In this approach, an animal's sensitivity to rewarding stimuli, which is influenced by previous positive and negative experiences, plays an important role. Because previous experiences determine the state of an animal in terms of welfare reward-sensitivity is likely to be indicative of this state and is therefore applied as a tool to *assess* animal welfare. If the state of reward-systems (i.e. reward-sensitivity) plays a central role in animal welfare and is rather flexible, maintaining this state within certain boundaries (thus counteracting stress) may be a way to improve (or prevent) poor welfare. Within this concept of welfare, announcing and providing rewarding stimuli will be put forward as tools to *improve* animal welfare in the sense that they may counteract stress and prevent or reverse *in*sensitivity of the reward-system (a result of chronic stress and a core symptom of depression). Because the biological background of the proposed tool can probably be generalized to all (vertebrate) species, the obtained information is considered to be applicable to other captive animals as well.

This chapter starts with a brief overview of existing definitions and indicators of animal welfare and continues with an explanation of the approach and background of the present study. The biological background of welfare in terms of the importance of motivational aspects, efficiency of behaviour, and reward-senstivity for the concept of animal welfare will be discussed. Especially, the relevance of reward-sensitivity as an indicator of the welfare state of an animal will be explained. Furthermore, it will be argued that an animal's behavioural expression in anticipation (i.e. expectation) of a reward is a general expression of the animal's sensitivity to this reward and that this anticipatory behaviour can serve several potential purposes in relation to welfare assessment and improvement. This chapter will also highlight the importance of environmental enrichment for animal welfare in the sense that it is considered to result in rewarding activities which is proposed to counteract stress. Some notes on terminology are presented in Box 1 (p.26) This chapter will be closed with a summary of the aim, approach and outline of this thesis.

2. ANIMAL USE & ANIMAL WELFARE

Animals have been exploited by man for all kinds of purposes. The dominion of man over animals implies that we determine how and where they will live. According to many, society should therefore be concerned about the welfare of these animals. Since animals are thought to have less mental capacities than man, it has been questioned whether animal welfare concerned more than physical health alone. However, as early as the 18th century, Jeremy Bentham (1789) [25] addressed this question in his classic dictum: 'The question is not, Can they reason? Nor, Can they talk? But, Can they suffer?'. Nowadays, it is assumed that similar to man, chronic stress may induce mental suffering in animals, which is not necessarily associated with physical health problems.

Using animals for certain purposes implies keeping them in captivity, which means that we are responsible for their housing conditions. The design of these housing conditions is mainly based on economical and ergonomical factors with little attention for behavioural needs of animals. For laboratory animals, not only economical and ergonomical factors

played a role in the design of animal cages, but also standardization requirements. These factors led to more and more impoverished housing conditions that prohibit the performance of a large part of the natural behavioural repertoire [270]. Due to this behavioural deprivation the current housing systems are inadequate in creating an environment that guarantees the welfare of animals [107][410], a statement which is often acknowledged in the guidelines [6]. As long as the use of animals for research is necessary, society should be concerned about their welfare. In addition, a lack of adequate environmental stimulation is shown to cause a range of brain- and behavioural deficits which do not only jeopardize animal welfare but also the validity of research results. Poole [299] stated for instance: 'If welfare is not guaranteed, the validity of laboratory animals as a research model is questionable.'
Currently, there is an urgent need to be able to assess and improve animal welfare. Several scientific studies have addressed this issue during the past decades and have made substantial progress. However, because of the complexity of the concept of animal welfare, and despite of the extensive amount of relevant research results, animal welfare experts failed to reach a clear consensus on how animal welfare should be defined and measured. Until now, animal welfare has mostly been addressed as inversely related to stress and its related parameters. This is not in line with the scientific delineation of stress and is also different from the approach of human welfare (see section 3.2).

An important aspect of animal welfare includes brain processes which are involved in the animal's subjective evaluation of its internal state and its environment. Thus, to be able to determine animal welfare we need to find ways to 'read their minds'. In other words: we need a tool to 'ask' an animal how it 'feels' and be able to understand the 'answer'. It is important that the answer to this question can be scrutinized in the phase *before* the animal's adaptive mechanisms are chronically challenged and subsequently fail over a longer period of time allowing the early detection and solving of problems.

For successfully realizing improvements in animal welfare, we need: 1) to know how these improvements are perceived by the animals and 2) to be able to monitor the effects of these improvements. Webster [407] has illustrated the necessity of understanding the animal's appraisal of its own situation by describing a rabbit that was housed in isolation, without food, in a cold box on dirty, wet litter. He argued: 'If we are to do our best for the welfare of the rabbit, whatever our ultimate intention may be and however good or bad that may make us feel, we have no option but to do our best to understand how the rabbit perceives and interprets its world and adjust our actions accordingly.'

The ultimate 'product' of various complex interacting mechanisms in the brain is behaviour. A behavioural response can be described as being the 'answer' of an animal to challenges in its external and internal environment. Recently, cognitive ethologists and psychologists have developed new methods to study the animal mind by means of combining knowledge of evolutionary biology with behavioural and brain research. In the present thesis such a multidisciplinary approach has been followed in order to develop a tool to *asses*s the state of animals by means of posing 'questions' to animals and deduce the 'answers' from their behaviour. This tool is based on how an animal evaluates its own situation, adapts its behaviour and optimizes its energy use under natural conditions. Furthermore, new insights and methodologies are proposed to *improve* animal welfare and assess an animal's appraisal of certain conditions. Before going into detail concerning the tools and methodologies

applied in this thesis, this chapter will now continue with a general overview of welfare research. Subsequently, the biological background of welfare in relation to efficiency of behaviour will be presented because this is the basis of the concept of welfare that is applied in this thesis.

3. ANIMAL WELFARE: DEFINITIONS AND INDICATORS

To investigate and discuss animal welfare a comprehensive and practicable definition and analysis of welfare is required. It should not only be defined by how an animal feels at a certain time within a continuum that ranges from suffering to pleasure but also by its ability to cope with environmental changes and challenges over a longer period of time. Up until now, several definitions and indicators of animal welfare have been forwarded of which some will be discussed in this section. However, despite the extensive literature on related topics and individual facets of animal welfare, consensus on how it should be defined and measured has not been achieved (for a review see [239][74][75]).

3.1 Definitions of welfare

One of the first definitions of welfare was put forward in 1965 by the Brambell Committee [45] that was constituted after the publication of 'Animal Machines' in which Ruth Harrison [171] described the loss of identity of animals kept in commercial husbandry systems. This definition was devised as a checklist of minimal requirements for farm animals but was considered to serve equally well for other captive animals [407]. These minimal standards became to be known as the 'Five freedoms'. The 'Five Freedoms' have evolved somewhat with time and have been revised by FAWC in 1993 (UK Farm Animal Welfare Council)[7]:

(1) *Freedom from thirst, hunger and malnutrition* – by ready access to fresh water and diet to maintain full health and vigor.
(2) *Freedom from discomfort* – by providing a suitable environment including shelter and comfortable resting area.
(3) *Freedom from pain, injury and disease* – by prevention or rapid diagnosis and treatment.
(4) *Freedom to express normal behaviour* – by providing sufficient space, proper facilities and company of the animal's own kind.
(5) *Freedom from fear and distress* – by ensuing conditions that avoid mental suffering.

Although these five standards are easily memorized, comprehensible and will definitely improve quality of life if complied with, the main flaw in the concept of the five freedoms is that it is not necessary to the welfare of an animal (or man) to have absolute freedom from hunger, cold, pain, fear etc.; only that the animal should be able to cope with these problems by taking effective action to avoid suffering. Therefore, Webster [407] argued that perhaps a sixth freedom should be added: the freedom to exert control over the quality of life. This seems in line with Broom [49][52] who defines welfare of an animal as its state as regards its attempts to cope with its environment (and thus control the quality of its life). Carpenter [1] proposed that the welfare of managed animals relates to the degree to which they can adapt without suffering in the environments designated by man. Correspondingly, many others have characterized animal welfare as a state of mental and physical health indicating living in harmony with its environment [185][129][417]. Thus, the abovementioned definitions

indicate that the features relevant for animal welfare relate to how well the animal is coping, how well it adapts, or whether it is in harmony with its environment. This was biologically translated by Koolhaas and Wiepkema [417] in: 'welfare is present when an individual can reliably predict or control relevant events by means of species-specific signals or means.' Predictability and controllability are key concepts in this respect. Another somewhat tentative but nevertheless important conclusion Wiepkema and Koolhaas [417] draw is that for optimal welfare some uncertainty (unpredictability and uncontrollability) is of great positive significance (see also [11b]). This implies that welfare is not fully dependent on complete control, certainty and solely positive experiences that might result in a rather insensitive animal. Under natural conditions, animals are exposed to both negative and positive stimuli and 'living in harmony with its environment' probably implies that it must be possible to keep a (positive) balance between these stimuli. This is in line with the ideas of Toates [362] who argues that an animal can never be 'perfectly' adapted to its environment since the latter is always subject to change but that animals develop behavioural strategies to approach 'optimality' and maintain homeostasis. Toates [362] indicates that behaviour has been shaped by evolution to take both physiological and environmental cues into account. A threatened homeostasis can be defined as the result of the difference between the actual and the expected (or preferred/desired) state [364] and the (stress) responses of animal to minimize this difference can be interpreted as reflecting its efforts to cope. In this sense, 'homeostasis' does not only refer to a physiological state but is also related to the environment and thus to the abovementioned expression 'living in harmony with its environment'.

Overall, it seems that animal welfare can be regarded as being related to both positive and negative experiences. Measuring welfare by focusing on only one of these categories will probably yield an insufficient picture. It is therefore proposed here that it is the *state* of the balance between positive and negative experiences that eventually determines welfare. This will be further explained in section 3.3.

3.2 Welfare indicators: classic criteria based on stress responses

It is obvious that animal welfare cannot be simply measured as such; rather, welfare research must focus on variables and criteria that are relevant to animal welfare [150]. Previously, animal welfare has been mainly addressed as inversely related to stress-induced physiological and behavioural changes. The classic criteria utilized in welfare research appear to concern mainly measures of non-welfare that are based on the *presence* or *absence* of stress. The acute stress response is generally considered to facilitate the functional adaptation of an organism in order to cope with a challenge [20]. Failure in the attempt to cope is often seen after chronic stress and is considered to indicate that welfare is severely impaired.

3.2.1. Physiological and behavioural parameters

Changes associated with the stress response have been widely used as physiological indicators of (poor) welfare. The increased secretion of hormones from the adrenal cortex (e.g. cortisol) and medulla (e.g. adrenaline) in the initial alarm phase are designed to condition the animal for immediate action by switching the flow of blood and nutrients from long-term goals like growth towards immediate problems like fight or flight. Since increased secretion of adrenocortical hormones, typically cortisol, is a constant feature of the alarm response, concentrations of these hormones in the blood or saliva of animals are regularly

used as an index of stress. However, this approach can fail to distinguish between alarm as a potential source of suffering, and excitement as a potential source of pleasure [419][407])(see section 3.2.2 for further explanation). Several other physiological measures, related to increased corticosteroid levels, are traditionally used as indicators of poor welfare as well. For instance: a change in plasma concentrations of glucose, urea or protein, indicating a significant metabolic cost to the animal, and immuno-suppression, indicating a potential health risk [20].

Similar to the classic physiological criteria, most behavioural indicators of welfare also rely on showing evidence of changes that are indicative of (chronic) stress. Particularly, the occurrence of abnormal behaviour has been used as an indicator of stress, c.q. (non)welfare [20]. Stereotypies, for instance, are traditionally considered to indicate reduced welfare [48][416] and are typically observed in situations of conflict or frustration [100][237]. The performance of stereotypic behaviour may be regarded as a mechanism that helps animals to cope with and adapt to environmental changes [20][92][93]. However, is essential to emphasize that it is generally accepted to represent an animal's response to an inadequate environment [91][237][3]. Thus, stereotypies may not be directly indicative of the welfare of the individual animal that performs it (the expression of stereotypies has been argued to be rewarding via a positive feedback effect of sensory stimulation on their underlying control systems) but is certainly indicative for the insufficiency of housing conditions in which they occur.
In some cases the presence or absence of certain natural behaviours are used as indicators. For instance, play behaviour is argued to be a reliable indicator of good welfare in mammals [53] since one of the common characteristics of play behaviour is that it is absent under stressful conditions [300][427].

3.2.2. Disadvantages of stress-related parameters

As mentioned in the previous section, changes associated with the stress response have been widely used as physiological and behavioural indicators of poor welfare. However, as Duncan [131] argued: 'The absence of stress does not necessarily indicate good welfare and the presence of stress does not necessarily indicate poor welfare'. In line with this, Wiepkema and Koolhaas [417] suggested that mild stress might even improve welfare by optimizing alertness and preventing boredom. Similarly, Haller and Halasz [169] demonstrated that daily mild stressors have protective effects against the effects of isolation. The acute stress responses of an animal are suggested to reflect its efforts to minimize the difference between the actual and the desired state and, thus, attempting to cope with the situation and maintaining homeostasis [20][364]. Therefore, acute stress responses can also be interpreted as beneficial or having rewarding properties to the individual.

Furthermore, as mentioned in section 3.2.1, concentrations of cortisol can be misleading. Namely, corticosteroid release is also seen in the absence of behavioural evidence of aversion and the HPA-axis is also activated by novelty in rats, which can hardly considered to be stressful since rats often show a preference for novel environments. It is argued that this might be related to 'sensation-seeking' [419][295] which can also be observed in humans and suggests that corticosteroid release may be related to a state of arousal, rather than to psychological stress as defined in terms of aversive behaviour. This is in line with the recent findings of Salvador and colleagues [326] who suggested that neuroendocrine responses to competition in young men are associated with cognitive appraisal. Such a relationship is also

suggested by the finding that the size of the corticosteroid response to novelty is predictive of the ease with which rats acquire amphetamine self-administration behaviour [294] and is also consistent with notion that acute administration of corticosteroids usually elevates mood in human subjects [157]. This indicates that stress seems hard to define and lacks specificity: the responses can be elicited by neutral or even pleasant events as well. Thus, it appears that animal welfare cannot simply be addressed as being inversely related to stress and the physiological and behavioural responses to it. However, this does not mean that the stress parameters are useless for welfare research; they may be very valuable in combination with other parameters.

3.2.3. Relation to human welfare

It is important to note that animal welfare is traditionally defined on the basis of other criteria and indicators than those used for the definition of human welfare. However, animal welfare research and human welfare research are more closely related than is recognised so far. Animal models are often employed for elucidating human (neuro) psychology or psychopathology based on the homology and analogy between them. An extensive amount of research results point to mutual neural circuits underlying experience and expression of emotions in both man and animal (see [117]). Thus, it should be self-evident that there should be no discrepancy in the general definition of emotional states of humans and animals. Where animal welfare is mostly approached as being related to the presence or absence of stress, it is acknowledged in humans that the absence of negative symptoms alone is not a guarantee for the presence of welfare. The absence of behavioural expressions of positive experiences and 'low mood' are acknowledged in humans to be an indicator of poor welfare and is probably equally important for the assessment of animal welfare. Thus, not only a general definition for both animal and human welfare might be useful for welfare research in general, but also existing knowledge from either human or animal welfare research might be applicable for the benefit of both. Furthermore, animal welfare is related to the quality of animal models that are used to improve human welfare and is thus related to the validity of the data [316a].

3.3. A concept of welfare based on the balance between positive and negative experiences

As proposed in section 3.1, welfare is related to both positive and negative experiences in the sense that the outcome of the integration of these experiences eventually determines welfare. Impaired welfare does not refer to acute positive or negative experiences, but refers to a chronic imbalance between these experiences reflecting a chronic failure to cope.
It is argued here that as long as signs of satisfaction are in balance with signs of stress, the situation is not hopeless. In this thesis, welfare is conceptualized as the state of the balance between positive and negative experiences. This concept of welfare is based on the hypothesis that rewarding experiences can be compensated by aversive experiences (and vice versa) and that such a compensatory mechanism serves the organization and efficiency of behaviour in all vertebrate species.

Although welfare is considered to be a subjective experience, it has a biological function that is related to the fitness and survival of organisms. Therefore, the following sections will first discuss the biological background of welfare (section 4) and subsequently continue with further explaining the approach and background of this thesis (section 5).

4. BIOLOGICAL BACKGROUND OF WELFARE

4.1.Efficiency of behaviour and motivational states

Animals appear to be capable of high efficiency in the use of environmental resources and the avoidance of potentially harmful stimuli and situations. The nervous system of an organism receives information on internal and external events, processes this information, and selects the most efficient behavioural response. Such efficiency of behaviour is based on the relationship between the investment of an organism and the consequences of its actions (see [346]). This can be translated in terms of costs and benefits that are related to the economy of behaviour [107][108][184]. This means that animals adapt their behavioural responses in such a way that a maximum benefit is achieved with a minimum of effort (investment of energy or taking risks) [215][247]. Efficiency requires a continuously changing sensitivity to stimuli dependent on the actual situation. For instance, under stressful circumstances both the sensitivity to rewarding and aversive stimuli increases [293][63] whereas repeated exposure to rewarding stimuli induces insensitivity to these stimuli in terms of behavioural and neurophysiological reactions (see for instance [396] for a review on the variable sensitivity to drug-reward (morphine)). Thus, the variable reward-sensitivity may be part of the adaptive repertoire that allows the animal to reduce reactivity in case of abundantly present rewards or increase its attempts to obtain rewards in their absence.
A general theory that exists about behaviour is that its primary goal is to obtain rewards (and avoid punishment) [77] and thus is - from an evolutionary perspective - an instrument to reach ultimate goals (maximal fitness) (e.g. [348]). The general adaptive significance of the capacity for goal-directed action allows man and animal to control their environment to satisfy their needs [122]. Goal-directed actions are controlled via activation of motivational systems that signal differences between actual and preferred states. Goal-directed behaviour is defined as behaviour controlled by representation of a goal or an understanding of a causal relationship between behaviour and capture of a goal [332]. The induction of expectation (i.e. anticipation) through the acquisition of associations between certain (environmental) stimuli and its consequences is regarded as having high adaptive value since an animal can evaluate and regulate the necessary investment in order to achieve a goal [132]. This means that an animal must be able to evaluate its own state and the significance (rewarding value) of certain stimuli for this state before goal-directed action is performed. This implies that motivation consists of an appetitive phase in which this evaluation takes place and a consummatory phase in which the goal is captured and consumed.

The relationship between internal physiological changes on one hand and behavioural changes on the other in relation to the availability of different commodities in living organisms are captured as motivational systems [380]. Motivation is the tendency of an animal to perform certain behaviour, whereby its motivational state is determined by the interaction of internal and external factors [248]. Motivational systems can be defined as feedback mechanisms that are activated by a certain class of stimuli and deactivated following specific events or behavioural patterns [186][191][192]. The various motivational systems can be distinguished by the type of responses or events that terminate the activation of a specific motivational system. Every behavioural response that diminishes the difference between the actual and the expected/preferred state, and thus terminates the activation of the motivational system, can be regarded as having rewarding properties.

The analyses and evaluation of external stimuli, which is essential for subsequent behavioural output, has its counterpart in the organization of the central nervous system. The central nervous system may be conceptualized as a hierarchically organized series of feedback mechanisms in which stimuli are processed in different levels and in which each level adds its specific component or programming rule to ongoing behaviour [380][346]. At the highest levels different types of information are evaluated and integrated to be able to elicit the most beneficial response (decision-making mechanism). This processing is not very fast and elicits delayed responses. In case immediate action is required, such as fight or flight [65], processing of stimuli needs to be direct and fast and thus solely involves the lowest levels (see for instance [363]).

4.2.Evaluation and integration of positive and negative experiences

Efficiency of behaviour implies a continuous evaluation and integration of positive and negative experiences that result in activation of motivational systems to reduce the difference between actual and desired states. Selection of the most efficient (rewarding) response implies that a common 'evaluation' system is available that functions at a higher level than specific motivational systems to be able to compare costs and benefits in a case of conflicting motivations. The largest possible reduction of the signalled difference will then lead to the activation of the motivational system involved. For the process of evaluation, a "common currency" must be available to be able to compare different motivational systems and their respective responses (e.g. hunger versus thirst). Because the reduction between the actual and the expected state can be considered to be rewarding, the type of response (e.g. eating or drinking) that causes the largest reduction between the actual and the desired state is the criterion for the response selection. Therefore, reward may be considered as the common currency of this evaluation system, which is supported by Cabanac's theory [61] that 'pleasure' is the common currency of the brain. Earlier, he had already captured the importance of reward in his classic dictum: 'Pleasant is useful' [60].

4.2.1. Biological function of subjective states

Psychological stress and behavioural and mental disorders are features that are likely to be present in both man and animals, at least higher vertebrates [101][132][407][63][131][12]. Negative subjective states and experiences such as stress and fear play a role in the survival of an animal. These negative subjective states can be viewed as proximate mechanisms registering the organism's problem and triggering appropriate motivational systems and consequential courses of action to cope with the problem [19]. As mentioned in the previous section, successful reduction of the difference between the actual and the preferred state is considered to be rewarding. On the other hand, a failure in reducing this difference induces a negative/aversive experience (i.e. a state of stress).

Fraser and Broom [151] have stated that an animal may use behavioural strategies that alter motivational states to try to counteract adverse environmental conditions. In line with this, it can be argued that stress may be counteracted by rewarding activities [26][401] and thus, that stress increases the motivation and subsequent sensitivity for rewards. The fundamental role of reward in the survival and welfare of organisms ranges from the control of autonomous functions to the organization of voluntary, goal-directed behaviour [332](see also section 4.1). Rewards have several basic functions: they may induce subjective feelings of pleasure and contribute to positive emotions, they can act as positive reinforcers by increasing the

frequency and intensity of behaviour that leads to the acquisition of goals, and they can act as goals in their own right and elicit approach and consummatory behaviour (for a review see [332]). Via these mechanisms rewards influence behaviour and play an important role in the survival and welfare of animals. This is also indicated by the abovementioned variable sensitivity to reward, which has adaptive value in the sense that it can be utilized to counteract negative experiences. The importance of reward for survival can also be illustrated by the fact that some behaviours seem to be rewarding in itself without necessarily obtaining an immediate goal at that particular moment, such as exploration, sexual behaviour and grooming. These behaviours, also indicated as ethological needs, are not immediately followed and reinforced by the goal (which is the case for proximate mechanisms) but are essential in the long run (ultimate mechanism) for the animal's survival and that of the species. It may be argued that the course of evolution has attributed rewarding properties to the display of these behaviours to guarantee their expression.

4.2.2. Interaction between stress and reward systems

It is known that previous experiences such as stress can alter the sensitivity to rewarding and aversive stimuli in both man and animal [293][63]. Much information has been collected over the years concerning the consequences of acute and chronic stress on reward-sensitivity. For instance, post-weaning isolation rearing causes, besides a range of other behavioural and physiological changes, an increase in the behavioural responses to both primary reinforcers and reward-related stimuli [194]. Acute or short-term stress, such as isolation, foot shock or tail shock, is reported to lead to an increased motivation for rewards [29][241][260]. Continuously changing reward-sensitivity is an adaptive response that allows the organism to fulfill its needs and maintain a balance between negative and positive experiences. For instance, stress urges an animal to react more eagerly, c.q. be very sensitive, in case of the presence of a valuable reward to compensate for the negative state of the balance. In case of deprivation of certain essential stimuli an animal can become very sensitive for non-related or relatively neutral stimuli as well. The rewarding properties of these stimuli will increase due to the deprived condition of the animal. This can for instance be seen in individually housed rats that display an increase in preference for environmental novelty [321][166] or in play-deprived rats that show an enhanced preference for sucrose and food [376]. Under less stressful circumstances the sensitivity to rewards will decrease again. Via this variable reward-sensitivity a balance between positive and negative experiences can be achieved and this way welfare is guarded.

If the attempts to cope fail over a longer period of time – and the balance is too heavily loaded on the negative side – the animal will enter a depression-like stage that is, among other symptoms, reflected by insensitivity to rewarding stimuli. It is known that chronic stress leads to a strong decrease or even a total loss of reward-sensitivity [118][403]. Chronic stress is applied in animal models of depression since it is known that stress has a precipitating effect on the development of this disorder [433]. Insensitivity to rewards in chronically stressed animals is reminiscent of reward alteration in human depression. This stressor-induced insensitivity to rewards simulates anhedonia (loss of the ability to experience pleasure), which is considered to be a major symptom of human depression [4]. Insensitivity to reward may therefore indicate that welfare is severely impaired.

Thus, because of its flexibility that is affected by previous experiences such as stress, reward-sensitivity may be a useful tool to assess the state of an animal in terms of welfare. Furthermore, animal models that are used to study stress-related disorders and their

symptoms might be very useful for animal welfare research (see [401]) since they investigate the consequences of previous experiences that are relevant for animal welfare as well.

4.2.3. Underlying substrate of reward and reward-sensitivity

The dopaminergic and opioidergic reward pathways of the brain are critical for survival since they provide the 'pleasure' drives for, for instance, food consumption and rewarding activities such as sexual behaviour. Rewarding events are known to be accompanied by activation of the mesolimbic dopamine system [64][424] and in particular by the release of dopamine from the nucleus accumbens [73][289][324]. It is often suggested that processing of reward is mediated by both the mesolimbic dopamine system and the opioid system via an interaction between these systems [27][127][46][292]. This idea was based on studies indicating that the opioid-system is involved in the regulation of the reward-system (see for instance [397]) and the fact that opiates are reported to stimulate dopamine turnover and release [216][244].
According to Berridge [27] opioid systems are involved in the mediation of 'liking' (pleasure/palatability) related to food reward whereas dopamine systems are involved in the mediation of 'wanting' (appetite/incentive motivation). In line with this concept, it has been recently discovered that dopamine release (in the ventral striatum) is triggered by the expectation of a reward and not by the actual receipt of the reward [331][111][112]. Furthermore, it has become apparent that dopamine neurons react to a novel reward but their activation will be transferred from this primary reward to a predictive stimulus during the course of association learning [333]. Also, dopamine neurons are activated when a reward is better than predicted and depressed when a reward is worse than predicted [334][331]. Thus, the activity of dopamine neurons is related to signaling the difference between the actual and the expected state and is therefore important for the activation of motivational systems that aim to reduce this difference. This is in line with the observation that dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra send their axons to brain structures involved in reward, motivation and goal-directed behaviours (see [331][34][199][370]) each of which are important factors in the efficiency of behaviour and maintaining homeostasis.

5. A CONCEPT OF WELFARE BASED ON REWARD-EVALUATING MECHANISMS IN THE BRAIN

In this thesis, welfare is conceptualized as the transient balance between positive and negative experiences. This concept implies that, to assess welfare, it is important to assess the state of the balance in terms of the outcome of the integration of past en present positive and negative experiences. It is proposed here that an animal is continuously monitoring its own welfare state in the sense that when it encounters a challenge/stimulus, an internal evaluation-system determines how to respond depending on its previous (positive and negative) experiences. Thus, by offering a challenge to an animal its state is internally evaluated and this state can be assessed via analysis of its response. In other words, via the presentation of a certain challenge/stimulus an animal can be 'asked' to evaluate its own state and the 'answer' can be deduced from its response.
It is argued in this thesis, that a way to 'ask' an animal about its own welfare state is to present a rewarding stimulus and to investigate the reward-related behavioural response to this stimulus. In case of a large number of previous negative experiences, an animal will show an increased motivation for (i.e. increased sensitivity to) rewards in order to compensate the negative state of the balance. Therefore, quantification of reward-sensitivity is considered as a potentially valuable tool to assess animal welfare. Because this measure of

sensitivity reflects the outcome of the integration of positive and negative experiences it may not only detect a long-lasting process of failure to cope (the balance is (too) heavy-loaded on the negative side) but may also detect positive welfare states. In the latter case, an animal will have less need for reward and, thus, will be less motivated to obtain a reward.

This thesis focuses on the quantification of reward-sensitivity in order to assess the welfare state of animals. Thereby, it is not only aimed to measure the absence but also the presence of welfare. Another aim, following the concept of compensatory mechanisms of stress and reward, is to prevent the development of chronic stress symptoms by means of rewarding stimuli. This will be further explained in the next sections.

5.1. Reward-related behaviour as an indicator for the sensitivity of the reward-system

Reward-sensitivity can, amongst other parameters, be measured by the behavioural response to expectation (i.e. anticipation) of a reward. In several studies it has been noted that rats display an increase in activity prior to the arrival of a reward. According to Dum and Herz [127], this state of behavioural arousal results from endorphinergic modulation of neural reward systems. Mesolimbic dopaminergic areas are involved in mediating appetitive aspects of motivated behaviour (see [34][274][269]) and the activity (sensitivity) of this system is related to quantitative aspects of motivation. Thus, the level of activation depends on the incentive value of the reward (appraisal) [145][57] [146] but, as explained in section 4.2.3, is also dependent on the internal state of the animal which is influenced by its previous (positive and negative) experiences [165][376][403])(see also section 4.2.2). The relation between sensitivity of the reward-system, the consequent behavioural response to rewards, the state of an animal that is influenced by its history, and the characteristics of a reward is represented in Figure 1. In short, this figure indicates that the sensitivity of the reward-system, which determines the level of behavioural activation in anticipation of a reward (i.e. anticipatory behaviour), is related to the welfare state of an animal, which is in its turn determined by previous positive and negative experiences. This indicates that anticipatory behaviour might be a useful tool to assess welfare (see also [348] [380]). Furthermore, the fact that the level of activation is also influenced by the rewarding properties of the reward indicates that anticipatory behaviour might also be used to assess the perception (appraisal) of certain stimuli. Before further explaining the utilities of anticipatory behaviour in relation to animal welfare (section 5.1.3), the characteristics and the experimental control of anticipatory behaviour will be discussed (section 5.1.1-5.1.2).

5.1.1. Characteristics of anticipatory behaviour

Anticipatory behaviour was as early as 1918 described by Craig [89] as a typical arousal with goal-directed activity that occurs in the appetitive phase when the actual reward is not present yet. Thus, anticipation requires the ability to internally represent expectations of a forthcoming reward during the appetitive phase [346] that precedes the actual consumption of the reward (consummatory phase) [28][188]. In several experiments conducted with rats anticipatory behaviour prior to the arrival of, for instance, food, water or sexual contact has been described as an increase in activity (for example: [38][183][253][271]. In some studies it was mentioned that rats showed an increase in alertness, grooming, exploration and running [33][35][36]. Furthermore, Dum & Herz [127] suppose a state of behavioural arousal resulting from endorphinergic modulation of neural reward systems to be a part of

the anticipatory response. In some studies the 'spontaneous' behavioural response in anticipation of a reward is shaped by the test situation, for instance, the number of level changes in a bilevel box as a measure of motivation for sexual contact [249][389] or the performance of an operant task in an instrumental conditioning paradigm [103]. As a consequence of an association between a certain stimulus and a reward rats display enhanced locomotor activity in anticipation of the delivery of the reward, which is defined as being an expression of biologically significant preparatory behaviours [243]. Thus, anticipatory behaviour can also be referred to as 'preparatory' behaviour, which is indicative of the function that has been ascribed to this response. It is said to prepare an animal for a forthcoming change and leads to and facilitates consummatory behaviour [35]. Historically, terms such as approach behaviour and goal-directed behaviour have been used as well [89][122][85].

Although anticipatory behaviour has been observed on several occasions, in neither of the aforementioned studies the behavioural profile of the spontaneous anticipatory response was further quantified since it never had the prime interest. Therefore, one of the first steps that need to be taken in the present thesis is analysing the profile and quantitative aspects of anticipatory behaviour. Subsequently, this information can be used to reach the main goal of the present study: investigating its relevance as a tool to assess and improve the welfare of laboratory rats.

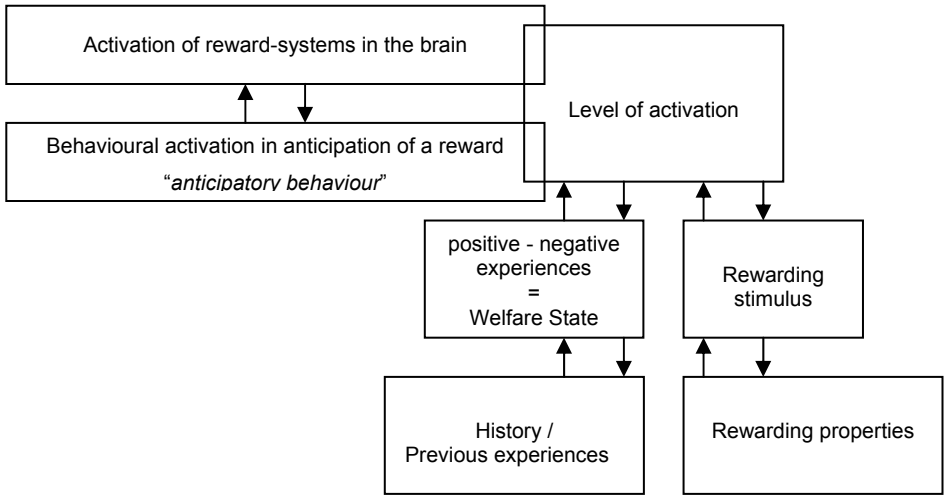

Figure 1. Relation between the sensitivity (and consequent activation) of the reward-system and the consequent behavioural response to rewards (anticipatory behaviour) that are influenced by both the welfare state of an animal and the properties of the reward.

5.1.2. Experimental control of anticipatory behaviour

Anticipatory behaviour can be induced in conditioning paradigms. These paradigms involve motivation and reinforcement in relation to rewards that are anticipated via acquisition of associations between certain stimuli (or context) and the reward. In a classical conditioning paradigm, an animal will learn to associate an initially neutral (visual or auditory) stimulus with the delivery of a reward (unconditioned stimulus; US)(Pavlovian conditioning [283]). Via repeated pairing of the stimulus (conditioned stimulus; CS) with the US the initial (unconditioned) appetitive behavioural response to the reward will shift towards the presentation of the CS and thus becomes a conditioned response. This transfer coincides with the activation of dopamine neurons that shifts from just after the time of reward delivery (consummatory phase) to the time of CS-onset (appetitive phase) after repeated pairings of this CS and the reward [331][334]. The involvement of dopamine in the display of appetitive (anticipatory) behaviour and Pavlovian conditioning has been shown by various authors [197][288-290][28][276].

5.1.3. Utilities of anticipatory behaviour in relation to animal welfare

As depicted in figure 1, anticipatory behaviour is related to the activation of reward-systems in the brain and it reflects the way an animal perceives stimuli from the outside world, which is in its turn influenced by its history. Therefore, it is hypothesized that anticipatory behaviour can serve several purposes in relation to animal welfare (Table 1):

(i) Assessment of welfare

It is argued that anticipatory behaviour reflects the activation of the reward system, and thus reward-sensitivity, and is influenced by previous experiences. Acute or mild stress appears to cause an increased reward-sensitivity whereas chronic or severe stress appears to cause a decrease or even a total absence of reward-sensitivity. It was previously shown that social isolation in rats caused an increase in anticipatory activity for a sucrose reward [376] and that a chronic and more severe stressor caused a total absence of anticipatory activity in rats [403]. Thus, it is argued that anticipatory activity may be indicative of the state of animal in terms of welfare.

(ii) Assessment of appraisal of stimuli

Because it is argued that the level op anticipatory behaviour is influenced by the (rewarding) properties of the forthcoming stimulus/event, the level of anticipation could be indicative for the perception of these stimuli/events in terms of appraisal. In this way, the anticipatory response may be used to investigate the rewarding or aversive value of certain housing conditions and experimental procedures (provided that the welfare state of the animals is constant at that moment).

(iii) Improvement of welfare: counteracting stress

In line with the presently used concept of welfare as being a transient balance between positive and negative experiences and the consequent hypothesis of compensatory mechanisms, it is posed that rewards are able to compensate stress. Because it is known that stress affects the sensitivity of the reward-system and even leads to a total loss of reward-sensitivity if this stress is chronic and severe and an animal fails to cope, regular activation of the reward-system may be a tool to counteract these effects, and thus, improve welfare. Because it is known that dopaminergic activation shifts to the expectation phase after several encounters with a certain reward and its predictive stimuli, it is hypothesised that inducing expectation via a Pavlovian schedule may be of additional value for the therapeutic efficacy of rewards.

Table 1. Several utilities of anticipatory behaviour that can be investigated in a conditioning paradigm in which an initially neutral stimulus is repeatedly paired with the delivery of a reward (unconditioned stimulus). (i) The state of animals with different previous experiences such as housing conditions or stressful events (A,B,C) that influence their welfare can be investigated by announcing a reward (I) and observing anticipatory behaviour in the period before delivery of the reward. (ii) Perception of different stimuli or events (I,II,III) can be investigated by announcing these stimuli to animals with the same previous experiences (A). (iii) The welfare state of animals can be improved by regularly activating their reward-system by means of reward-announcements, thereby counteracting stress.

	Previous experiences (positive / negative)	**Unconditioned stimulus**	**Utility**
i	A B C	I I I	Assessing the state of animals by means of their reward-sensitivity (reflected by the level of anticitipatory behaviour)
ii	A A A	I II III	Assessing the perception of different stimuli/events by means of the level of anticipatory behaviour
iii	D or (E F)	I or (II III)	Improving the state of animals by regularly activating the reward-system via announcements of rewards

6. ETHOLOGICAL NEEDS, ENVIRONMENTAL ENRICHMENT, AND ANIMAL WELFARE

Another way to maintain the balance between positive and negative experiences may be to increase the biological relevance of a captive environment to allow an animal to satisfy its ethological needs. In the past, standardization efforts led to more and more impoverished housing conditions for laboratory animals that are stimulus-poor and in which the performance of a large part of the natural behavioural repertoire is not possible [270]. The ability to satisfy ethological needs should be an important factor in improving and evaluating housing conditions of animals. Enriching the housing environment of animals by the addition of objects in their cage increases the complexity of this environment and the ability to perform a more extensive repertoire of their natural behaviour including the possibility for activity and control at a social and a spatial level [266]. If an enriched environment allows the animal to satisfy its ethological needs [191][298] and thus results into rewarding activities, stress is counteracted continuously. Furthermore, the stimulation of a variety of physiological and behavioural responses provides the animals with an extended adaptive repertoire of responses in case a challenge is encountered. Thus, enrichment may contribute to improved welfare in two ways that both counteract the effects of stress: (i) activating the reward-system through the display of natural behaviour and (ii) increasing the adaptive capacity of the animals.

6.1. Ethological needs

Typically a distinction is made between physiological and ethological (i.e. behavioural) needs of animals. Physiological needs such as nutrition, and climatic influences on the health, productivity, and survival of animals have been recognised for decades. Conversely, ethological needs, although already noted in 1965 (Brambell Report [45]; *freedom to express normal behaviour*), have only been concentrated on since the last decennium. Ethological needs are those activities of which the display is guaranteed by their rewarding properties [348]. Sometimes the term 'psychological needs' is also used which refers to the need to have the possibility, for instance, to hide from conspecifics or frightening external stimuli. In this case, the display of the behaviour (hiding) is not rewarding but the presence of the *possibility* to do so is important. In this thesis, these needs are considered to be a part of the previously mentioned ethological needs. Concerning the rewarding properties of behaviour, Spruijt and colleagues [348] have argued that not only those behaviours that lead *indirectly* to positive feedback (i.e. reinforcement via obtaining (food) reward) but also behaviour that *directly* causes positive feedback through its mere display rather than through its consequences, are rewarding. The latter is especially relevant for animals that are kept under restricted conditions [186] because – in terms of earlier mentioned compensatory mechanisms – it might be that under deprived conditions certain rewarding behaviours may be excessively displayed and develop into stereotypies.

6.2. Enriched housing: effects on brain, behaviour and research

In the last decades much research has been devoted to the subject of environmental enrichment (for a review see [209][395]). It is well-established that housing rats in a stimulating, enriched environment (e.g. large cages with stimulus objects) compared to housing in a non-stimulating, impoverished environment (e.g. housing in barren cages or isolation) induces a number of neurochemical, neuroanatomical and behavioural alterations. Although the principle of brain alteration due to experience can be traced back to 1928 [305], it was Hebb [173] who made this a central feature of his neuropsychological theory. Hebb was the first to study the consequences of enriched rearing on the behaviour of the rat [172] by means of investigating the effects of problem-solving capacity in aged rats that were reared as pets. Environmental enrichment remained an important experimental manipulation since then, and many studies used enriched housing, of mainly laboratory rats, as an experimental tool to study the facilitation of physiological and physical functions of animals. Brain anatomy, plasticity and functions like learning and memory, development, as well as recovery after brain and spinal cord damage were the centre of interest [398][344][120][277][428]. Although these studies did not focus on the welfare of experimental animals, their results contribute to the common idea that enrichment of the living-environment of laboratory rats improves their welfare. Because of the effects of environmental enrichment on brain, behaviour and animal welfare, results of scientific studies conducted with animals are also affected. Enriched housed animals are reported to have a larger behavioural repertoire and to be more efficient in assimilating stimuli from the environment [399]. Hence, these animals are probably better able to cope with and are less sensitive to stressful experimental situations [366][428][221] resulting in more adequate responding [71]. It is therefore expected that enriched housed animals will be more suitable models for many kinds of research questions which increases the scientific validity of the experimental results [304][44][23].

6.3. Enriched housing: assessment of appraisal, welfare, and therapeutic efficacy

6.3.1. Assessment of appraisal of an enriched cage using anticipatory behaviour

In the last decennium, several studies have been conducted that investigated environmental enrichment in relation to animal welfare [72][267][153][278]. The animal's *appraisal* of environmental enrichment, however, is mainly considered by preference studies [37][373] [233][279][374]. As Duncan[130] has indicated, these studies are difficult to interpret since measuring time spent with objects provides only limited information and is dependent upon the choices that are offered (see also [128][37]). Furthermore, the results do not conclusively indicate whether the enrichment is actually perceived as rewarding and this information might be useful in addition to the results of preference tests. Few attempts have been made to present a quantitative measure of the perception of an enriched environment in terms of appraisal [234][233][238][109][217]. These studies aimed to investigate how much an animal is willing to invest to get access to a certain environmental feature. This method is based on operant techniques to establish demand functions by which the motivation of an animal to perform a specific behaviour is measured. However, these studies mainly involve species other than rats and mainly focus on only one feature. Furthermore, during these operant tasks the animals have to perform activities such as pushing or lifting a weight or pressing a lever, which are not always related to their natural behavioural repertoire. For these reasons an animal may not always be able to learn an operant response [128]. It is important that they associate the required activities with the goals to be reached, and this might be easier if the behavioural response required for expressing the preferences is reasonably natural for the type of reward [150][375]. One of the aims of the project described in this thesis was to verify that rats perceive an enriched cage that was developed in our laboratory, as a rewarding stimulus using a quantitative and objective parameter. The natural behavioural response of animals to a certain commodity is very likely representative for the perception of this commodity. Therefore, anticipatory behaviour, could be very useful for the assessment of the perceived appraisal of environmental enrichment.

6.3.2. Assessment of welfare of differently housed animals using anticipatory behaviour

Behavioural deprivation as is the case in the commonly used standard housing systems for laboratory rodents [107] and most other captive animals is considered to be stressful for these animals. An enriched cage that provides increased possibilities to display more natural behaviour should therefore be less stressful. Deprivation of essential stimuli urges an animal to react more eagerly, c.q. be very sensitive, in case of the presence of a valuable reward. For instance, social deprivation [194][260] can cause increased sensitivity for rewards. It is therefore expected that animals housed in enriched conditions are less sensitive for rewards since they are kept under less deprived conditions. As argued in this thesis, the sensitivity for reward can be reflected by the anticipatory response and, thus, to validate the effect of improved housing conditions it could be useful to study the anticipatory response of differentially housed animals. This way, the improvement of housing conditions may be validated via a decreased need (sensitivity) for rewards. In addition to assessment of reward-sensitivity as a parameter for the deprivation-induced increased need for a reward, the effects of the differential housing conditions on behaviour in general are also important. Enriching the environment should have a positive influence on behaviour in the home-cage. For instance, increased complexity and compartimentalisation is assumed to offer the ability to

avoid aggressive encounters and it should be established that an enriched cage actually causes a decrease in aggression rather than an increase as is for instance seen in mice [394]. Furthermore, assessing the coping-capacity of enriched housed animals may also be important to assess the effects of enriched housing on the welfare of these animals since coping-capacity appears to be strongly related to welfare (see section 3.1).

6.3.3. Therapeutic efficacy of an enriched cage: counteracting stress

As mentioned at the start of section 6, in an enriched environment animals should be able to counteract stressful experiences via the display of rewarding activities. This way, the animals have more control over their own situation which is likely to result in less stressful situations for the animal. Environmental enrichment can then also be used as a therapy to counteract stressful experiences. This therapeutic efficacy can for instance be investigated by using an animal model that is validated for its stress effects to find out what effects an enriched cage may have on the persistence of these stress effects. If the therapeutic efficacy of environmental enrichment is validated it can be utilized as an easy tool to counteract stress in laboratory and other captive animals. Furthermore, as argued in section 5.1.3, prolonging the activation of the reward-system via announcement of an enriched cage may have an additional effect on the therapeutic efficacy.

7. AIM, APPROACH AND OUTLINE

The experiments described in this thesis aim to validate tools to assess and improve welfare of laboratory rats. This is approached in two ways:

(1) Assessing *and* improving their welfare by means of announcing and providing rewarding stimuli

(2) Improving their welfare by means of environmental enrichment.

In this approach, animal welfare is conceptualized as the state of the balance between positive and negative experiences. It is argued that in the presence or the expectation of a reward (or other challenge), the state of the animal is internally evaluated via an 'evaluation-system' and its behavioural response is adapted according to the sensitivity to (need for) the reward. This sensitivity depends on previous experiences with positive and negative stimuli (e.g. stress increases the sensitivity to rewards). Thus, reward-sensitivity is argued to be an indicator of the state of the balance and thus of the state of the animal in terms of welfare. Reward-sensitivity is measured by means of the reward-related behavioural response in anticipation of a reward that is evoked in a Pavlovian conditioning paradigm. Furthermore, this anticipatory behaviour is argued to be dependent on the rewarding properties of the announced stimulus and is therefore also utilized to investigate the appraisal of housing conditions. Additionally, the induction of anticipation is argued to be activating the reward-system, which is used as a therapy to counteract negative experiences. Thus, anticipatory behaviour is argued to have 3 utilities: (i) assessment of welfare, (ii), assessment of appraisal of certain stimuli/conditions, and (iii) counteracting stress. The main aim of this thesis is to validate these 3 utilities. In addition, it is argued that the counteraction of negative experiences via rewarding activities can also be induced by environmental enrichment since the satisfaction of ethological needs is considered to be rewarding. Considering the fact that improving housing conditions of laboratory animals should be the first step to take to improve welfare of these animals, this thesis also pays special attention to environmental enrichment. Via the combination of anticipatory behaviour and enriched housing it is aimed

to investigate the 3 hypothesized utilities of anticipatory behaviour and also to validate the applied type of enrichment as a tool to improve welfare.
The experimental part of this thesis starts with addressing the improvement of welfare in terms of housing conditions by investigating a relatively simple type of enrichment that was developed at our laboratory. This is done to be able to use this enriched cage for the rest of the experiments. In this chapter it was explained that it is important that this enriched cage should be perceived as rewarding by the animals and also influences behaviour in a positive way. This is investigated in Chapters 2 and 3:
In **Chapter 2**, it was aimed to investigate the rewarding value of the developed enriched cage by means of analysis of the anticipatory response evoked by announcement of the transfer to such a cage. For this, the transfer to an enriched cage was announced via a Pavlovian conditioning paradigm in which an initially neutral stimulus (sound/light) was repeatedly paired with this transfer. A time-interval between the announcement and the transfer was applied in which the anticipatory behaviour could be monitored. In this study, the anticipatory response was investigated in detail to characterize and quantify this behavioural response and be able to utilize it for subsequent experiments.
In **Chapter 3**, the effects of a relatively simple enriched cage on home-cage behaviour and behaviour during a behavioural test for anxiety and exploration was investigated. To that end, rats were housed in a standard or an enriched cage for one year and subsequently observed in their home-cage and subjected to an elevated plus maze test. Total duration of agonistic behaviour, activity, inactivity and exploration in the home-cage were assessed. In the elevated plus maze test, the activity (as reflected by the number of arm entries) and time spent on open areas were analyzed as parameters for exploration (approach-avoidance conflict) and anxiety.
Because it was expected that standard housed rats would be more sensitive for rewards than enriched housed rats due to the behavioural deprivation and consequent increased need for rewards to compensate this deprivation-induced stress, reward-sensitivity was investigated in these differentially housed animals. This is described in **Chapter 4**, in which the level of anticipatory activity for an announced sucrose-solution of standard and enriched housed rats was analyzed as a measure of reward-sensitivity. A similar approach was used in **Chapter 5** by means of a different method. In the study described in this chapter, instrumental conditioning was used to investigate the differences in reward-sensitivity between standard and enriched housed rats. This was done to get more insight in the common features and possible differences between instrumental and Pavlovian conditioning to be able to use the available knowledge on instrumental conditioning for the interpretation of our results on Pavlovian conditioning and to be able to determine whether both conditioning methods can be used for welfare research at equal merits. Instrumental conditioning was conducted in fully automated operant chambers and to be able to compare both conditioning paradigms, the animals were trained in these chambers via a Pavlovian schedule as well.
To further increase our knowledge about a possible common underlying mechanism a second instrumental conditioning experiment was performed, which is described in **Chapter 6**. In this study, it was investigated whether the presentation of a conditioned stimulus (CS), which has been paired with a reward in a Pavlovian conditioning phase, would enhance the performance of an independently acquired instrumental response. In this 'Pavlovian-to-Instrumental-Transfer' experiment, the relation between the CS-induced anticipatory activity during the Pavlovian conditioning phase and the CS-induced number of lever presses during the transfer-test was analyzed.

Because it is hypothesized that the induction of anticipation can also be applied to improve welfare in terms of preventing insensitivity of the reward system in chronically stressed animals, **Chapter 7** describes a study that aimed to investigate the therapeutic efficacy of anticipation to rewards. For this, an animal model of depression was used that concerns inducing chronic social stress via repeated defeat and subsequent individual housing of rats. These chronically stressed rats were subjected to a therapy of repeated announcements of a sucrose-reward during the long-term isolation-period. After several weeks it was investigated whether the previously reported chronic-stress induced impairment of the expression of reward-related behaviour in these defeated animals had been prevented by this behavioural therapy. In **Chapter 8** it was investigated whether regular stay in an enriched cage or inclusion of an announcement of the transfer to this cage could reverse the depressive-like symptoms of chronically stressed rats. In this chapter, the effects of the therapy on the stress-induced impairment in the expression of reward-related behaviour and the stress-induced impairment of hippocampal synaptic plasticity were investigated.
In **Chapter 9**, the results of the separate studies as described above are integrated and discussed in relation to the implications for animal welfare and scientific research.

NOTE: The format of the chapters may vary due to the different guidelines of the specific journals in which they will be or are published (or are submitted to).

BOX 1: NOTE ON TERMINOLOGY

Balance

To conceptualize welfare as the balance between positive and negative experiences can cause some confusion since the word 'balance' is not only synonymous to 'weighing scale', but also to 'equilibrium' which implies that the weighing scale is stable/in balance. The word 'balance' used in this thesis refers to the first synonym and by using the terms 'transient' and 'state of' in relation to 'balance' it was tried to avoid the abovementioned confusion. Furthermore, when it is posed that an animal will try to maintain a balance between positive and negative experiences it is not meant that an animal will also search for negative stimuli. Negative stimuli are always present in nature and besides trying to avoid those stimuli an animal will search for positive stimuli to compensate the unavoidable negative stimuli.

Appetitive-consummatory versus wanting-liking

Berridge [27] poses that reward contains distinguishable psychological or functional components - 'wanting' (appetite / incentive motivation) and 'liking' (pleasure / palatability). It is suggested that 'wanting' refers to the behaviours that are used to consume and to anticipate the reward and that 'liking' refers solely to the palatability of a reward as measured by taste-reactivity patterns [28]. This implies that the distinction between wanting and liking is not synonymous to the distinction of appetitive and consummatory behaviour. In this thesis, anticipatory behaviour is defined as reward-related behaviour occurring during the appetitive phase indicating a maintained state of attention that reflects the need/sensitivity for reward ('wanting') and leading to and facilitating consummatory behaviour which is also dependent on the appraisal of the reward ('liking').

Sensitivity and motivation

It is important to notice that in the present study we do not make a clear distinction between motivation and sensitivity to the stimulus-properties. Reward-value can be influenced by the characteristics of the reward (e.g. the concentration of a sucrose solution), previous experiences with the reward, and the internal state that can be influenced by both physiological factors (hunger and thirst) and 'emotional' factors ('wants' and 'needs'). In this thesis it is argued that the latter factors are influenced by previous experiences that can cause increases in the sensitivity (need) for a reward to compensate negative experiences and maintain a balance between stress and reward. It is argued that an increased need for a reward is reflected by an increased motivation to obtain the reward, and therefore, consider motivation and sensitivity to be interacting factors that cannot be distinguished easily. Following the same line of reasoning, 'need' and 'sensitivity' are used as synonyms.

Antromorphistic vocabulary

The abovementioned explanation of terminology of motivation and sensitivity is an example of the difficulty to avoid antropomorphistic vocabulary and concepts. Words as 'emotion', 'wants' and 'needs' are widely used but also widely avoided by authors that regard them to be subjective. Cabanac, who uses the word 'pleasure' very often, has addressed the concern of ambiguity in a footnote in one of his papers [62]. He states that the use of the word 'utility' instead of 'pleasure' is ambiguous as well since it can refer to the stimulus as well as the mental experience of a stimulated subject. Thus, instead of removing the ambiguity by using all kinds of different detailed adverbs and thus creating difficult terminology he rather uses 'pleasure' and 'pleasantness'.

In this thesis, I tried to avoid or clearly explain antromorphistic vocabulary, but I did not avoid it 'at all costs' since some of these words most clearly convey what is meant.

Perception and appraisal

In this thesis, 'perception' and 'appraisal' are sometimes used as synonyms although one might argue that perception is synonym to 'detecting' or 'sensing'. However, 'perception' is more than just sensing internal or external stimuli; it involves some processing or interpretation by other areas of the brain (the secondary and tertiary cortical sensory areas). Duncan & Petherick [132] characterize 'perceiving' as a, although one of the simplest, cognitive process and categorize it as being distinct from 'sensing/detecting'.

Standard housing

For a gregarious species such as rats, social housing is probably the first need [281]. In rats, social isolation causes a variety of behavioural and neurochemical changes [165] and is extensively used as an animal model to study mental disorders such as schizophrenia and depression [328][354][403]. In this thesis, individual housing is considered to be stressful for a gregarious species such as rats and is only applied as a part of experimental procedures to induce stress. Thus, in the experiments described in this thesis, 'standard housing' is defined as social housing in a stimulus-poor environment, although I believe that 'impoverished housing' would be a better term for this type of housing condition. However, I used the term 'standard' to refer to the conditions that are considered to be standard in most laboratories.

CHAPTER 2

ACCESS TO ENRICHED HOUSING IS REWARDING TO RATS AS REFLECTED BY THEIR ANTICIPATORY BEHAVIOUR

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Animal Behaviour, In press

ABSTRACT

The main aim of the present study was to verify the general assumption that enrichment of the housing environment is rewarding to laboratory rats. The behavioural response in anticipation of a forthcoming reward was used as a measure for the rewarding property of a simple enriched cage. For this, a Pavlovian conditioning schedule was applied to announce the oncoming transfer to an enriched cage. The response of rats in anticipation of this transfer was compared to that of rats that expected sexual contact, transfer to a standard cage or forced swimming. A second aim was to characterize the behavioural profile of the anticipatory response since up until now only general descriptions of this behaviour are available.

The strong increase in activity in anticipation of both an enriched cage and sexual contact and the similar response concerning the analysed behavioural elements indicate that the appraisal of access to an enriched cage shares a common denominator with the perception of sexual contact. Since the latter is generally accepted to have highly rewarding properties to rats it is concluded that the enriched cage is highly rewarding as well. The anticipatory response for sexual contact or an enriched cage was significantly different from the anticipation for access to a standard cage or a forced swim session indicating that this response is related to the positive nature of the stimulus. In general, anticipation appears to be quantifiable by the level of activity measured by the total frequency of displayed behavioural elements. Additionally, some behavioural elements such as exploration, locomotion, arousal and grooming seem to be more specifically related to the nature of the forthcoming stimulus.

INTRODUCTION

In the last decades much research has been focused on environmental enrichment [209][395]. In most of these studies, enriched housing of rats has been used as a tool to study the behavioural and physiological adaptive capacity, i.e.: brain anatomy and plasticity, learning and memory, as well as functional recovery after brain and spinal cord damage. Although a different goal is pursued, these studies contribute to the common idea that environmental enrichment improves the welfare of laboratory animals. Recently, several studies have been conducted that considered environmental enrichment in relation to animal welfare [278]. An enriched cage allows animals to display a more extensive repertoire of natural behaviour and may provide appropriate stimulation to facilitate coping with physiological and ethological needs [298]. It has been argued in Chapter 1 that successful coping is maintaining a balance of stress and reward systems (see also [348]), which is in line with Broom's [52] definition of welfare as the result of successful and unsuccessful coping. This implies that rewarding events or activities may counteract the effects of stress (Chapter 1; see also [26][348]). If an enriched environment allows satisfaction of ethological needs [191] and thus results into rewarding activities, stress is counteracted de facto. The general assumption that satisfying ethological needs is rewarding raises the question to what extent enriched housing is rewarding. So far, the animal's appraisal of environmental enrichment is mainly considered by preference studies [233][279][375]. Although preference tests are valuable in animal welfare studies, the results are difficult to interpret [128][130] and do not conclusively indicate whether the enrichment is actually perceived as rewarding. Up until now, few attempts have been made to present a quantitative measure of the perception of an enriched environment in terms of appraisal (e.g. [217][238]) and these studies mainly involve species other than rats and focus on only one commodity.

The main aim of the current study was to verify that rats perceive an enriched cage (with increased complexity and opportunity to explore and hide) as rewarding using a quantitative and objective parameter. The behavioural response of animals to a certain commodity is likely to be representative for the perception of this commodity since the neuronal substrates of behavioural activation and the perception of reward are remarkably similar [199]. In line with this, Spruijt and colleagues [348] have argued that behavioural activation in anticipation of the arrival of a reward represents the activation of reward centres in the brain. The level of activation depends, among other parameters, on the incentive value of the reward (e.g. [211][310]). Therefore, a behavioural parameter based on this response could be very useful for the assessment of the appraisal of environmental enrichment. In the present study the intensity of the behavioural activation occurring in the time-window between the announcement and the arrival of a reward is used as an indicator of the perception of an enriched cage. This anticipatory response is induced by a Pavlovian conditioning schedule in which an initially neutral stimulus is repeatedly paired with the transfer to an enriched cage. Anticipatory behaviour was as early as 1918 described by Craig [89] as a typical arousal with goal-directed activity that occurs in the appetitive phase when the actual reward is not present yet. Later, it has been generally described in several studies (e.g. [38][252]) but it was never further quantified. Therefore, the first step in this study is to analyse the profile and quantitative aspects of anticipatory behaviour. Subsequently, this information can be utilized to reach the main goal of the present study: assessment of the perceived appraisal of access to environmental enrichment to laboratory rats.

METHODS

In situations where a cue (conditioned stimulus; CS) is repeatedly presented prior to the acquisition of a reward (unconditioned stimulus; US), animals associate these two events (Pavlovian conditioning [283]). Consequently, the mere presentation of the cue can provoke a reaction, which is called the conditioned response. This response consists of a reward-specific response, such as salivation in case a food reward is expected, and an independent activation of behaviour. This behavioural response can for instance be seen if a time interval exists between the CS and US (see for instance [339]). Based on this knowledge, the present study utilizes a conditioning paradigm with an interval between the offset of the CS and onset of the US to investigate the rewarding property of an enriched cage. This was realized by comparing the level of anticipation in the CS-US interval for this commodity to that of groups which receive a strong positive US, a neutral US, a negative US or no US. Different behavioural elements displayed in the time-interval between CS and US are carefully analysed in order to characterise and subsequently quantify the anticipatory response. Furthermore, by using both rewarding and aversive stimuli it was attempted to determine that the behavioural response typical for so-called positive anticipation is different from so-called negative anticipation. From fear-conditioning studies it is known that rats show an increase in anxiety-related behaviour such as freezing and consequently decreased locomotor activity when a foot shock is expected (e.g. [69][163]). Therefore, it is expected that positive and negative anticipatory responses can be distinguished by behavioural activation versus behavioural suppression.

The experiments have been performed in adherence to the legal requirements of The Netherlands concerning research on laboratory animals, and have been approved by the Ethical Committee of the Utrecht University.

Experiment 1

In this experiment the anticipatory response of rats to the expected transfer to an enriched cage is compared to that of groups receiving either a strong positive US (sexual contact), a neutral US (standard cage) or no US (stay in home-cage) after the presentation of the CS (see 'Procedure' and 'Unconditioned stimuli' for details).

Subjects

Forty-eight experimentally naive male Wistar rats (U:WU, (CPB), GDL, Utrecht University) served as subjects. These animals were 10-11 weeks old and had a mean body weight of 392 ± 4 gram at the onset of the experiment. Thirty-six receptive female Wistar rats (U:WU, (CPB), GDL, Utrecht University) acted as unconditioned stimulus for one of the control groups. Two weeks before participation in the experiment these females were bilaterally ovarectomized under a combined anaesthesia of a neuroleptic (Hypnorm®) and a sedativum (Dormicum®). At the onset of the experimental procedures they had a mean body weight of 209 ± 11 grams and were 10-11 weeks old. All animals (males and females) were sexually experienced at the start of the experimental procedures.

Housing and husbandry

Male rats were socially housed in cohorts of three rats in a Makrolon type IV cage (1875 cm²; height: 18 cm; Tecniplast, Milan, Italy). When recovered from ovarectomization, female rats were housed in cohorts of 3 animals in type IV cages that were enriched with a shelter and gnawing sticks. Males and females were housed in separate temperature-controlled rooms (21 ± 2ºC) and background music was present 24 hours per day. Bedding material type 3-4 (Lignocel 3/4 ®, Rettenmaier & Söhne, Ellwangen-Holzmühle, Germany) was provided and lab chow (RMH-B®, Hope Farms, Woerden, The Netherlands) and water were available ad libitum. The animals were housed under a reversed dark/light cycle (dim light (25W): 8:00h- 20:00h; bright white light: 20:00-8:00) since rats are mainly active at dusk/night and behaviour should therefore be observed in this active period. The animals were allowed to adjust to the room and light regime for two weeks and were handled daily during this period. Cages were cleaned and animals were weighed once per week. This was always done after the experimental tests to prevent influence of this disturbance on behavioural parameters.

Procedure

In the present study a conditioning paradigm was used by which a reward or other forthcoming event (unconditioned stimulus; US) was announced repeatedly by an initial neutral stimulus (conditioned stimulus; CS) in the form of a bell. A time-interval of ten minutes between the offset of the CS and onset of the US was applied in which the anticipatory response could be investigated. The subjects were divided at random into four experimental groups of twelve animals each. These four groups were randomly assigned to receive one of the unconditioned stimuli. The animals were trained once a day for four days and tested on every fifth day during the weekdays of 5 consecutive weeks. The training and test sessions were conducted in an experimental room to which the animals were transported per experimental group in their home-cage on a cart. The animals were trained and tested in their home-cage where they had continuously access to water and food. After transportation the animals were left undisturbed for several minutes. Subsequently, the CS was presented and after a time-interval of 10 minutes the US was offered. The US-period lasted for 30 minutes and after that the animals were transported back to the housing room. Timing of training and testing was counterbalanced among the experimental groups in order to avoid the acquisition of an association between time and order of testing and the presentation of the US. During the test sessions (every fifth day) behaviour of the animals in the time-interval between CS and US was recorded on tape and analysed afterwards. The subjects were housed with dim (white) light in the dark period in order to be able to test under these conditions and make clear recordings of the displayed behaviour with a normal camera. Each experimental group of 12 animals received a different unconditioned stimulus except for the general control group to which only the CS was presented.

Unconditioned stimuli

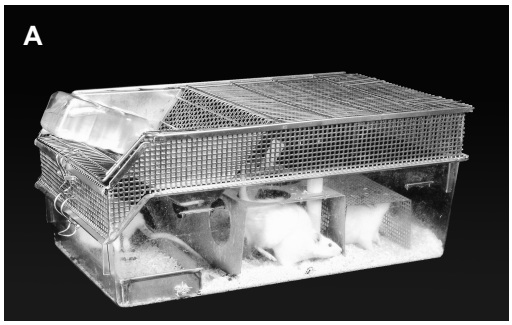
The main experimental group was transferred to an enriched cage (EC) after the presentation of the CS. Animals that gained access to receptive females (SX) served as the positive control in this experiment since sexual behaviour belongs to a class of naturally occurring behaviours that are generally considered to be rewarding (see for instance [137]). A third group was transferred to another standard cage (SC) to control for general arousal effects caused by the transfer. The general control group stayed in their home-cage (HC) after the presentation of the CS in order to prevent the forming of any association. This group serves

as a control for a possible (unconditioned) behavioural reaction to the CS. The US was offered in the same room and lasted for 30 minutes.

The cages to which the animals of the SC- and EC-group were transferred were labelled in the same way as the home-cages so that every cohort of cage-mates was always transferred to the same cage. Moreover, these cages were not cleaned during the course of the experiment. In this way the anticipatory response could not be related to a novel environment. For each animal of the SX-group one female was available. To accomplish this, 3 females were placed in each home-cage of the males at the start of the US period. Sexual receptivity was induced in the females by subcutaneous injections of oestradiol benzoate (20 mg) and progesterone (500mg). These hormones were subcutaneous administered 48 hr and 3 hr, respectively, before each test.

Environmental enrichment

The enriched cages (Fig.1A) that were used as an unconditioned stimulus for the EC-group were developed at our laboratory and consisted of a standard Makrolon type IV cage (see Fig. 1B) with several extensions (a rim, 3 objects and gnawing sticks). The general characteristics of these cages are: an increased height, increased dimensions, increased space and compartmentalization. These features improve the ability to display natural behaviour such as rearing, climbing, jumping, hiding, and avoiding each other and also reduce the chance of under-stimulation. Furthermore, the presence of a toilet (containing a large amount of droppings) made it possible to clean the cage partly and, thus, reduced disturbance.



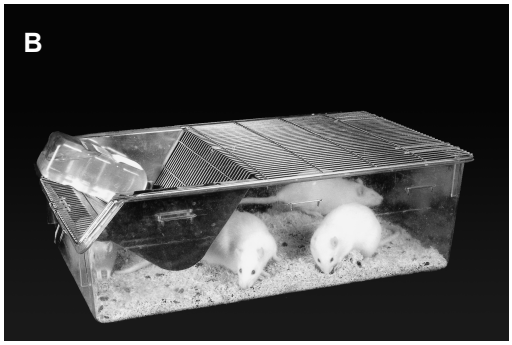


Figure 1.
The enriched cages (A) consist of a standard Makrolon type IV cage (B) (1875 cm^2; Tecniplast, Italy) with several extensions:

1) Rim (8 cm) to increase the height;
2) Shelter (10x11x24.2 (hxwxl));
3) tunnel-shaped compartment (14.5x16x32 (hxw xl)) with passages at the sides and on top and small holes with gnawing sticks inserted;
4) Toilet (5x12x32 (hxwxl)).

Objects 2 and 3 increase the utilizable area by app. 45%.

Photo's: Patrick Fermont

Observational procedure.

Every fifth day the behaviour of the subjects in their home-cage during the CS-US interval was recorded on videotape by two cameras (Sony Hi-8 30x). A set of 2 x 2 cages was recorded simultaneously. In that way, each individual animal of every separate experimental group was tested at the same time. The recordings were analysed with commercial software ('The Observer', Noldus Information Technology b.v., Wageningen, The Netherlands) using the focal animal sampling method (continuous observation per animal). By following the procedure as described above, a complete data set was obtained on the performed behaviour of a whole experimental group at exactly the same time-period.

Behavioural elements

An ethogram of 30 behavioural elements was used for behavioural observations (Table 1). The behavioural elements were mainly derived from Timmermans [361], Grant [158], and Draper [125]. When necessary a short description of the behavioural elements will be given in the present section. A detailed description of most behavioural elements scored in this study can be found in the aforementioned papers. A distinction was made between 'mobile' (12) and 'immobile (13) exploration'. When the rat is exploring a certain area by which it does not move or just moves a little around on the same spot the activity is scored as 'immobile exploration'. If the rat clearly alters this activity by moving forward (with all four feet) in a certain direction while exploring, the activity is scored as 'mobile exploration'. In case of a 'head raise' (26), the animal lifts up its head for a moment and sniffs or looks around before continuing the foregoing activity or another behaviour. A sudden convulsive movement of the head and/or body is called 'jerking' (29).
'Yawning', 'shaking' and 'jerking' (27-29) is expressed during the display of another behavioural activity and is therefore recorded as event. The computer program registers an event as a point on the time axis while the duration of the other behavioural activity is still registered. 'Scanning' (25) is defined as the left-right movement (swinging) of the head and sometimes the front part of the body while the rest of the body is immobile. The behavioural element 'gnaw/nibble' (11) is directed towards the sawdust (or the sometimes big particles in it), the walls of the cage or the wires of the lid.

Table 1. Ethogram: behavioural elements recorded during the observations

1	Drink	12	Mobile exploration	23	Circling/chase tail
2	Eat	13	Immobile exploration	24	Freeze
3	Walk	14	Rear	25	Scan
4	Sit	15	Root/dig	26	Head raise
5	Lie	16	Social sniff	27	Yawn
6	Huddle	17	Anogenital inspection	28	Shake
7	Groom	18	Defensive behaviour	29	Jerk
8	Scratch	19	Offensive behaviour	30	Hop
9	Groom genitals	20	Keep down	31	Run
10	Groom other animal	21	Keep off lying	32	Hang on lid of cage
11	Gnaw/nibble	22	Mount		

Data analysis.

Mean frequencies and durations were calculated for the behavioural elements and categories. Since the individuals within one cage could not be considered to be independent in the analysis mean values per cage were calculated for each test. Subsequently, the mean value of five experimental tests was calculated for each experimental group of four cages. These data were compared using the non-parametric Kruskal-Wallis test followed by a Mann-Whitney-U test, as the data were not normally distributed for all tests. Certain behavioural elements assumed to be related to a state of arousal were analysed as one category. These 'Arousal Factors' are presented in Table 1 with the numbers: 8, 23, and 28-32. Also the elements of 'Exploratory Behaviour' (12-15), 'Locomotion' (3, 12, 30, 31), 'Grooming' (7-9), 'Social Behaviour' (6, 10, 16-22) and 'Resting' (4-6) were combined and analysed as categories. Since the category 'Arousal' consists mostly of behavioural elements that are registered as so-called events (points on the time-axis with no duration) the analysis of the duration is not performed for this category.
The anticipatory activity in the ten-minute observation period was established by adding the scored frequencies of all individual behavioural elements specified in Table 1. Behavioural data are presented as group means ± SEM. The Statistical Package for the Social Sciences (SPSS, version 9.0) was used for all statistical calculations.

Ethical Note

It was necessary to ovarectomize (OVX) the female rats and subsequently control their hormone-level by administration of exogenous hormones since it was important for the success of the conditioning procedure of the SX-group that the females were indeed sexually receptive at the particular moment they were introduced to the males. It would not have been possible to be sure that the male rats were anticipating sexual behaviour if the female rats were not receptive since their behaviour is highly dependent on their hormone-level. Each female was only used as stimulus 1 or 2 times per week. In addition to the above-described anesthesia, post surgical analgesia was applied via administration of TemgesicÒ. Subsequently, the animals received a subcutaneous injection with 1 ml of saline. After surgery the animals were placed on a heated mat until they got conscious again and started to move around. At that moment they were placed in their home-cage. The animals were weighed every day for several days and their food and water consumption was monitored. The females would not be used as stimulus-animals before they weighed more than their pre-surgical weight and the wounds were completely closed.
After completion of the experiments a part of the males rats was kept for educational purposes and permanently housed in enriched cages. Since no other purpose was found for the rest of the males they were euthanised. Since it is not desirable to subject new animals to surgical procedures (OVX) if not necessary, all females were kept for other experiments. They remained housed in enriched cages.

Experiment 2

This experiment aimed at investigating whether the anticipatory response, displayed by the animals receiving a positive US (enriched cage), is specifically related to the positive character of such a stimulus. Therefore, the anticipatory response of rats to the expected transfer to an enriched cage is compared to that of rats that expect a negative US (forced swimming). Forced swimming is chosen for its similarity to environmental enrichment concerning physical activity and natural characteristic (in the wild, rats can encounter water

and may need to cross it). To further minimize variability and the number of animals needed a different method is applied: the animals serve as their own control and are therefore tested at different time-points (before, during and after several training sessions). Furthermore, the animals are tested individually in a different context (observation cage) than they were trained in (home-cage) (see 'Procedure' and 'Unconditioned stimuli' for details).

Subjects

Twenty-four experimentally naive male Wistar rats (U:WU, (CPB), GDL, Utrecht University) served as subjects. These animals were 8-9 weeks old and had a mean body weight of 301 ± 5 gram at the onset of the experiment.

Housing and husbandry

Housing and husbandry procedures were the same as in experiment 1 except for the weight monitoring; the animals were weighed several times per week during the course of the experiment. This was done to ensure that the condition of the group that received a stressor as unconditioned stimulus would not decline dramatically.

Procedure

The subjects were divided into two groups of twelve animals each. These groups were randomly assigned to one of the experimental conditions (EC: enriched cage or FS: forced swimming). Similar to Experiment 1, a conditioning paradigm was used in which a forthcoming event was announced repeatedly by a stimulus (once a day for a total of 17 trials). In this experiment a combination of an auditory and visual stimulus was used (bell and light flash). The time-interval between the CS and US was gradually increased over the training sessions from 0 to 10 minutes. The animals were trained once a day in their home-cage (thus, per 3 animals) and tested during trial 0 (pre-training; CS-US: 10 min), trial 10 (CS-US: 5min) and trial 17 (CS-US: 10 min) for which they were (individually) transferred to an observation cage. During the habituation period the animals were transported twice to the observation room and placed in the observation cage to habituate them to the experimental procedures and to ensure that novelty would have no effect on the results. The first test at trial 0 was conducted to set a baseline for behavioural activity in the observation cage for 10 minutes after presentation of a (at that moment) meaningless stimulus. In this manner, the animals serve as their own control in the sense that an increase or decrease in behavioural activity in the CS-US time-interval of 10 minutes after training (thus, after an at that moment meaningful stimulus) can be compared within subjects. Training was conducted in the same room as the animals were housed, for that reason the two experimental groups were housed in separate rooms. Test sessions were conducted in a different context (individually in an observation cage). For this, the animals were transported in their home-cage to an experimental room.

Unconditioned stimuli

The main experimental group was subjected to a forced swim session (FS) following presentation of the CS. Forced swimming was chosen since it is proven to be stressful to rats (see for instance [9301]), and, similar to environmental enrichment, consists of physical activity that might be displayed under natural conditions. The relevance of this was indicated by Suarez and Gallup [355]: they noted that most of the applied stressors such as electric shocks are highly atypical under natural conditions and may therefore not be very relevant. Following a certain time-interval after the CS animals were transferred to a water-filled glass

cylinder (diameter: 20cm). The temperature of the water and the duration of the swim session was varied between 24°C-34°C and 2-6 minutes respectively. This variation was applied to prevent total predictability and possible subsequent habituation that might influence the perceived severity of the stressor [96]. Clean water was used for each session. Animals that were transferred to an enriched cage (EC; 30 minutes) served as a positive control in this experiment. The US was offered in the same room as where the training or testing took place.

Observational procedure

At trial 0, 10 and 17 the behaviour of the individual subjects during the CS-US interval was recorded on tape. A set of 2 x 3 Plexiglas observation cages (62x26x33 cm; h x w x l) was recorded simultaneously. In that manner, six individual animals were tested at the same time. The recordings were analysed with commercial software ('The Observer', Noldus Information Technology b.v., Wageningen, The Netherlands) using the focal animal sampling method (continuous observation per animal).

Behavioural elements

The observations were carried out using an adjusted ethogram since in this experiment the animals were placed individually in an observation cage. Consequently, no social behaviour would be observed and these elements (Table 1: 6, 10, 16-22) are therefore excluded. In addition, 'Immobile exploration' was now subdivided in 'sniffing' and 'attention' since it appeared from Experiment 1 that immobile exploration could consist of sometimes-rapid changes between 'sniffing' and 'attention' (personal observation). Hanging on the lid of the cage was not possible in the observation cages and is therefore excluded from the ethogram.

Data analysis.

Behavioural data were expressed as group means with standard error of the mean (SEM). Mean frequencies and durations of the behavioural elements and categories were analysed per minute. Similarly, activity (represented by the mean frequency of all behavioural elements) was analysed per minute. Differences in behaviour between the experimental groups over the course of the training trials were analysed with an ANOVA for repeated measurements (between subjects factor: type of unconditioned stimulus; within subject factor: trial). Differences in behaviour between the experimental groups at the separate trials were analysed by a t-test for independent samples. The Statistical Package for the Social Sciences (SPSS, version 9.0) was used for all statistical calculations.

Ethical Note

The reason for not choosing a less aversive stimulus was that a pilot-experiment in which transfer to a mesh-wire cage acted as unconditioned stimulus (data not shown) yielded inconclusive results. It appeared difficult to distinguish the anticipatory response to this US from the response to a familiar standard cage (similar group as SC in Experiment 1). It was not clear whether that stimulus was perceived as stressful and therefore a more convincing stimulus, that was proven to be stressful in several studies, was chosen. The behaviour and physical condition of the animals was closely monitored. The animals were weighed several times per week to be sure that the condition of the forced-swim group in particular would not decline dramatically. Before the actual protocol of the experiment was determined an in-depth study of the available literature on forced swimming was conducted. Based upon the results of this survey the range of water temperature was chosen between 24°C-34°C since

the stressfulness of forced swimming appears to be a U-function of the water temperature; Exposure at extreme temperatures about below 18°C and above 48°C is very stressful [955] and not acceptable for our experiment. Testing animals in soiled water, i.e. water previously swum in by other rats thus containing an alarm substance, is more stressful [8] and also considered too stressful for this experiment; the cylinders were filled with clean water for each session. The duration of the swim sessions were kept relatively short to prevent fatigue and to moderate the severity of the stressor. Immediately after the pre-determined duration of the swim session all animals were transferred quickly to a 'drying cage'. Cage mates were placed together in the same drying cage. Infrared lamps were hanging above these cages and the bottom was covered with paper towels. Once all animals were placed under warmth lights they were blotted dry one-by-one with a towel and placed back in the drying cages. The animals where allowed to dry further under the infrared lamps for approximately fifteen minutes. A part of the drying cage was covered to give the animals the opportunity to move to the shade. After completion of the experiments the males of the EC-group were kept for testing enrichment and food reward items. Since the forced swimming procedure is considered to be stressful for the rats and the long-term consequences for the welfare of these animals are not clear, the FS-animals were euthanised immediately after completion of the experimental procedures.

RESULTS

Experiment 1

Table 2 presents the mean values and standard errors of the mean (SEM) of the analysed behavioural elements or categories displayed by animals that received one of three unconditioned stimuli (sexual contact: SX; Enriched cage: EC; Standard cage: SC) or no US (stay in home-cage: HC).

Behavioural elements/categories

Analysis of the frequency of behavioural elements and categories revealed that no significant differences were present between EC and SX except for the frequency of grooming (Mann-Whitney *U* test: U=1.0, N_{SX}=N_{EC}=4, P<0.05). Similarly, no significant differences were detected between SC and HC. In contrast, several differences were detected when comparing EC and SX with SC and HC. It became apparent that SX-animals displayed a significantly higher frequency of exploratory behaviour than both SC- and HC-animals (U=0, N_{SX}=N_{SC}=N_{HC}=4, $P_{(SX\text{-}SC)(SX\text{-}HC)}$<0.05). For this category, EC-animals showed a significantly higher frequency than HC-animals (U=1.0, N_{EC}=N_{HC}=4, P<0.05) but no significant difference was detected between EC- and SC-animals (U=2.0, N_{EC}=N_{SC}=4, NS). Similar differences between groups were found when investigating the frequency of locomotion (SX-SC: U=1.0, P<0.05; SX-HC: U=0, P<0.05; EC-SC: U=5.0, N.S.; EC-HC: U=0, P<0.05). For the category of grooming behaviours a significant difference was present between EC- and HC-animals (U=0, P<0.05) and, except for the abovementioned difference between EC and SX, not between other experimental groups. In case of the frequency of social behaviour, no significant differences were detected between either of the experimental groups. When the category of arousal factors was analysed, it appeared that SX-animals as well as EC-animals displayed significantly more of these behavioural elements than HC-animals (SX-HC & EC-HC: U=0, P<0.05). Concerning the arousal frequency of SC, the difference with EC reached significance (U=1.0, P<0.05) whereas a trend towards significance was present for the

difference with SX (U=2.0, P<0.1). Similar to the results of the analysis of social behaviour, no differences were present between the experimental groups concerning scanning, freezing and resting behaviour.

Table 2. Frequencies and durations of behavioural elements (mean±SEM) displayed in the CS-US time-interval[1]

	Unconditioned stimulus (US)			
	Enriched cage (EC)	**Sexual contact (SX)**	**Standard cage (SC)**	**Home cage (HC)**
Frequency				
Exploration	42.35 ± 1.53 a	48.29 ± 2.63 b c	39.20 ± 0.93 b	35.15 ± 1.59 a c
Locomotion	28.44 ± 2.75 a	32.33 ± 1.09 b c	24.85 ± 2.06 b	20.06 ± 2.01 a c
Grooming	8.40 ± 0.23 a b	7.00 ± 0.57 b	7.62 ± 0.70	6.06 ± 0.59 a
Social behaviour	7.31 ± 2.50	3.25 ± 0.95	3.92 ± 1.37	3.21 ± 0.86
Arousal	39.01 ± 3.95 a b	34.69 ± 3.38 c	21.35 ± 1.58 a	27.83 ± 1.04 b c
Scan	0.56 ± 0.09	1.15 ± 0.41	1.13 ± 0.13	0.44 ± 0.20
Freeze	0.83 ± 0.31	0.27 ± 0.15	0.31 ± 0.15	0.25 ± 0.13
Rest	2.06 ± 0.16	2.83 ± 1.01	1.94 ± 0.45	1.77 ± 0.30
Total	117.6 ± 5.45 a b	112.77 ± 3.89 c d	95.05 ± 3.99 a c	83.33 ± 4.19 b d
Duration				
Exploration	315.55 ± 14.45 a	374.5 ± 19.26 a	338.33 ± 10.80	314.35 ± 25.03
Locomotion	50.19 ± 4.14	53.09 ± 2.83	45.75 ± 3.52	45.54 ± 5.38
Grooming	82.32 ± 2.97	76.46 ± 7.59	85.27 ± 10.78	71.50 ± 4.16
Social behaviour	17.57 ± 4.75	8.43 ± 1.96	8.72 ± 3.29	8.20 ± 3.21
Scan	1.19 ± 0.22	3.61 ± 2.22	3.77 ± 0.54	1.37 ± 0.74
Freeze	6.40 ± 1.42	2.35 ± 1.49	2.11 ± 1.20	3.50 ± 1.91
Rest	5.74 ± 0.18	9.47 ± 3.81	8.19 ± 2.10	16.69 ± 10.38

[1]Similar characters in one row indicate significant differences between groups at α=0.05 as analysed by Mann-Whitney U tests (N_{EC}=N_{SX}=N_{SC}=N_{HC}=4 cages of 3 animals per group).

Analysis of the total duration of the displayed behavioural elements and categories indicated that no significant differences were present between the experimental groups in case of grooming, locomotion, social behaviour, scanning, freezing and resting behaviour. In case of exploratory behaviour it appeared that SX-animals spent significantly more time exploring than EC-animals (U=1, P<0.05). All other comparisons between the experimental groups for exploratory behaviour did not reveal significant differences.

Activity

The analysis of the activity represented by the total frequency of all behavioural elements displayed in the time interval between CS and US revealed significant differences between several experimental groups (Fig.2). EC- as well as SX-animals displayed a significantly higher level of activity than both SC- and HC-animals (Mann-Whitney U test: N_{EC}=N_{SX}=N_{SC}=N_{HC} =4; EC-SC & EC-HC: U=0, P<0.05; SX-SC: U=1.0, P<0.05; SX-HC: U=0, P<0.05). EC-animals appeared to display an equal level of activity as compared to SX-animals (U=7.0, NS). Similarly, no difference was detected between SC- and HC-animals (U=3.0, NS).

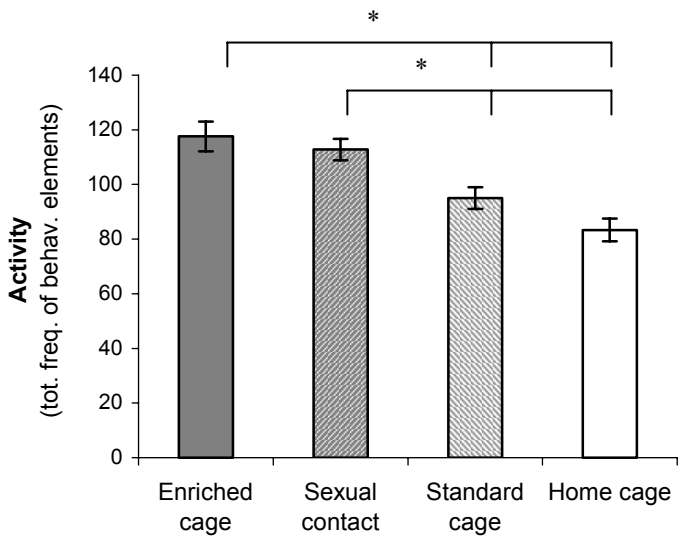


Figure 2.
Activity represented by the total frequency of displayed behavioural elements in the CS-US interval of animals that received different types of US:

-transfer to an enriched cage,
-sexual contact,
-transfer to a standard cage
-no US (stay in home-cage))

Expressed as the mean frequency of 5 tests (± SEM); n=4 cages of 3 animals per group (*: $P<0.05$).

EXPERIMENT 2

Table 3 presents mean values ± SEM of the analysed behaviours displayed by the animals that received either transfer to an enriched cage (EC) or forced swimming (FS) as unconditioned stimulus. Values are presented for three trials (0, 10, 17) during the course of training since the animals serve as their own control in this experiment. Furthermore, Table 3 contains the results of *t*-tests (*P*- and *t*-values) at these separate trials.

Behavioural elements/categories

An ANOVA for repeated measures reveals that EC-animals display a significantly higher frequency of exploratory behaviour ($F_{2,44}=6.28$, $P<0.01$), locomotion ($F_{2,44}=65.16$, $P<0.01$), and arousal ($F_{2,44}=5.89$, $P<0.01$) than FS-animals. For groom, scan and attention no differences between FS and EC are detected ($F_{2,44}=0.06$-0.34, NS).

Analysis of the mean time spent on the different behavioural elements and categories indicates that a significant difference between EC- and FS-animals exists for exploratory behaviour ($F_{2,44}=7.18$, $P<0.01$) and grooming ($F_{2,44}=4.73$, $P<0.05$). It appears that the duration of exploratory behaviour does not change over the trials in EC-animals whereas in FS-animals the mean time spent on exploration seems to decrease over the trials. In case of grooming, the duration seems to decrease over the trials in EC- whereas it seems to increase over the trials in FS-animals. For attention a trend towards significance is detected for the difference between EC- and FS-animals ($F_{2,44}=2.49$, $P<0.1$). EC and FS do not differ significantly concerning the duration of locomotion ($F_{2,44}=0.74$, NS) and scanning ($F_{2,44}=0.25$, NS).

Freezing and resting behaviour were almost never observed. Therefore, analysis of frequencies and durations of these behavioural elements are not included.

Table 3. Frequencies and durations (per minute) of behavioural elements (mean±SEM) displayed in the CS-US time-interval at trial 0, 10,17[1] and the results of *t*-tests at these trials.

		Unconditioned stimulus (US)			
	trial	**Enriched cage (EC)**	**Forced swim (FS)**	t_{22}	**P**
Frequency					
Exploration**	0	8.79 ± 0.37	8.13 ± 0.56	-0.099	0.335
	10	12.59 ± 0.62	8.93 ± 0.93	-3.301	0.003
	17	11.35 ± 0.79	6.61 ± 0.42	-5.320	<0.001
Locomotion**	0	2.45 ± 0.22	1.97 ± 0.29	-1.311	0.203
	10	3.02 ± 0.32	2.04 ± 0.32	-2.148	0.043
	17	3.43 ± 0.37	1.35 ± 0.17	-5.150	<0.001
Grooming	0	0.72 ± 0.12	0.54 ± 0.07	-1.259	0.221
	10	0.91 ± 0.11	0.86 ± 0.17	-0.231	0.819
	17	0.83 ± 0.13	0.74 ± 0.12	-0.545	0.519
Arousal**	0	0.13 ± 0.03	0.07 ± 0.03	-1.246	0.226
	10	0.20 ± 0.07	0.09 ± 0.05	-1.225	0.233
	17	0.57 ± 0.11	0.14 ± 0.04	-3.776	0.001
Scan	0	0.44 ± 0.16	0.76 ± 0.14	1.557	0.134
	10	0.88 ± 0.19	1.31 ± 0.38	0.996	0.334
	17	0.89 ± 0.18	1.03 ± 0.19	0.536	0.597
Attention	0	2.35 ± 0.21	2.56 ± 0.17	0.790	0.438
	10	3.13 ± 0.28	3.21 ± 0.31	0.186	0.854
	17	2.06 ± 0.24	2.73 ± 0.23	0.219	0.829
Total **	0	15.66 ± 0.64	14.78 ± 0.68	-0.942	0.356
	10	24.31 ± 1.11	19.34 ± 1.48	-2.686	0.013
	17	22.73 ± 1.29	14.81 ± 0.84	-5.139	<0.001
Duration					
Exploration**	0	38.58 ± 1.68	38.59 ± 2.32	0.006	0.995
	10	37.50 ± 1.76	27.99 ± 2.74	-2.920	0.008
	17	38.58 ± 2.52	23.54 ± 1.84	-4.814	<0.001
Locomotion	0	6.53 ± 0.17	5.98 ± 0.98	-0.444	0.661
	10	5.04 ± 0.61	3.71 ± 0.48	-1.700	0.103
	17	4.48 ± 0.47	2.54 ± 0.39	-3.163	0.005
Grooming*	0	10.25 ± 1.62	5.95 ± 1.06	-2.226	0.037
	10	7.95 ± 1.53	10.30 ± 1.70	1.027	0.316
	17	6.93 ± 1.13	11.09 ± 1.81	1.955	0.063
Scan	0	1.73 ± 0.81	3.90 ± 0.97	1.723	0.099
	10	4.82 ± 1.45	9.62 ± 2.84	1.505	0.147
	17	5.92 ± 2.10	9.01 ± 3.40	0.771	0.449
Attention	0	6.98 ± 0.95	9.99 ± 2.19	1.264	0.220
	10	8.92 ± 1.04	11.62 ± 1.69	1.359	0.188
	17	6.24 ± 0.63	15.37 ± 2.40	3.681	0.001

*[1]An asterisk indicates a significant difference at α=0.05 between the experimental groups over the course of the training trials (ANOVA for repeated measures; *P<0.05; **P<0.01).*

Activity

Analysis of the total frequency of all behavioural elements indicates that the activity over the course of the trials is significantly different in EC-animals as compared to FS-animals (Fig. 3; ANOVA: $F_{2,44}$=7.27, P=0.002). It seems that EC-animals show an increase in activity

from trial 0 to trial 10 and remains about that high level of activity for the subsequent trials. Conversely, the level of activity of FS-animals remains about the same level as the first test (trial 0) at which no association between CS and US could have been formed yet.

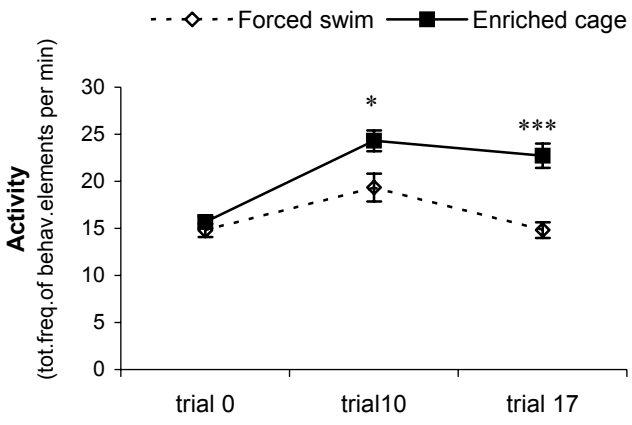


Figure 3.
Activity represented by the total frequency of displayed behavioural elements in the CS-US interval of animals that received different types of US (transfer to an enriched cage or a forced swim session); Baseline activity (before training) is measured at trial 0 and CS-induced activity is measured after 10 and 17 trials of training. Data are expressed as the mean frequency per minute; *N*=12 per group (*: $P<0.05$; ***: $P<0.001$).

DISCUSSION

Behavioural elements/categories

An enriched cage (EC) and sexual contact (SX) seem to share a common denominator regarding the behavioural response of rats to the announcement of these commodities. No differences are present between EC- and SX-animals except for the frequency of grooming and the duration of exploration. Similarly, no differences were detected between the animals that were transferred to a standard cage (SC) or stayed in their home-cage (HC) after the presentation of the CS. The main difference between EC and SX that seems to be present concerns the comparison with SC and HC. Namely, regarding the frequency of exploration and locomotion SX-animals displayed a higher level than both SC- and HC-animals whereas EC-animals only differed from HC-animals. Conversely, for the frequency of arousal factors EC-animals displayed a higher frequency than both SC- and HC-animals whereas SX-animals only differed from HC-animals. This might be indicative for the occurrence of a stimulus-specific increase in certain behavioural elements. For instance, SX-animals might be more exploring in search for the receptive females whereas EC-animals show an increase in the level of arousal since this is more related to the behaviour that they display in the enriched cage. A similar phenomenon was described by Rosenwasser and colleagues [315] for the difference between ‘spontaneous’ locomotor behaviours and explicit food-appetitive behaviours during the anticipatory phase. Concerning the stressful event forced swimming (FS), the frequency of exploration, arousal and locomotion is significantly lower as compared to the behavioural response in anticipation of an enriched cage. In addition to the results of Experiment 1, this confirms a possible relationship of these behavioural categories with anticipatory behaviour . However, this is probably not very useful to assess the positive

nature of a stimulus since the neutral-stimulus group (SC) does not in all cases significantly differ from the EC- and SX-group concerning these behaviours.
The fact that resting and freezing is almost never observed in Experiment 2 is probably caused by the experimental set-up; the animals are tested individually in an observation cage which probably increased the basal level of activity [355] and indicates that in Experiment 1 (home-cage observation) freezing was probably only displayed in the social context (agonistic interactions).

Activity

Analysis of the activity indicates that the experimental groups of Experiment 1 can be divided into two clusters: one with a relatively high level of activity to which SX and EC belong, and one with a significantly lower level of activity to which SC and the HC belong. The strong increase in activity in rats after the announcement of transfer to an enriched cage is seen in both experiments and indicates this is a consistent anticipatory response. The fact that this anticipatory activity significantly differs from the announced transfer to a standard cage or to a water-cylinder indicates this is not just caused by the transfer-procedure. The expectation that behavioural suppression in anticipation of an aversive situation might manifest itself in a decreased activity as a result of increased freezing was not met. This might be due to the experimental set-up since in contrast to fear conditioning studies (e.g. [139]) the actual aversive stimulus is offered outside the cage in which it was announced. Apparently, conditioned freezing is only induced if the aversive stimulus (such as foot shock) arrives in the same context in which the animal is observed. It is known that the conditioned freezing response to a discrete stimulus such as a tone is short lived whereas the conditioned response to context can last for much longer [69]. This indicates that the context is very important (see also [412]|). Another explanation might be that the predictability of the forthcoming aversive event caused some kind of tolerance (e.g. [179][417]). Also, the fact that the exposure to the swim procedure and the subsequent termination of the session after several minutes is repeated for several days might have caused habituation [84][96]. For the aforementioned reasons forced swimming might not have been perceived by the animals as a very stressful stimulus after all. The fact that FS-animals did not show a lower body weight than EC-animals at any moment during the course of the experiment (data not shown) is in accordance with this line of reasoning.

Anticipatory response

In most cases the frequency of behavioural elements differed between the experimental groups whereas the total duration did not. This confirms the earlier assumption that general activity is probably related to anticipatory behaviour. During anticipation of a forthcoming positive event the animals spent the same amount of time on certain behavioural elements but the duration of each bout is much shorter which implies a higher frequency and thus a higher level of reactivity on the presentation of the conditioned stimulus (i.e. more behavioural transitions). The frequency of certain specific behavioural categories such as locomotion, exploration and arousal also seem to be related to the anticipatory response. But, as stated before, these elements are probably more related to the specific characteristics of the forthcoming stimulus since the results are not consistent regarding the significant differences between the experimental groups. Results of the analysis of the activity seemed to be easier to interpret since clear and highly significant differences between the experimental groups were present. This is in line with previous studies [194][376][403] in which (hyper) activity was used as an index for the incentive value of social contact and

sucrose. However, in case of a stressful stimulus, the activity remains about the same over the training-trials (Exp. 2) and it is difficult, therefore, to be sure that a distinction in response for a neutral and a negative stimulus exists. It appeared that the expected behavioural suppression in anticipation of forced swimming was mainly expressed in terms of a decrease in exploration. On the other hand, an increase in mean duration of grooming also seemed characteristic for anticipation of this negative event. This seems to be in line with the notion that stress influences grooming [349][388] and inhibits exploratory behaviour [69].

Rewarding value

The similar responses of SX- and EC-animals indicate that access to an enriched cage is likely to be of similar rewarding value as sexual contact. Since sexual contact is generally accepted to be a very strong rewarding stimulus for rats (e.g. [10][287][391] the relatively simple enriched cage used in this study appears to have highly rewarding properties.
The presence of certain similarities between SC and both EC and SX might be indicative for an intermediate rewarding position of SC. General arousal related to the experimental set-up or an anticipatory response to the expected transfer to another cage might be the cause of this. It is possible that SC-animals anticipated the contact with the experimenter, since Davis & Perusse [105] suggested that contact with a familiar human being could be rewarding (see also [56]). If this is the case, it is probably perceived as less rewarding than sexual contact or and enriched cage since the SC clearly shares a different denominator than EC and SX concerning the activity and the display of several other behavioural elements. The analysis of the activity indicates that rats display a relatively high frequency of behavioural elements when they anticipate a future event with a rewarding character. It has been suggested earlier that an increase in activity would be specifically related to the food-anticipatory response [315]. However, the present results indicate that an increased activity is not just related to food-stimuli but also to other types of stimuli such as sexual contact or an enriched cage. This is in line with the findings of Van den Berg et al. [376] showing that previously isolated rats display (hyper) activity in anticipation of social contact. Thus, an increased activity seems to be characteristic for a rewarding stimulus in general.
The stimulus-specific increase in certain behavioural elements and categories that also seems to exist is not very useful for the assessment of the rewarding or aversive value (perception) of different types of stimuli. Namely, the relative difference cannot be established in that way and therefore the general activity might be a more valid method. However, it should be noted that the anticipatory response to different types of US is investigated in different groups. Offering both SX and EC to the same group of animals might render more information about the quantitative difference in perception of these positive stimuli. Furthermore, in case anticipation of an aversive stimulus is expected, it might be necessary to, besides general activity, also analyse frequency and duration of separate behavioural elements for correct interpretation of the perception of the stimuli offered.

Environmental enrichment

The relatively simple enriched cages that are designed with consideration for both animal welfare and ergonomical aspects provide the ability to display a more extensive repertoire of behaviour including rearing, climbing, jumping, gnawing, and hiding that are essential parts of the natural ethogram of rats [21][59][125]. Furthermore, the enrichment objects increase the dimensions and total surface in the cage, which is preferred by laboratory rodents [43][152][337]. The results of the present study indicate that the aforementioned

characteristics of the enriched cage are perceived as highly rewarding by rats. Additionally, the subdivision of the cage into several compartments caused by the different objects allows the animal to search for or to avoid contacts [351], and is thus likely to decrease the frequency of aggressive behaviour [71]. Therefore, enriched housed animals are probably less stressed by the increased controllability and also due to the possible counteraction of stress by rewarding activities such as the display of natural behaviour. The stimulation of a variety of physiological and behavioural responses provides the animals with an extended adaptive repertoire of responses in case a challenge is encountered. In this way, their welfare will be improved [366] and they obviously serve as more valid models for research [299] perhaps requiring fewer animals [44]. All of the abovementioned and the fact that this type of enrichment is relatively easy to implement should stimulate the use of environmental enrichment for laboratory rats.

Animal welfare

The natural behavioural reaction of rats that associate certain stimuli and events has proven to render useful information for assessment of the perception of stimuli. The conditioned behavioural response can probably indicate what events or conditions are perceived as attractive or aversive. This way it might be used in the future to investigate preference for or appraisal of certain housing conditions or to establish the perception (aversiveness) of husbandry or experimental procedures. Since it has been found that anticipatory behaviour is influenced by previous experiences [376][403] it might also be used to assess the state of animals in terms of welfare. Therefore, as argued in Chapter 1, anticipatory behaviour might be a useful tool to both measure and improve welfare of laboratory animals. Further experiments that validate this in more detail are described in the following chapters.

Acknowledgements

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CHAPTER 3

A SIMPLE ENRICHED CAGE FOR LABORATORY RATS REDUCES AGGRESSION, ENHANCES ACTIVITY AND INFLUENCES BEHAVIOUR ON THE ELEVATED PLUS MAZE

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ABSTRACT

Animal welfare is currently one of the major topics of interest and research in this field is growing extensively. In the past, standardizations requirements for experimental purposes led to more and more impoverished housing conditions for laboratory animals. Nowadays, one of the main interests of animal welfare scientists is improvement of housing conditions by means of environmental enrichment. In the European guidelines for the use of experimental animals it is advocated to that cage enrichment should be provided unless there is justification against doing so. So, a need exists for an easy applicable method of enrichment that is thoroughly investigated for its consequences. The present study describes a new, simple method of enrichment for rats that can be easily applied in standard laboratory settings and is based on meeting the behavioural needs of these laboratory animals. In the current study, the effects of this new type of cage enrichment are investigated by means of ethological procedures. Wistar rats (males and females) that were socially housed under enriched or standard conditions have both been observed in their home cages and have been tested in a widely used test of anxiety and exploration (Elevated Plus Maze). In the home cage, the enriched rats showed an increase in exploration, mobility and general activity. Importantly, the level of aggression was significantly lower in rats that were housed under enriched conditions. Enriched males also moved more freely on the Elevated Plus Maze and spent more time on the open areas of the platform indicating a lower level of anxiety. We conclude that the enriched system, although simple in construction, is effective in positively influencing rats in their behaviour in the home cage. Combined with the declined expression associated with anxiety that was seen in enriched housed male rats, this increase in behavioural possibilities and social control is very likely to increase animal welfare. Consequently, these effects of the enriched housing on the laboratory rats are very relevant for their validity as an animal model.

INTRODUCTION

In the last few years there has been a lot of discussion about adapting the guidelines for housing and care of laboratory animals according to the changing viewpoints on animal welfare. In the past, standardization efforts led to more and more impoverished housing conditions that are stimulus-poor and in which the performance of a large part of the natural behavioural repertoire is not possible [270]. Due to this behavioural deprivation the current housing systems are inadequate in creating an environment that can guarantee the welfare of animals, a statement often mentioned in the guidelines [2](see also [107][410]). If welfare is not guaranteed, the validity of laboratory animals as a research model is questionable [298].
Nowadays, the use of environmental enrichment for laboratory rodents is more and more encouraged and is also incorporated in European legislation [5][6a]. Furthermore, in 1998, expert working groups were constituted by the Council of Europe to make a proposal for the revision of Appendix A of the Convention (accommodation and care of laboratory animals) [214]. In these future principles [6b] it is stated that gregarious species such as rats should be housed in groups whenever possible. Furthermore, it is advocated that cage enrichment should be provided unless there is a justification on experimental or welfare grounds against doing so.
Group housing of rats requires special features in contrast to solitary housing [223][406]. Rats have a social hierarchy and complex social behaviour accompanying this hierarchy [21][22][43][361]. Group housing systems should, therefore, allow a stable social structure by providing certain elements that offer the possibility to display specific behaviour seen in groups of rats [235][254]. For instance, individuals in a group should be able to initiate or avoid contacts with other cage mates thus gaining more control over their social environment [41].
The currently used standard housing systems consist in general of an empty environment without structure [42],[70]. A number of studies have been conducted on the enriching effects of a single object in a cage, or of very complex structures [39][113][278][327]. A need exists for a simple, structured social group environment that combines more objects in one cage and is applicable in standard laboratory settings. A structured environment with an increased height would provide rats with more complexity and division of space with subsequent improved behavioural possibilities, like rearing, climbing, hiding, upright defense (boxing), and avoiding each other which are essential parts of their natural ethogram [125][21][22][59][361]. Furthermore, the provision of objects in a cage increases the dimensions and total utilizable area in the cage, features that are preferred by laboratory rodents [43][153][337][204]. The subdivision of space that results from the presence of enrichment objects will enable the animals to reduce competitive situations adequately, e.g. decreasing the frequency of aggressive behaviour [70][351]. The abovementioned characteristics of enriched housing may offer appropriate stimulation to allow the animal to cope with physiological and ethological needs [298][235]. Satisfaction of ethological needs and the stimulation of a variety of physiological and behavioural responses provide the animals with an extended adaptive repertoire of responses in case a challenge is encountered [348]. Enriched housed rats probably respond more adequate to situations such as the novelty of an experimental task [70] since these animals are more efficient in assimilating stimuli from their environment [399]. Hence, these animals are less sensitive to stressful experimental situations [221] and are better able to cope with environmental variations [366][428]. It is therefore expected that enriched housed animals will be more suitable

models for many kinds of research questions and thus increase the scientific validity of the experimental results.
From a pilot-experiment with a very large enriched enclosure so-called key elements [350] were established for the development of the current more practical enriched housing system adjusted to the possibilities in normal laboratory animal facilities. The main characteristics of the newly developed system were an increased height, division of space and availability of a shelter and gnawing objects. Besides the ergonomical and economical aspects of applicability in existing laboratory animal facilities that were used as criteria in the design of the new system, two other aspects have to be considered when evaluating the enriched housing system: 1) the behavioural possibilities for the rats, including control over their social environment, and 2) the quality of the animals concerning the validity of their use as a model under experimental conditions.
The first aspect can be studied by observing home-cage behaviour of the enriched and standard housed animals. A lower level of agonistic behaviour in enriched cages compared to standard cages would indicate an environment more suitable for social housing. Since enrichment may have an effect on the time-budget of the animals – enriched animals are probably less passive through an increased stimulation by the various objects in the cage – increased activity and exploration in the home-cage may be a useful indicator for improved welfare [17][50][20][335].
The second aspect, concerning the validity of the animal model, can be assessed in a standard laboratory test. Since enrichment leads to, amongst many other effects, improved motor coordination [97][32][219] and decreased sensitivity for mild stressors such as novel environments and aversive conditions [366][221] it is likely that enriched housing of the experimental subjects affects the outcome of test results. In this study, the Elevated Plus Maze is chosen to investigate the effect of environmental enrichment on the outcome of this most widely used animal model of anxiety [285]. Anxiety on the elevated platform is related to approach-avoidance behaviour that results from a conflict between exploratory drive and fear drive that is generated in a novel environment [259][170]. The major determinant of behaviour in this test is the unconditioned aversion to heights and open spaces [285]. Besides the difference in response to novel and aversive environments the results of the Elevated Plus Maze test may also be influenced by the difference in the physical abilities of standard and enriched housed rats.
In the present study, both aspects concerning the evaluation of the new housing system will be investigated for both males and females since it is known that gender differences can exist for many parameters (see for instance [141][273][356]).
Summarizing, it is expected that rats housed in the newly developed enriched system show less aggressive behaviour because of the division of space and ensuing possibility to avoid each other. The addition of objects in the cage will probably stimulate exploration and activity in general inside as well as outside the home cage (experimental conditions). Together with the decreased sensitivity for environmental stressors and the improved motor skills this will result in less signs of anxiety on the Elevated Plus Maze.

METHODS

Subjects and housing

Thirty-nine Wistar rats (U:WU, GDL, Utrecht University, The Netherlands) were used of which 16 (8 males, 8 females) were housed in standard conditions and 23 (12 males, 11 females) in enriched conditions. Exposure to the differential housing systems began at birth; at weaning animals were housed in groups of four (except for one female group of 3). Observation and testing was performed at 12 months of age. Cages were cleaned once every week and during this procedure all animals were weighed and examined to obtain a clear view on their condition and possible weight differences occurring between the groups.
In both housing systems food (Hope Farms™ standard rat chow) and water were provided ad libitum. All animals were housed under a reversed light/dark cycle (lights on at 9.00 p.m.); in the dark period red light was provided to be able to observe behaviour and carry out standard laboratory procedures (weighing, cage cleaning). The animals were kept in a temperature-controlled room (21 ± 2 °C).
The standard housing system (SH) consisted of a type IV Makrolon cage (A=1875 cm²; Tecniplast, Italy) (see Chapter 2; Fig. 1B) with a metal wire lid and standard bedding (Lignocel 3/4 ® , Rettenmaier & Söhne, Germany). The enriched housing system (EH) is a relatively simple system that consisted of a standard type IV Makrolon cage (as described above) with some extensions (see Chapter 2; Fig.1A): 1) A rim that increased the height of the cage with 8 centimetres to improve the possibilities for rearing and boxing; 2) A large tunnel shaped object (14.5x16x32 cm; hxwxl) that extended from one side of the cage to the other thus providing division of the available space. This object contained passages at the sides and on top and small holes in which pieces of wood were inserted; 3) A small mesh-wire shelter (10x11x24.5 cm); 4) A low bin beneath the food-hopper that was filled with old bedding when the cage was cleaned thus providing a constant familiar odour to the animals. Since the animals could also sit on top of elements 2 and 3 the enriched housing system offered an increased utilizable area (an increase of approximately 45 % compared to the standard type IV cage).

The experiments were approved by the Ethical Committee of the Utrecht University.

Observations

After 12 months of differential housing the animals were observed in their home cage. For this behavioural observation the Focal Animal Sampling technique [14] was used: each animal was observed in six separate five-minute periods, resulting in 30 minutes observation per animal. The animals were observed during the dark period using a specialised computer-program for behavioural observations and analysis ('The Observer'; Noldus Information Technology, Wageningen, The Netherlands). This program enables the experimenter to analyse both frequency and duration of behavioural elements. All observations were performed live in front of the home cage of the animals in a rotating observation-scheme to ensure that each animal was observed during different times of the day thus avoiding time dependent effects. Behavioural categories that were examined are: *Agonistic behaviour* (divided in aggression (pinning down, chase and fight) and submission (freeze and flee)), *Mobility* (consisting of the elements climb and walk,), *Inactivity* (sit, sleep, lie, sit together, lie together and huddle), and *Exploration* (sniff (objects) and gnaw (objects)). Detailed descriptions of the monitored behavioural elements can be found in [21][22][125][361].

Behavioural test of anxiety

All animals were subjected to the widely used Elevated Plus Maze test of anxiety ([286], adapted from [170]). The plus-shaped apparatus with two open and two enclosed arms was 80 centimetres high and had a 40-Watts white light bulb above the middle of the platform. Before each trial the apparatus was cleaned with a sponge and warm water, than dried off with a paper towel. Each animal was placed onto the middle platform facing one of the open arms. Immediately thereafter the observation program was started and the animal was observed for five minutes after which the animal was taken off the apparatus and returned to its home-cage. In case an animal fell off, the observation was ended and the animal was immediately returned to the home cage. The data of these animals have been excluded from the final analysis. Parameters recorded were the duration and frequencies of the visits to each of the five areas. In the analysis, the frequencies of visits to the open and closed arms were added to obtain the total number of arm entries. The total number of arm entries was taken a measure for activity and is indicative for the level of anxiety that the animal experiences on the apparatus. Furthermore, the level of anxiety was also measured by calculation of the total time spent on the open areas.

Analysis

In addition to the formation of the behavioural categories as described above a category *Activity* is created as well. This overall activity is established by adding the total frequency of all scored behavioural elements. The differences between groups concerning *Agonistic behaviour*, *Mobility*, *Inactivity*, and *Exploration* were analysed by means of comparing total time spent on these behavioural categories. For each group and behavioural category the data were tested for normal distribution with the 1-sample Kolmogorov-Smirnov test. In case of a normal distribution a two-way Analysis of Variance (ANOVA, factors: gender and housing) has been used to compare the experimental groups for differences. In case gender-effects were detected, the data file was split and analysed separately for the genders with a one-way ANOVA (factor: housing). A rejection-criterion of 0.05 has been set for all statistical tests.
Similar statistical analysis as described above for the behavioural categories was applied to the data of the Elevated Plus Maze. All results are presented as mean values per group ± standard error of the mean (SEM). The Statistical Package for the Social Sciences (SPSS, version 9.0) was used for all statistical calculations.

RESULTS

Home-cage behaviour

Housing conditions and genders were compared for differences after assessment of normal distribution of the data. Concerning the behavioural categories observed in the home cage no effect of gender was detected except for the general activity. Similarly, no interaction effect between gender and group was established for these categories. Therefore, to compare both housing systems concerning the home-cage behaviour of the rats, the data-files of males and females were analysed as one dataset except for *Activity*.

The category *Agonistic behaviour* is divided in two sub-categories: *aggression* and *submission*. Figure 1 shows that standard housed rats spent more time on aggressive behaviour (28.15 ± 6.96 s) than enriched housed rats (9.3 ± 3.79 s; $F(1, 35)= 6.27$, $p< 0.01$). Analysis of the sub-category *submission* yields no difference between standard and enriched housed animals ($F(1, 35)= 0.49$, n.s.).

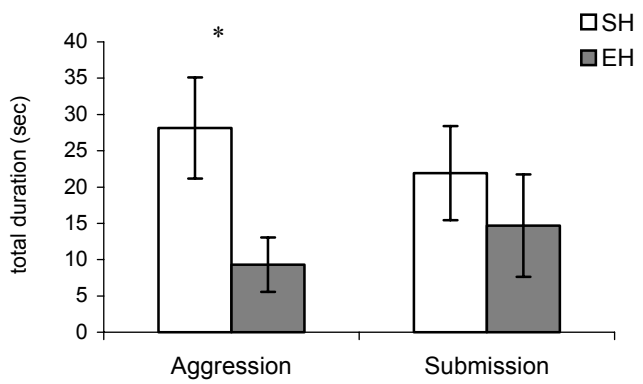

Figure 1.
Effect of standard (SH) and enriched (EH) housing on the time spent on agonistic behaviour (*aggressio*n and *submission*) in the home-cage. The data are presented as mean values per group (in seconds ±SEM) during the total observed time per animal (6 sessions of 5 minutes = 30 minutes)(*: p< 0.05).

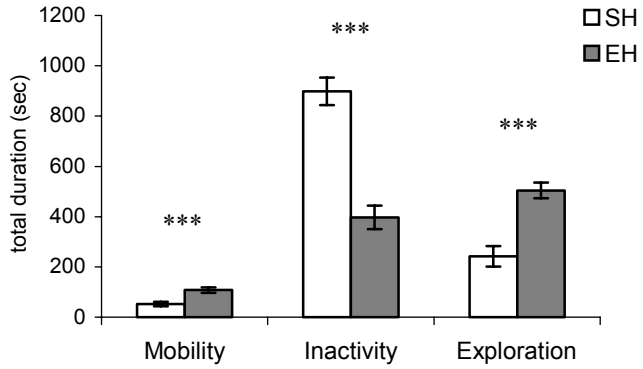

Figure 2.
Effect of standard (SH) and enriched (EH) housing on the time spent on three behavioural categories displayed in the home-cage: *Mobility, Inactivity, and Exploration*. The data are presented as mean values per group (in seconds ±SEM) during the total observed time per animal (6 sessions of 5 minutes = 30 minutes)(***: p<0.001).

Figure 2 presents the time spent on mobile, inactive and exploratory behaviour. For the category *Mobility* a difference has been detected between de standard and enriched housed rats. It appeared that standard housed animals were less mobile (51.89 ± 8.6 s) than enriched housed animals (107.78 ± 10.51 s) ($F(1, 35)= 14.8$, $p< 0.001$). Similarly, enriched housed rats spent less time on inactive behaviour (898.35 ± 54.94 s) than standard housed rats (397.21 ± 47.44 s; $F(1, 35)=53.71$; $p<0.001$) (see *Inactivity* in figure 2). Interestingly, the difference in this category is the result of only one of its many elements: in the standard housing system rats huddled significantly more than in the enriched housing system ($F(1, 35)= 55.11$, $p< 0.001$); no significant differences between both housing systems were detected for either of the other elements (sit, sleep, lie, sit together, lie together; $p>0.2$ in all cases). Concerning the duration of *exploratory behaviour* it became apparent that enriched housed rats spent more time exploring (504.39 ± 40.77 s) than standard housed rats (241.68 ± 31 s; $F(1, 35)= 25.78$, $p< 0.001$).

Figure 3 shows the level of activity represented by the total frequency of displayed behavioural elements of males and females in the standard and enriched housing system. A two-way ANOVA indicated that females expressed a higher level of activity (125.19 ± 6.62)

than males (95.9 ± 8.25) ($F(1, 35)= 15.08$; $p<0.001$). Also, the enriched housed rats display a higher level of activity (129.91±6.04) than the standard housed rats (83.50± 8.14) ($F(1,35)=30.31$, $p<0.001$). This holds true when analysing the genders separately (females EH vs.SH: $F(1,17)=9.56$, $p<0.01$; males EH vs.SH: $F(1,18)=22.74$, $p<0.001$). When comparing all four groups individually by an one-way ANOVA (factor: group (EH-males, SH-males, EH-females, and SH-females)) and subsequent Post-Hoc analysis (Scheffé) it appeared that the difference in activity between males and females under standard housing conditions (SH males vs females: $p<0.05$) was not present when the animals are housed in the enriched system ($p>0.1$). Furthermore, it became apparent that the standard housed males were less active than any other group ($p<0.05$ in all cases).

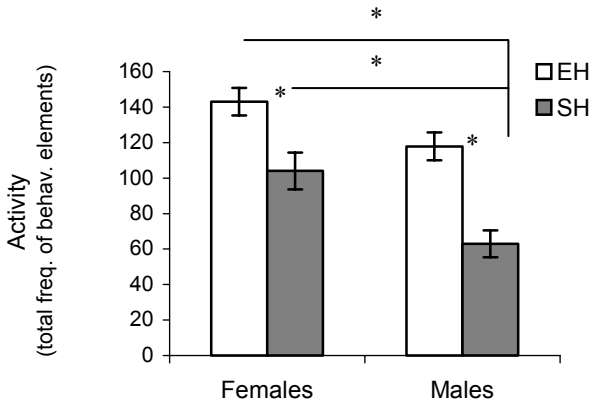

Figure 3.
Effect of standard (SH) and enriched (EH) housing on general activity in the home-cage for males and females. Activity is represented by the total frequency of the observed behavioural elements during the 30 min observation period (*: $p< 0.05$)

Elevated Plus Maze

Similar to the data of the home-cage behaviour, the data for the Elevated Plus Maze (EPM) have been checked for normal distribution and interaction effects. Concerning the activity, represented by the total number of arm entries, an interaction effect was present between gender and group ($F(1, 34)= 4.34$, $p< 0.05$). This was also the case for total time spent on the open areas of the apparatus ($F(1,34)= 4.28$, $p<0.05$). Therefore, for both parameters the data have been analysed separately for the genders by a one-way A/NOVA (factor: housing). Figure 4 shows a clear difference between females and males concerning their activity on the Elevated Plus Maze. That is, females were not influenced by the housing system in the number of arm entries (SH: 3.25 ± 0.86; EH: 3.7 ± 0.72; $F(1, 16)= 0.164$, n.s.) whereas a significant difference between housing systems was present for the males. Namely, enriched housed males showed higher rates of arm entries (4.67 ± 0.65) compared to standard housed males (1.38 ± 0.26; $F(1, 18)= 15.99$, $p< 0.01$).

Concerning the total time spent on the open areas it became apparent that, again, the type of housing system does not influence the results of the females (figure 5; EH: 85.66 ± 25.88 s; SH: 91.48 ± 38.83 s; $F(1, 16)= 0.017$, n.s.). Males, on the other hand, appeared to spend more time on the open areas when they had previously been housed in an enriched environment (157.07 ± 39.52 s) as compared to males that were standard housed (24.23 ± 5.54 s; $F(1, 18)= 7.33$, $p< 0.05$).

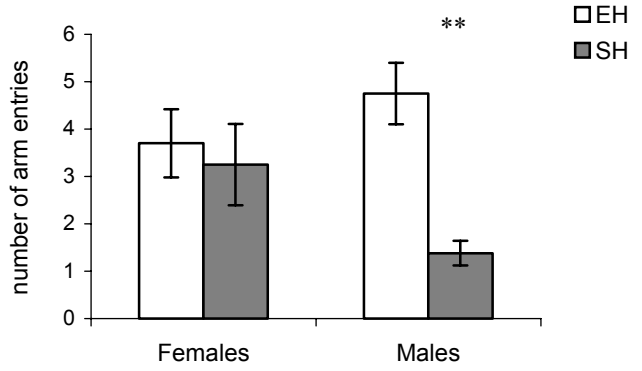

Figure 4.
Effect of standard (SH) and enriched (EH) housing on the activity of male and female rats on the Elevated Plus Maze. The Activity is represented by the total number of arm entries during the 5 minute-test (**: p<0.01).

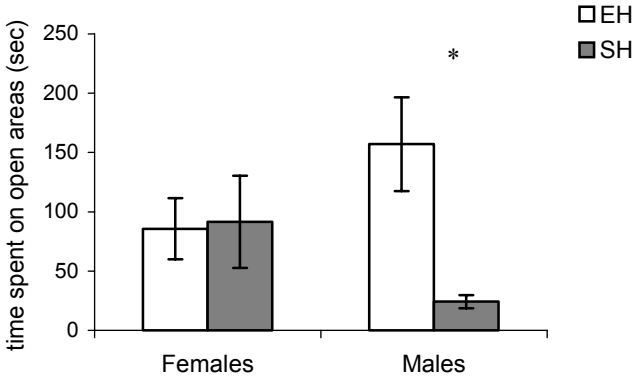

Figure 5.
Effect of standard (SH) and enriched (EH) housing on the total time spent on the open areas of the Elevated Plus Maze. Data of the 5 minute-test are presented separately for the genders (*: p<0.01).

Body weight

Figure 6 shows the mean weight of each group and each gender at the age of 12 months. Since an interaction effect was detected between gender and group ($F(1,35)=18.48$, $p<0.001$) the data file was split and analysed per gender (one-way ANOVA, factor: housing). It became clear that male rats housed in the enriched system weighed less (572 ± 12.6 g) than male rats housed in the standard system (663.5 ± 14.7 g) ($F(1, 18)=21.78$; $p<0.001$). This difference is not present for females: enriched housed female rats weighed 340.8 ± 8.7 g and standard housed female rats 331.25 ± 8.6 g which was not a significant difference ($F(1,17)=0.58$, n.s.).

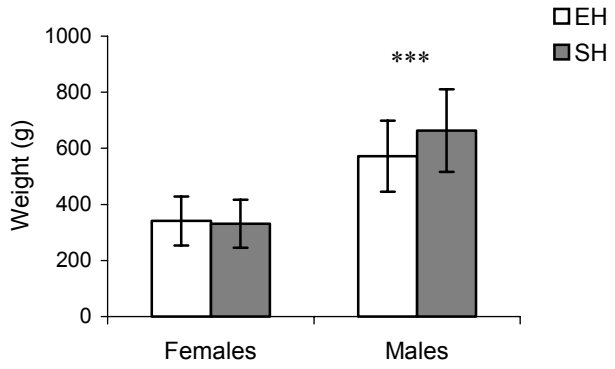

Figure 6.
Effect of standard (SH) and enriched (EH) housing on the bodyweight of male and female rats at the age of 12 months. (***: p<0.001)

DISCUSSION

Behaviour

Since laboratory rats spend most of their time in the home-cage with their cage mates, regulation of social behaviour is very important to these animals. Therefore, concerning the welfare of laboratory rats, the most important effect of the currently studied simple enriched housing system is the facilitation of management and control of agonistic behaviour. Enriched housed (EH) animals showed less aggression than standard housed (SH) animals. The total time spent on submissive behaviour appeared to be equal for the differentially housed animals. This might suggest that in enriched cages the submissive behaviours are more effective in terminating agonistic interactions. This implies that the frequency of aggression would be the same for both groups, but that bout-lengths are much shorter in the enriched housed animals. However, this is not the case: the frequency of aggressive behaviour appears to be significantly lower in enriched housed rats and the bout-length similar for both groups. So, the occurrence of aggression is lower in enriched housed animals but not the durations of separate aggressive encounters. Further analysis of submissive behaviour reveals that again the enriched housed animals display a lower frequency and similar bout-length compared to the standard housed rats. This is probably the logical result of the lower frequency of aggression in the enriched housing system and is not related to the aforementioned possible effectiveness of terminating agonistic interactions.

The total frequency of displayed behaviours is representative for the rate of behavioural transitions and indicates that enriched housing results in activating both males and females. Accordingly, enriched housed rats display a higher level of Mobility and *Exploration* whereas the level of *Inactivity* is lower. This may be caused by the increase of explorable objects, utilizable area and dimensions in the cage. The number of behavioural transitions can also be indicative for variability of behaviour and it is often suggested that this can be considered as an indicator of welfare [17][20][42][50][51][125][335]. The presence of objects and gnawing sticks provide the possibility for a more extensive behavioural repertoire and induces variability of behaviour and reduces the chance of under-stimulation [107][224].

Notably, the difference between groups that was present for the category *Inactivity* was mainly caused by the fact that standard housed animals huddled more than enriched housed rats. Probably, in standard housing conditions the animals try to compensate the absence of a shelter.

It must be noted that although the present study focuses on socially housed animals and the importance of enrichment for the control of agonistic behaviour, it is very likely to be equally important for individually housed animals (e.g. increased possibilities for a larger repertoire of natural behaviour).

Elevated Plus Maze

According to literature, the time an animal spends in the open or closed areas represents the level of anxiety the animal experiences on the apparatus [285][286][90][262]: an animal with a high level of anxiety will spend less time on the open areas as compared to an animal that is less anxious. Furthermore, the number of arm entries as a measure of locomotor activity is also one of the most often used indices of the level of anxiety [285][405].
The enriched housed males moved more freely on the apparatus as reflected by a higher number of arm entries and they spent more time in the open areas than in the presumed "safer" closed areas as compared to standard housed males. Thus, enriched housed males show less signs of anxiety which is very likely to be caused by the fact that they are less sensitive to stressful events [221] and are better able to cope with environmental variations [366][428]. In addition, the increased physical abilities caused by enriched housing might cause the animals to be less uncertain on the elevated platform, and as a consequence, to express less signs of anxiety. It appeared that enrichment did not have a similar effect on females concerning their response on the Elevated Plus Maze: no significant differences were detected between standard and enriched housed female rats. This will be discussed in the next section.

Gender

Remarkably, the enrichment had different effects on males and females with regard to some of the investigated parameters. The gender-effect that was present for overall activity in the home cage is probably caused by the fact that females are more active than males in general [98]. The effect of enrichment on the activity, however, was similar in males and females: for both genders enrichment caused a significant increase in activity. Interestingly, the commonly existing gender difference concerning the activity that exists under standard housing conditions is not present under enriched housing conditions. Enriched housed females and males are equally active in contrast to standard housed rats for which the females are more active than the males.
Also concerning the results of the Elevated Plus Maze test (EPM), pronounced gender differences are present. In contrast to the activity in the home cage for which only an effect of gender was detected, a significant interaction effect between gender and group is present for the EPM data. In males, enriched housing caused an increase in activity (number of arm entries) on the apparatus and a prolonged time spent on the open areas. Differentially housed females, on the other hand, did not show a difference in their response to the EPM test. It is often found and well described that females show less fear/anxiety than males [16][159][225][141][302]. This might be explained by the natural difference in activity and exploration between genders [198][393][340]: females tend to be more active and exploratory than males. This sex-difference in behaviour can influence the parameters used

to measure anxiety. A similar explanation was used by Van Haaren and colleagues [392] for previously observed behavioural differences between the sexes in different complex maze procedures. Pellow and colleagues [285], on the other hand, showed that in an EPM test the parameters indeed reflected anxiety and could not be explained by competing behaviours such as exploration. It is possible that females have reached a minimum concerning their level of anxiety on the elevated platform; enriched housing might be of no additional value for females in that respect. Another reason might be that the type of enrichment used in the present study is not complex enough to induce differences in females. This would imply that males are more sensitive for minor changes in their environment than females. It has been found that behaviour of females is less dependent on previous experiences such as aversive stimulation [353][352][126] and this might also be similar for experiences in different housing conditions.
Separate analysis within each housing system revealed that a trend towards significance exists for the difference between males and females that are housed in standard cages concerning the activity on the platform. This gender difference that is often found in other studies as well (see for instance [193][189]) is not present in enriched housing conditions.
Apparently, enrichment can lead to the confinement of the difference between males and females concerning their activity on the EPM and also in the home cage. Thus, the gender differences in activity that are often present and hinder interpretation of scientific results might be restrained by enriched housing of the subjects.

Body weight

The fact that enriched housed males had a significantly lower bodyweight than standard housed males is probably a result of the increased physical activity the enriched animals express in the home cage [265]. Enriched housed males did not spent less time on eating in the active period of the day (data not shown) and the amount of consumed food is therefore not likely to be the reason for the fact that they are less obese than standard housed males. Concerning the females, it appeared that the increased activity in the enriched cages had no significant effect on bodyweight. This is very likely to be caused by the fact that females are in general less obese than males. It must be noted, however, that beside activity several other factors such as time spent on huddling (temperature control) can influence bodyweight. It is therefore not possible to draw a definite conclusion about the reason for the weight differences between animals in the two housing systems.

Model

The results from the home-cage observations show that enriched housed animals are more active in general which might indicate better welfare of these animals [335]. Even more important, the level of aggression is significantly lower in the enriched housing system. Group housed animals in an enriched cage may, therefore, be more appropriate models for "normal" functioning organisms since they live in a less turbulent social environment in which they have more control through the possibilities to display species-specific (social) behaviour. The ability to hide in case of general distress (sounds, noise, movements in the housing room) probably also creates more control. Furthermore, the presence of the objects stimulates the animals, which leads to the display of more active and exploratory behaviour. Increased control, stimulation and the possibility to satisfy ethological needs through the display of species-specific behaviour will cause improved coping capacities [366]. Consequently, enriched housed animals are likely to respond more adequately to novel experimental situations [70]. This can be verified by the EPM data: the enriched housed

males moved more freely on the apparatus and showed less signs of anxiety. Further proof that enriched housing leads to more suitable research models can be deduced from the fact that the difference between males and females can be confined or even abolished by enriched housing of the subjects.
If the animals serve as models for studies of 'normal functioning' organisms the enriched environment may be a more suitable housing condition thus resulting in a higher scientific validity of the experiments. It must be noted, however, that for studies concerning, for example, aggressive behaviour, sensitivity to stress or anxiogenic/anxiolytic drugs the standard system might be more relevant for housing the experimental subjects. In that case, impoverished housing should be incorporated as a part of the animal model.

CONCLUSION

In general it can be concluded that the enrichment used in this study is a simple but effective means of providing an environment for social stability in groups of laboratory rats as shown by the reduction of aggression. Furthermore, the relatively simple enriched cages activate the animals thereby reducing the chance of under-stimulation, as shown by the reduction of inactive behaviour and the increase of exploration, mobility and overall activity. Combined with the declined expression associated with anxiety that was seen in enriched housed male rats, this increase in behavioural possibilities and social control in the enriched cage is very likely to increase the welfare of the animals. Consequently, these effects of the enriched housing on the laboratory rats are very relevant for their validity as an animal model for behavioural and brain research.

Acknowledgements

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CHAPTER 4

STANDARD HOUSED RATS ARE MORE SENSITIVE TO REWARDS THAN ENRICHED HOUSED RATS AS REFLECTED BY THEIR ANTICIPATORY BEHAVIOUR

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ABSTRACT

The present study was designed to investigate the effects of potentially stressful standard housing conditions for laboratory rats on the sensitivity to rewards as reflected by their anticipatory behaviour for sucrose. This anticipatory response is evoked in a conditioning paradigm in which a sucrose-reward is repeatedly announced by a stimulus. The underlying neurocircuitry of this anticipatory response in expectation of a reward involves mesolimbic dopaminergic systems of which it is known that they can be sensitised by stressors. The results show that the anticipatory response for the sucrose-reward is stronger in the standard housed animals which indicates that these animals are more sensitive to the reward than the enriched animals. From this, it is concluded that standard housed rats are stressed which is likely to be caused by deprivation of the ability to satisfy behavioural needs in these impoverished housing conditions.

INTRODUCTION

In the past, standardization efforts led to more and more impoverished housing conditions for laboratory animals that are stimulus-poor and in which the performance of a large part of the natural behavioural repertoire is not possible [270]. Behavioural deprivation as is the case in the commonly used standard housing systems for laboratory rodents [107] and most other captive animals is considered to be stressful for these animals. Since stress, such as for instance social deprivation [194][260], can cause increased sensitivity for rewards it is expected that standard housed rats are more sensitive to rewarding stimuli as compared to enriched housed rats. Changes in reaction to rewarding and also to aversive stimuli cause sensitisation of mesolimbic dopaminergic systems and may be long lasting [27].
In general, sensitivity to rewards is affected by stress in both man and animal [63] [156][175][296][403]. It is known that in case of stressful circumstances the sensitivity to rewarding as well as aversive stimuli increases [293]. According to Spruijt and colleagues [348] this might be explained in terms of economy of behaviour. They argue that adaptive behaviour requires a continuously changing sensitivity to rewarding and aversive stimuli in order to allow the organism to fulfill its needs with a minimum of effort. Deprivation of essential stimuli urges an animal to react more eagerly, c.q. be very sensitive, in case of the presence of a valuable but rare reward. Thus, the sensitivity for reward determines the threshold for launching appetitive behavioural responses. The underlying neurocircuitry of these appetitive responses involves mesolimbic dopaminergic systems of which it is known that they can be sensitised by stressors [63]. The variable sensitivity of dopaminergic systems determines the behavioural effort an animal is willing to perform to obtain a reward (‘wanting’, see: [28]) [290][348]. This is behaviourally recognized by an altered appetitive response.
The present study was designed to investigate whether standard housed rats are stressed due to chronic deprivation of the ability to satisfy behavioural needs in these impoverished housing conditions. For this, the sensitivity to rewards in standard and enriched housed rats was chosen as a parameter. In a previous study (Chapter 2) it has been shown that the enriched system used in this study has rewarding properties for rats [384]. In that paper it is argued that this rewarding property is likely to be a result of the ability to display a more extensive natural behavioural repertoire. The sensitivity to rewards of the differentially housed rats was determined by focussing on the anticipatory response (expectation) - an early component of appetitive behaviour - to an announced sucrose reward. It has recently been found that the expectation of a reward triggers dopamine release in the ventral striatum and not the reward itself [15][112][343]. In previous studies we showed that rats display an increase in activity in the interval between (the offset of) the conditioned stimulus (CS, cue) and the (onset of) the unconditioned stimulus (US, reward) in a conditioning paradigm [376][403]. This anticipatory increase in activity could be measured by the frequency or transitions of behavioural elements [381][384] and is mediated by the mesolimbic dopaminergic system [28][288][290][334][348]. Therefore, it is expected that the increase in reward-sensitivity caused by stress-induced sensitisation of the dopaminergic system due to impoverished housing will be reflected by an increase in anticipatory activity for an announced reward.

METHODS

The experiments have been performed in adherence to the legal requirements of The Netherlands concerning research on laboratory animals, and have been approved by the Ethical Committee of the Utrecht University.

Subjects, housing, and general procedures

A total number of 60 male Wistar rats (HsdCpb:WU, Harlan, The Netherlands) weighing approximately 200 g at their arrival (age: 7-8 weeks) were socially housed in cohorts of 3 animals in either a standard cage (n=36) or in an enriched cage (n=24). The enriched cages (see Chapter 2; Fig. 1A) were developed at our laboratory and consisted of a standard Makrolon type IV cage (see Chapter 2; Fig. 1B)(ground area: 1875 cm²; height: 18 cm; Tecniplast, Milan, Italy) with some extensions: a 8-cm rim, a shelter (10x11x24.2; h x w x l), a large tunnel shaped object (14.5x16x32; h x w x l) with passages at the sides and on top, and a low bin beneath the food hopper which was filled with old bedding when the rest of the cage was cleaned. Furthermore, the tunnel-shaped object contained small holes in which pieces of wood were inserted. The presence of the enrichment objects increased the utilizable area inside the cage with 45%. The animals were housed under a reversed dark/light cycle (bright white light: 20:00 h- 8:00 h; dim light: 8:00h- 20:00h) since rats are mainly active at dusk/night and behaviour should therefore be observed in this active period. Background music was present 24 hours per day. Bedding material type 3-4 (Lignocel 3/4 ®, Rettenmaier & Söhne, Ellwangen-Holzmühle, Germany) was provided and lab chow (RMH-B®, Hope Farms, Woerden, The Netherlands) and water were available ad libitum. The animals were allowed to adapt to the room and light regime for two weeks and were handled daily during this period. Also during this habituation period the animals were transported to the observation room on a regular basis to allow them to habituate to this procedure. During 2 of these habituation-to-transport sessions the animals were placed in an observation cage to prevent the effect of novelty on the anticipatory response during the test session in the observation cage (see section 'Observations'). Home cages were cleaned and animals were weighed once per week. This was always done after the experimental tests to prevent influence of this disturbance on behavioural parameters. Experimental procedures started after 2 weeks of differential housing and habituation.

Conditioning procedure

The standard housed and enriched housed group were each subdivided in 2 groups of 12 animals each that were either subjected to the conditioning training (cue+sucrose-reward paired) or to the control procedure (cue-only)(Fig.1). To control for possible effects of sucrose-consumption on the activity of the animals an additional control group of 12 standard housed animals was subjected to a so-called yoked procedure (Fig.1). This group received both the cue and the sucrose-reward but not paired. The conditioning procedure consisted of repeated pairing of a combined light/bell stimulus (conditioned stimulus, CS) with a sucrose reward (5% solution, 5 minutes). The reward (unconditioned stimulus, US) followed after a certain time-interval that was gradually prolonged over 32 trials to 10 minutes (see Box 2, p.68). In this time-interval the anticipatory response to the announced reward could be investigated. To the control groups solely the CS was presented without the reward. The animals were trained (CS + US) or subjected to the control treatment (CS-) 3-5 times a day for a total of 42 trials. The yoked control group received sucrose once per day for a total time that was equal to the total time the conditioned group had access to sucrose (15-

25 minutes, depending on the number of training trials that was given to the conditioned groups). The intertrial intervals (US-CS interval) were varied from a minimum of 45 minutes up to a maximum of 2 hours. Timing of training and testing was counterbalanced among the experimental groups in order to avoid the acquisition of an association between time and order of testing and the presentation of the US.

Standard housing	Conditioning training:	CS + US *paired*	(n=12)	**ST**
	Control procedure:	CS -	(n=12)	**SC**
	Control procedure:	CS / US *unpaired*	(n=12)	**SY**
Enriched housing	Conditioning training:	CS + US *paired*	(n=12)	**ET**
	Control procedure:	CS -	(n=12)	**EC**

Figure 1. Scheme of the subdivision of the experimental groups. The animals are either housed under standard (**S**) or enriched (**E**) conditions. Rats that are subjected to the conditioning training (**T**) receive the conditioned (CS, cue) and unconditioned stimulus (US, reward) paired whereas to the rats of the control group only the CS (**C**) is presented or the CS and US unpaired (**Y**).

Observations

Behaviour displayed in the interval between CS and US was observed at trials 0 (baseline activity) and 39 in the home-cage (social) and at trial 42 in an observation cage (individual). These different test conditions were used to investigate whether the housing-effect is consistent and to verify that the response is indeed elicited by the cue that announces the reward and is not context dependent. The observational trials were conducted in a different room to which the animals were transported per experimental group on a cart. During these observational trials the animals were recorded on videotape during the CS-US interval. In this manner all animals of each group could be observed in exactly the same period of time. An ethogram of 23 (home-cage: including social behaviour) or 17 (observation-cage) elements was used to record the behaviour of the rats in the CS-US interval from videotape using the programme The Observer (Noldus Information Technology B.V., Wageningen, The Netherlands). A short description of the recorded behavioural elements can be found in Table 1.

Table 1. Ethogram of the observed behavioural elements. The last category, 'social behaviour', is only used during home-cage observations (trial 0 and 39).

BEHAVIOUR	DESCRIPTION
Mobile exploration	Exploring the surroundings (sniffing, attention) while moving forward or around
Walk	Moving forward in a certain direction (more than 3 steps) without obvious exploration
Rest (lie&sit)	Lying or sitting without obvious exploration
Groom	Washing the muzzle or grooming the fur by means of licking, chewing or scratching
Shake	Shaking the head or whole body
Yawn	Yawning
Drink	Licking at the spout of the water bottle
Rear	Exploring while standing in an upright posture (leaning with its front paw against on object or unsupported)
Scan	Slow sideways swaying of the head and anterior part of the body
Attention	Alertness (listening and/or looking around)
Sniffing	Sniffing in the air, on the sawdust or walls of the cage
Root/dig	Rooting with the muzzle or digging with its front paws in the sawdust
Gnaw/nibble	Gnawing or nibbling on the sawdust, droppings or at the walls or floor of the cage
Hop/Jump	Hopping (moving forward with small hops) or jumping (big forward or upward jump(s))
Circling/chase tail	Circling around its own axis or chasing its tail
Jerk	Sudden convulsive movement with the head or whole body
Freeze	Stiffening of the whole body, including immobility of the whiskers and auricles
Social behaviour	Sniffing, grooming, chasing (a) conspecific(s) or fighting, playing, huddling with (a) conspecific(s)

Analysis

Anticipatory activity displayed in the CS-US interval (reflected by the frequency or transitions of behavioural elements) was used as a measure for the level of reward-sensitivity. For this, the total observed frequency of all behavioural elements was calculated. The data were tested for normal distribution by means of 1-sample Kolmogorov-Smirnov analysis. An ANOVA for repeated measures was conducted to analyse interaction effects between training and housing over the trials. For analysis of differences between groups within each trial an independent-samples t-test was used. A paired-samples t-test was used for analysis of differences between trials within the groups. Effects of housing and training at trial 39 (home-cage) and trial 42 (observation cage) were analysed by means of a MANOVA. Differences were considered to be significant if $p \leq 0.05$.

RESULTS

Figure 2 shows that both groups that were subjected to the conditioning training (ST and ET) display a significant higher activity in the CS-US interval after 39 training trials as compared to their baseline-activity before training (ST $_{\text{trial 0 vs 39}}$: $t_{(11)}$=9.12, p<0.001; ET $_{\text{trial 0 vs 39}}$: $t_{(11)}$=5.03, p<0.001). This is confirmed by an ANOVA for repeated measures that reveals a significant training effect (trial x training: F(1,44)= 56.39, p<0.001). In Figure 2 it can also be seen that enriched housed animals seem to be less active than standard housed animals. This is confirmed by an ANOVA for repeated measures that reveals a significant housing effect on activity (trial x housing: F(1,44)= 6.29, p=0.016). When comparing the displayed number of behavioural transitions before training (trial 0) with that after training (trial 39) within the trained groups, it seems that the increase in activity is larger in the standard housed animals. Analysis of this increase (total frequency (trial 39 – trial 0)) indicates that this difference is significant (ST: $\Delta_{\text{trial 0-39}}$ = 75.25; ET: $\Delta_{\text{trial 0-39}}$=35.67; $t_{(22)}$=3.64, p=0.001; Table 2). Thus, standard housed rats seem to show a stronger anticipatory response for the sucrose-reward than enriched housed rats. This is also indicated by an ANOVA for repeated measures that reveals a trend towards significance for the interaction between trial, housing and training (F(1,44)=3.51, p=0.068). The fact that significance is not fully reached for the interaction-effect is probably caused by the high transition rate of the standard housed control group at trial 0.

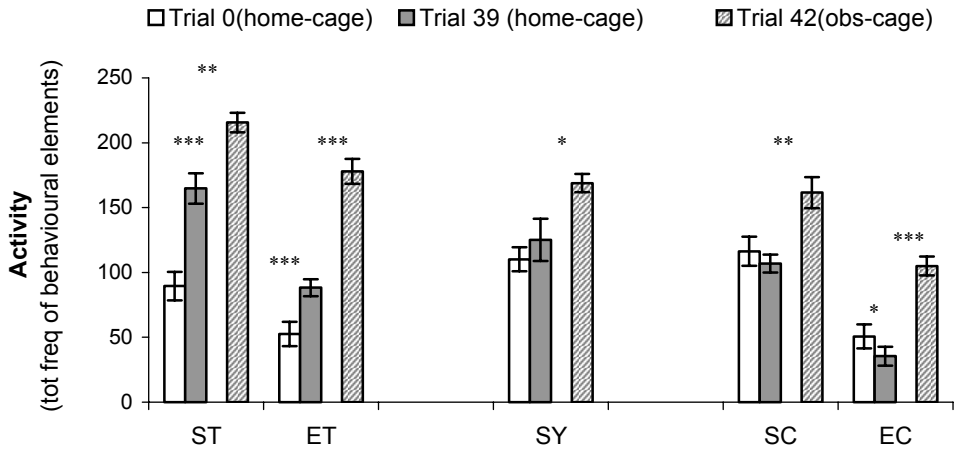

Figure 2. Activity in the CS-US interval (represented by the total frequency of all behavioural elements) (± SEM) of standard housed (**S**) and enriched housed (**E**) animals, which were subjected to the anticipatory training (**T**: CS+US paired), or to the control treatment (**C**: CS-, or **Y**: CS/US unpaired). Trial 0 represents the basal level of activity of the subjects before training. After 39 training-trials the animals were observed in their home-cage (with cage-mates) and at trial 42 (individually) in an observation-cage. Significant differences in activity between pre- and post-training and between the test conditions are indicated with an asterisk (*: p≤0.05; **: p≤0.01; ***:p≤0.001); p- en t-values of all possible comparisons between the experimental groups can be found in Table 2 (n=12 for each experimental group).

Analysis of the effect of the regular consumption of sucrose on the experimental tests, for which the yoked control group (SY: CS+US unpaired) was included, reveals that sucrose did not influence the activity level (ANOVA for repeated measures (trial 0 -39) with SC and SY, factor: sucrose (yes/no): $F(1,22)=1.83$, $p=0.190$). This is also shown by analysis of a training-effect in ST and SY (ANOVA for repeated measures (trial 0 -39), factor: training (yes/no): $F(1,22)=16.61$, $p=0.001$). This means that the training caused the significant increase in activity and not just the regular consumption of a high-caloric reward such as sucrose.

Figure 2 also presents the activity in the CS-US interval in a different context, the observation cage (trial 42). It became apparent that all groups expressed a significantly higher activity level in the observation cage as compared to their activity in the home-cage ($t_{(11)}$=-2.30 to -6.98, $p \leq 0.05$-0.001). When analysing the overall effects of housing and training in both test conditions with a MANOVA it appears that the significant housing effect is present in both test conditions ($F(1,44)$, home-cage=25.03; observation cage=77.55, $p<0.001$ in both cases). Also the significant effect of training is present in both test conditions ($F(1,44)$, home cage=43.47; observation cage=46.09, $p<0.001$ in both cases) . Thus, concerning training and housing condition the effects are similar in both test conditions. So, these effects are reproducible independent of the context or condition they were tested in. Results of the several comparisons between separate groups per trial/test condition are presented in Table 2 (Independent-samples *t*-test).

Table 2. Results of independent-samples *t*-tests for all comparisons between the enriched/standard (**E/C**) and trained/control (**T/C - Y**) groups (n=12; df=22 for all comparisons). The results are presented by order of relevance: the first set (of 4) being relevant to the main research question whether ST responds differently from ET in relation to their control groups; The second set (of 2) being relevant to the question whether sucrose consumption has an effect on the activity of the animals; The last set (of 4) presenting the remaining comparisons.

Comparisons	TRIAL 0 home cage		TRIAL 39 home cage		TRIAL 42 observation cage		Δtrial 0-39	
	t	p	t	p	t	p	t	p
ST versus ET	2.55	0.018	5.67	<0.001	3.06	0.006	3.64	0.001
SC versus EC	4.51	<0.001	7.15	<0.001	4.02	0.001	0.40	0.694
ST versus SC	-1.71	0.101	4.25	<0.001	3.79	0.001	5.41	<0.001
ET versus EC	-0.15	0.886	-5.39	<0.001	-6.02	<0.001	-5.65	<0.001
SC versus SY	0.43	0.675	-1.04	0.311	-0.52	0.608	-1.35	0.190
ST versus SY	-1.44	0.165	1.97	0.061	4.50	<0.001	4.08	0.001
ST versus EC	2.69	0.013	9.37	<0.001	10.45	<0.001	9.09	<0.001
SC versus ET	4.379	<0.001	1.94	0.065	-1.06	0.299	-3.00	0.007
SY versus EC	4.53	<0.001	5.05	<0.001	6.27	<0.001	2.25	0.035
SY versus ET	4.38	<0.001	2.10	0.047	-0.77	0.452	-1.46	0.159

DISCUSSION

Overall, the results show that the anticipatory response is stronger in the standard housed animals which indicates that these animals are more sensitive to the reward than the enriched housed animals. Since stress affects the sensitivity for rewards [293] and the standard housed rats seem to be more sensitive for the sucrose-reward it is likely that the standard housed animals are more stressed as compared to their enriched counterparts. This stress in the standard housed rats might be caused by the deprivation of the ability to display a full natural behavioural repertoire. A standard cage consists of an empty environment without structure, stimulation or possibilities for, for instance, hiding and rearing. The enriched cages do provide more characteristics of a natural environment such as increased structure, dimensions, stimulus complexity and possibilities for hiding and rearing. It is therefore likely that the enriched housed rats are less (or even: not) stressed in their captive environment as compared to standard housed rats. This is also confirmed by a previous study (Chapter 2) that showed that the enriched housing had rewarding properties for rats [384].
Comparison of the standard housed groups that received only the cue (SC) or the cue and the reward paired (ST) or unpaired (SY) confirmed that the increase in activity in ST was the effect of training (repeated pairing of cue and reward) and was not caused by the regular consumption of sucrose. The significant housing-effect indicates that the general activity in the home-cage is lower for the enriched housed rats. The activity increases very strongly when the animals are tested in a different context (observation cage) than they were trained in (home cage) (Fig.2). This is likely to be an arousal effect caused by the different environment and the absence of conspecifics. However, again a housing-effect is present: similar to the home-cage activity, the enriched housed rats are still less active than the standard housed rats. More important, however, is the fact that the differences between groups concerning the anticipatory response are similar in both test conditions (Table 2) and appear not to be influenced by general arousal. Thus, the effects of housing condition on the reward sensitivity are consistent and independent of the context. Obviously, the association of the cue with the reward is very strong in the trained rats which makes it possible to elicit anticipatory behaviour in a different context than the animals were trained in. The only difference found between both test conditions is that the difference between ST and SY is more pronounced in the observation cage. Linking this to the fact that cage-mates cannot influence each other in the observation-cage condition, one might favour this test condition over home-cage testing.
The present study indicates that the reward sensitivity measured by the anticipatory response for a reward is influenced by previous experiences: rats that were housed in a stimulus-poor environment showed a higher level of anticipation for a sucrose reward than rats housed in an enriched environment. These results can be discussed in relation to the known effects of stress on reward sensitivity: mild, acute or short-term stress, such as isolation, foot shock or tail shock, leads to an increased motivation for rewards [29][194][241][260][376]. It is also known that severe or chronic stress leads to a decrease or even a total loss of reward-sensitivity [118][403]. So, if the level of anticipation for a reward is indicative for the state of an animal, it might be a candidate for the assessment of this state in terms of welfare of these animals [348][380].
In conclusion: I. Standard housed rats seem to be stressed as compared to enriched housed rats according to the increased reward sensitivity. II. According to the fact that the results are not influenced by different test conditions, (a) the increase in activity is elicited by the cue and is not context-dependent, and thus, (b) the effect of housing conditions on reward

sensitivity as measured by the anticipatory response is robust and not influenced by general arousal. III. According to the influence of previous experiences on the reward sensitivity, the anticipatory response for rewards might be a useful (non-invasive) indicator of welfare in animals.

Box 2 Training schedule of the conditioning procedure with 5% sucrose as reward

Day	Observation	Trial	Interval (min)
1	Home-cage	0	10
2		1-2	0
		3-4	0.5
		5	1
3		6-7	1.5
		8	2
		9	2.5
4		10-11	3.0
		12	3.5
		13	4.0
5		14-15	4.5
		16	5
		17-18	5.5
6		19-20	6
		21	6.5
		22-23	7
7		23-24	7.5
		25-	8
		26	8.5
8		27-28	9
		29-30	9.5
9		31	9.5
		32	10
		33-34	10
10		35-38	10
11	Home-cage	39	10
12		40-41	10
13	Observation cage	42	10

CHAPTER 5

EFFECT OF HOUSING CONDITION ON REWARD-SENSITIVITY AS ASSESSED BY BREAKING POINT TESTS AND ANTICIPATORY BEHAVIOUR

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ABSTRACT

In the previous study (Chapter 4) we have shown that standard (socially) housed rats were more sensitive to a sucrose reward than were enriched housed rats as reflected by their anticipatory (i.e. appetitive) behaviour in a Pavlovian conditioning paradigm. Because Pavlovian and instrumental conditioning are argued to share a common substrate (i.e. dopamine) and both involve stimulus-induced activation of behaviour during the appetitive phase, the main aim of the present study is to investigate whether instrumental responding and spontaneous (anticipatory) activity are related and are equally sensitive parameters for establishing effects of housing conditions.

In instrumental conditioning paradigms, progressive ratio (PR) procedures have been used to assess relative reinforcer value. The essential feature of the PR schedule is that the response requirement continues to increase until responding ceases altogether and reinforcements are no longer obtained. The final ratio completed is defined as the 'breaking point', which is used as a measure for the maximal effort a subject is will put forth to obtain the reward. It is argued that the breaking point is influenced by the 'need' for, or 'sensitivity' to reward and implies that an increased reward-sensitivity is likely to result in an increased willingness to invest and concomitant increased breaking point. It is hypothesized that a positive correlation exists between the breaking point as assessed by progressive-ratio instrumental conditioning and the anticipatory activity as measured in a Pavlovian conditioning paradigm. Furthermore, it is expected that the previously found increased reward-sensitivity in standard housed rats will be reflected by both an increased breaking point and an increased level of anticipatory activity as compared to enriched housed rats. To investigate this, standard and enriched housed rats were subjected to an instrumental conditioning paradigm with a progressive ratio (PR) schedule consisting of fixed ratio schedules with increasing ratio requirements. Subsequently, the animals were subjected to a Pavlovian conditioning paradigm in the same operant chambers.

Although some effects were quite subtle the parameters of both Pavlovian and instrumental conditioning pointed to an increased reward-sensitivity in standard housed rats as compared to enriched housed rats. Because anticipatory behaviour yielded the clearest results it might be that this parameter, which is based on the spontaneous behavioural response of the animal, is most sensitive to detect differences between animals with different experiences.

It appeared to be difficult to draw any firm conclusions concerning the correlation between the two forms of conditioning and to generalize instrumental and Pavlovian conditioning as common processes.

INTRODUCTION

In the previous study (Chapter 4) we have shown that standard (socially) housed rats were more sensitive to a sucrose reward than were enriched housed rats as reflected by their anticipatory (i.e. appetitive) behaviour in a Pavlovian conditioning paradigm [383]. It was argued that the difference in reward-sensitivity was caused by the fact that under standard housing conditions, rats are deprived of essential stimuli and the display of natural behaviour. In terms of behavioural economics [348], this behavioural deprivation prompts an animal to be very sensitive and willing to invest, in case of the presence of a valuable but rare reward. In this way the balance between positive and negative experiences, that underlies the economy of behaviour, will be maintained, and thus, welfare will be guaranteed [348]. In line with this, Rose and colleagues [314][313] have found that rats reared in an impoverished environment respond at significantly higher rates than do enriched environment-reared counterparts in a simple operant lever press training procedure with a food-reward. They posed that this performance difference is due to a differential reinforcement effect, thus, a difference in reward-sensitivity, rather than reflecting a difference in learning capacity.

Concerning the underlying substrate, it is known that the life history (i.e. previous experiences) of an organism influences mesolimbic dopamine functioning [63][115][154] and it is argued that the effects of housing conditions on anticipatory behaviour for a reward are caused through changes in the sensitivity of dopaminergic systems. This line of reasoning corresponds with the finding that the expectation (appetitive phase) of a reward triggers dopamine release in the ventral striatum but not the actual receipt of the reward (consummatory phase) [331][112]. Furthermore, anticipatory activity is known to be mediated by the mesolimbic dopaminergic system [288][290][197][28]. Thus, Pavlovian conditioning, which implies announcement of a reward and thus induces expectation (i.e. anticipation), involves activation of the dopaminergic system [276] during the appetitive phase. Likewise, instrumental conditioning occurs during the appetitive phase as well and is also known to be influenced by dopamine [324][322][68]. Moreover, Salamone and Correa [323] pose that principles of behavioral economics indicate that dopamine in the nucleus accumbens could be involved in the elasticity of demand in terms of the tendency to pay work-related response costs (see also [325]). According to the abovementioned suggested role of dopamine, Pavlovian and instrumental conditioning - that both involve investment of energy during the appetitive phase - appear to share a common substrate. Both Pavlovian and instrumental conditioning involve stimulus-induced activation of behaviour and a relation between both conditioning paradigms seems therefore plausible and has been acknowledged by others [309][123]. It is argued that lever pressing during instrumental conditioning is an appetitive response that is shaped by the test situation and, therefore, it is plausible that a correlation exists between the parameters of Pavlovian and instrumental conditioning. Both types of conditioning might involve the activation of a representation of the reward (e.g. [83][82]).

Instrumental responding has been widely used to study the reinforcing values of both natural and artificial rewards. Progressive ratio (PR) procedures have been used mostly to assess relative reinforcer value and were developed first with natural rewards such as food [144] and sweet solutions [181]. Later, PR schedules were modified to study artificial rewards such as electrical brain stimulation and drugs (for a review see [316b]). In PR schedules subjects must complete increasing fixed-ratio (FR) response requirements to obtain reinforcers. The essential feature of the PR schedule is that the response requirement continues to increase

until responding ceases altogether and reinforcements are no longer obtained. Thus, the 'cost' of obtaining a reward is progressively increased over a number of trials to determine the maximal effort the animal will emit for the reward [181]. The final ratio completed is defined as the 'breaking point' [181] and is said to refer to the relative strength of a reinforcer [182]. Brennan and colleagues [46] showed that this relative strength is not only related to the properties of the reward (e.g. the concentration of sucrose) but also to the sensitivity of the animal which is, among other things, influenced by previous experiences. This indicates that the breaking point is influenced by the sensitivity to (or 'need' for) reward and implies that an increased reward-sensitivity is likely to result in an increased willingness to invest and concomitant increased breaking point (see also [316b]). Following the above line of reasoning, animals that are less deprived of essential stimuli would be less sensitive to rewards and, thus, will put less effort in obtaining a reward, which results in a lower breaking point. This is in line with the findings of Green et al. [161] who showed that enriched housed rats responded less for a drug-reward.
The main aim of the present study is to investigate whether instrumental responding and spontaneous (anticipatory) activity are related and are equally sensitive parameters for establishing effects of housing conditions on reward-sensitivity. To this end, standard and enriched housed rats were subjected to an instrumental conditioning paradigm with a progressive ratio (PR) schedule consisting of fixed ratio schedules with increasing ratio requirements. This paradigm is used to determine when responding ceased and rewards are no longer obtained. Subsequently, the animals were subjected to a Pavlovian conditioning paradigm in the same operant chambers. In this paradigm, the level of anticipatory activity displayed in the time-interval between the offset of the stimulus that announced the reward (conditioned stimulus; CS) and the delivery of the reward (unconditioned stimulus; US) was investigated.
It is hypothesized that a positive correlation exists between the breaking point as assessed by progressive-ratio instrumental conditioning and the anticipatory activity as measured in a Pavlovian conditioning paradigm. Furthermore, it is expected that the previously found increased reward-sensitivity in standard housed rats [383] will be reflected by both an increased breaking point and an increased level of anticipatory activity as compared to enriched housed rats.

METHODS

The experiments have been performed in adherence to the legal requirements of The Netherlands concerning research on laboratory animals, and have been approved by the Ethical Committee of Utrecht University.

Subjects, housing, and general procedures

Twenty male Wistar rats (HsdCpb:WU, Harlan, The Netherlands) weighing approximately 200 g at their arrival were socially housed and randomly assigned to either a standard cage (2 x n=3; 2 x n=2 per cage) or an enriched cage (2 x n=3; 2 x n=2 per cage). The animals remained housed under these conditions for the total duration of the study. The enriched cages (see Chapter 2; Fig 1A) consisted of a standard Makrolon type IV cage (ground area: 1875 cm²; height: 18 cm; Tecniplast, Milan, Italy) with the following extensions: a 8-cm rim, a shelter (10x11x24.2 cm; h x w x l), a large tunnel shaped object (14.5x16x32 cm; h x w x l) with passages at the sides and on top, and a low bin beneath the food hopper which was filled with old bedding when the rest of the cage was cleaned. Furthermore, the tunnel-shaped

object contained small holes in which pieces of wood were inserted. The presence of the enrichment objects increased the utilizable area inside the cage with 45%. The animals were housed under a reversed light/dark cycle (bright white light 19:00-07:00h; dim light (25W): 07:00-19:00h) in a temperature-controlled room (21 ± 2 °C) with background music. Water and food (Hope Farms™ standard rat chow) were available ad libitum in the home-cage during the first five weeks. At the start of the experimental procedures, the animals were kept on a feeding schedule of 2 hours free feeding per day (14:30-16:30; after training sessions) and ad libitum feeding during the weekend. This was done to keep the rats motivated without actually reducing their weight to a certain percentage of their free feeding bodyweight. Bodyweights were monitored twice per week. Cleaning of the cages was conducted once per week after the experimental procedures to prevent potential effects on behavioural parameters. After 5 weeks of differential housing and concurrent habituation to regular handling and to the procedures (such as transportation to other rooms and placement in the operant chambers), the experimental procedures were started. At this time the animals had a mean bodyweight of 353 ± 3 grams. All experiments and procedures were conducted during the dark phase.

Apparatus

All behavioural testing was conducted in eight identical operant chambers (20 x 25x 61cm; h xw x l) equipped with two retractable levers, cue lights above each lever, speakers under each lever and a house light (24-V, 2.8-W) 24 cm above the floor. The house light was on during each entire session. A pellet dispenser delivered 45 mg sucrose pellets (BioServ Inc., Frenchtown, New Jersey; USA) into a food magazine that was positioned between the two levers. An infrared photo-beam located in the food magazine was used to detect magazine visits and/or pellet retrieval. A green light situated within the food magazine was used to signal reinforcement delivery and was turned off after 5 seconds or when the rat collected the pellet within 5 seconds. Each experimental chamber was enclosed within a light- and sound-attenuating box. A central computer using Delphi software controlled CS- and magazine light onset, the delivery of the pellets, and collected the data (lever pressing and food magazine entries).

Conditioning Procedures

The experiments were conducted in 2 phases: (a) instrumental training, and (b) Pavlovian training. A rest period of 2 weeks was applied between (a) and (b) during which the animals had free access to food. The animals were trained 5 days per week in cohorts of 8 animals, which had a fixed formation and consisted of 4 enriched (E) and 4 standard (S) housed rats (all from different cages). Timing of training was counterbalanced among the experimental groups in order to avoid interference by systematic factors. The operant chambers were located in a separate room to which the animals were transported on a cart.

Operant procedure (lever pressing)

Initially, the animals received two sessions of magazine training, in each of which one sucrose pellet was delivered on a fixed 60-s schedule, with the levers withdrawn. At sucrose delivery, the magazine was illuminated with a small green light. Pellet retrieval was detected by disruption of the infrared photobeam in the magazine. Once the mean reaction time of 5 consecutive trials was £ 1 s, the rats were trained to press the contingent lever (left or right; counterbalanced). This training consisted of 10 daily fixed ratio (FR)-1 sessions of 30 minutes, each of which started with the onset of the houselight and ended 30 min later with

the offset. The levers were extended at the beginning of each session and retracted at the end of each session. Throughout training and testing, presses on the non-contingent lever were never rewarded. After the 10 FR-1 sessions and determination whether all animals showed a stable response – defined as the ratio of collected rewards between two subsequent sessions to be between 0.8 and 1.2 – progressive ratio (PR) training started. One standard housed animal failed to emit stable responding and was therefore excluded.
The PR-schedule was somewhat different from the classically used PR-schedules since we used different ratios with a fixed number of sessions with a fixed duration of 30 minutes instead of gradually increasing the FR after a given number of collected rewards and a response limit of a given number of minutes. By doing so, all animals could be tested during a time-span of 1.5 hour, thus minimizing confounding factors such as differences in hunger and fatigue to affect the results. During PR training, subjects were reinforced for lever pressing under fixed ratio schedules with different ratio requirements: five 30-minute sessions of each FR 5, 20, 50, 100, 200, 300. Completion of the required number of lever presses was followed by pellet delivery in the magazine.

Pavlovian procedure (anticipatory activity)

The general characteristics of Pavlovian conditioning consist of repeated pairings of an initially neutral stimulus (3 pulses of a combined tone/light stimulus for 1.5 s with intervals of 0.5 s) with a reward (unconditioned stimulus; US). After acquisition of the association between the stimulus and the reward, the stimulus will elicit a conditioned response and is therefore referred to as conditioned stimulus (CS). The standard housed and enriched housed group were each subdivided in 2 groups that were either subjected to the conditioning training (T) or to the control procedure (C). This resulted in 4 experimental groups: (1) standard housed rats that received the CS and the US paired (ST, n=5); (2) standard housed rats to which solely the CS was presented (SC, n=4); (3) enriched housed rats that received the CS and US paired (ET, n=6); (4) enriched housed rats to which solely the CS was presented (EC, n=4). Training consisted of 50 trials over 7 sessions and started initially with a delay-conditioning procedure (20 trials) with an overlapping CS-US presentation. Subsequently, the interval between the offset of the CS and the onset of the US was increased (trace-conditioning; [229] p.104) to 180 seconds [381]. The training schedule is presented in Table 1. For the animals that received the training of repeated CS-US pairings a variable 3-minute interval (± 20%) between US and CS was applied; for the control animals a variable 6-minute interval was applied.

Table 1. Schedule of Pavlovian conditioning[1]

day	session		trial	interval (seconds)
1	1		1-10	0
2	2		11-20	0
3	3		21-28	30
4	4		29-33	60
5	5		35-38	120
6	6	observations	39-44	180
7	7	observations	45-50	180

[1]During the first 20 trials the sucrose pellet (US) is delivered at the second pulse of the CS (3 pulses of a sound/light stimulus with an interval of 1,5 s). During the subsequent trials the CS-US interval is gradually increased. Behaviour is recorded on videotape during session 6 or 7.

Behaviour displayed in the interval between CS and US of 2 animals per session was recorded on videotape during session 6 and 7. This way, behavioural data of 6 trials with a CS-US interval of 3 minutes was collected for each animal. An ethogram of 17 elements (see [383]) was used to observe and analyse the behaviour of the rats in the CS-US interval from videotape using the programme The Observer (Noldus Information Technology, Wageningen, The Netherlands).

Analysis and statistics

Data were expressed as group means with standard error of the mean (SEM). Group sizes differed due to the fact that 1 standard housed animal failed to press the lever during the first sessions and was therefore excluded from analysis. Furthermore, for the Pavlovian conditioning protocol each group was subdivided, resulting in different group sizes. The Statistical Package for Social Sciences (SPPS, version 9.0) was used for statististical analysis. Differences were considered to be significant if p £0.05. All statistics are two-tailed unless otherwise mentioned.

Operant Conditioning

Breakpoint (BP) per housing condition was established by calculating the mean last session in which the animals collected rewards. For this, a criterion of at least 3 subsequent sessions in which no rewards were collected was set. Also an 'estimated breakpoint' was computed, reflecting the maximum number of lever presses that an animal would emit for a reward. This estimation was done since some animals would still respond during the last FR. The breakpoint was estimated for each individual animal by means of the following procedure: the number of collected rewards during the last session of each ratio was plotted against the FR and subsequently an exponential trendline (1) was calculated. This trendline is established by means of regression analysis. In the equation for the trendline, c and b are constants and e is the base of the natural logarithm. The breaking point is established at the X-value in which the Y-value in the equation for the trendline is 0.1 (2). Y was set at 0.1 since 0 cannot be plotted on an exponential scale.

(1) Equation trendline: $Y = c.e\ b.x$

(2) Equation breaking point: $X = Ln(Y/c) / b$

Additional data analysed for each session were: number of magazine entries (counted whenever a rat disrupted the photobeam in the magazine), number of collected pellets, and percentage of presses on the non-rewarded lever.

Because the breaking point values are entities in a certain range and increase stepwise, differences in breaking point were analysed by means of the non-parametric Mann-Whitney U test that assigns a ranking order to the separate values. The data of the number of magazine visits and collected rewards were first checked for normal distribution and subsequently subjected to parametric analysis. Differences between the animals of both housing conditions in number of magazine visits and collected rewards were analysed by means of one-way analyses of variance (ANOVA; factor: housing). An ANOVA for repeated measures was used to detect possible differences between the groups in increases or decreases of magazine visits and collected rewards over the course of several sessions (factors: session and housing).

Pavlovian Conditioning

Anticipatory activity displayed in the CS-US interval, as reflected in the frequency or number of transitions of behavioural elements [384][381] was used as a measure of the level

of reward-sensitivity. For this, the total observed frequency of all behavioural elements was calculated. The data were expressed as mean frequency per minute.
Two-way analyses of variance (ANOVA; factors housing and training) were used to analyse differences in anticipatory activity and magazine visits between trained- and control-standard and enriched housed rats. Furthermore, to investigate the response to the presentation of the CS, activity and magazine visits during the pre- and post-CS period were analysed by means of paired-samples t-tests. Additionally, possible differences between groups in increases or decreases in activity and magazine visits over the course of time (pre- and post-CS) were analysed by ANOVA's for repeated measures (factors: trial, housing and training).

Correlations and comparisons

Only the animals that received the contingency training (ST and ET) during Pavlovian conditioning could be used for the comparison and correlation of the parameters of both conditioning procedures. The control groups would not anticipate a reward and would therefore not show a specific response that can be compared to the parameters of the instrumental conditioning. Furthermore, for the comparison of the number of magazine visits the second 5 sessions of the FR 1 schedule were used because visits during the subsequent FR schedules could have been affected by the increasing ratio. Within-subject analysis were conducted by means of paired-samples t-tests and for correlation-analysis the non-parametric Kendall's test or the parametric Pearson's correlation-tests were used, dependent on the distribution of the data and type of parameters.

RESULTS

Operant conditioning

Lever presses and collected rewards

To investigate a possible influence of housing condition on the acquisition of the instrumental task, the percentage of presses on the non-rewarded lever during the first 5 sessions (FR 1) was analysed. No difference between the standard and enriched group was present during acquisition: a one-way analysis of variance (ANOVA) did not detect a significant housing-effect during either of these first sessions ($p>0.1$ for all sessions);(data not shown). Similarly, no housing-effect was present for the number of collected rewards during the first 5 sessions of the FR 1 schedule ($p>0.2$ for all sessions).
The number of collected rewards over the 5 sessions of each of the FR schedules was similar for the animals of both housing conditions (ANOVA; session x housing: $F(4,68)=0.06\text{-}1.33$, $0.28<p<0.93$). This implies that the increase or decrease in number of collected rewards during each FR schedule is equal for both housing conditions);(see Fig.1A). Furthermore, the mean number of collected rewards during 5 sessions of each FR schedule appeared to be similar for both housing conditions (ANOVA: $F(1,17)=0.03\text{-}2.37$, $0.142<p<0.872$);(figure 1B). However, analysis of each separate session revealed that a significant housing-effect is present during the fifth session of FR300 (ANOVA: $F(1,14)=4.596$, $p=0.047$) and a trend towards significance during the second session of FR300 ($F(1,14)=4.08$, $p=0.063$); enriched housed animals collected less rewards during these sessions than did standard housed rats.

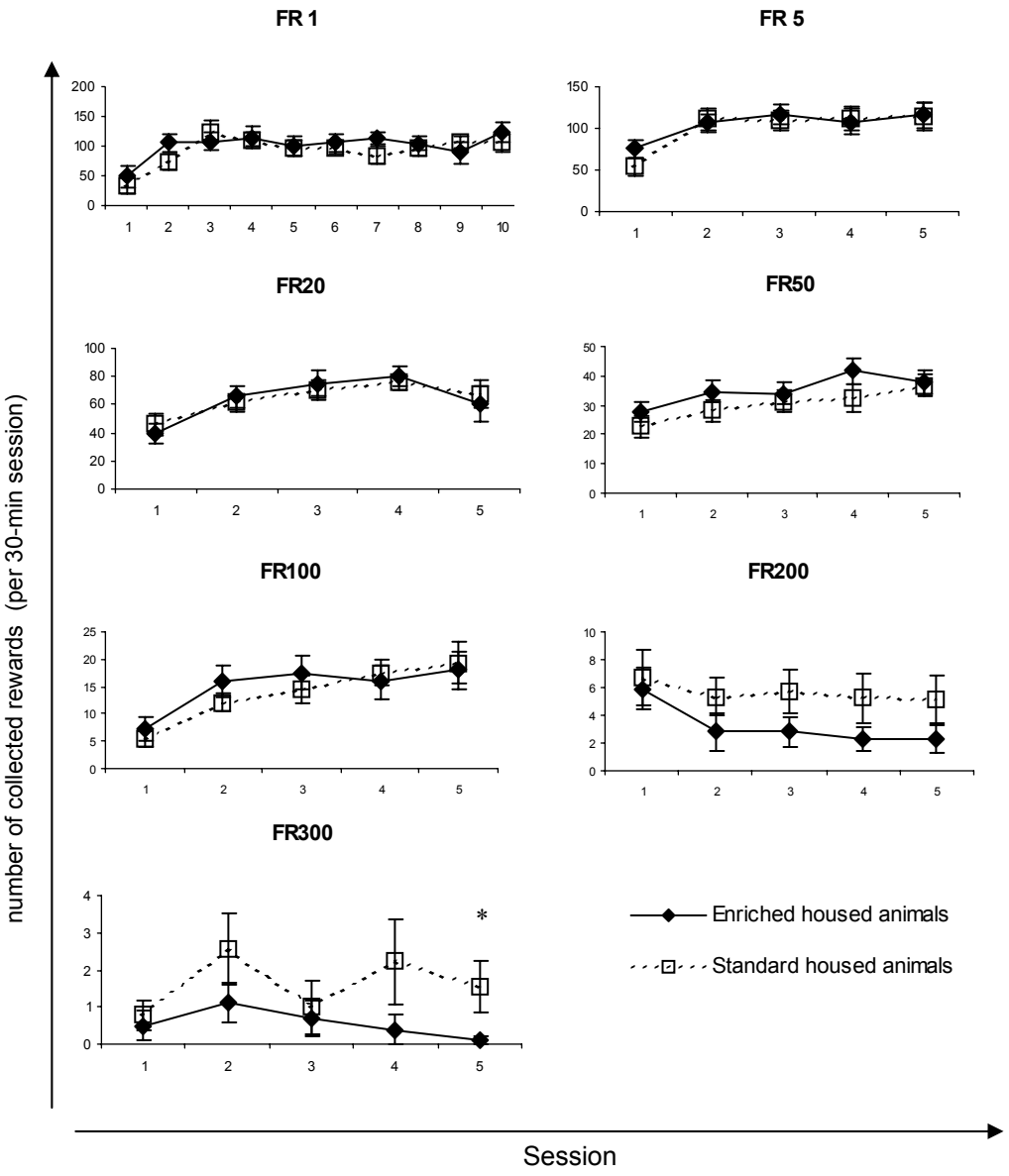

Figure 1A. Number of rewards that were collected by standard (n=9) and enriched housed (n=10) rats per session of 30 minutes for each FR-schedule. Data are presented as group mean ± standard error of the mean (SEM);(*p<0.05).

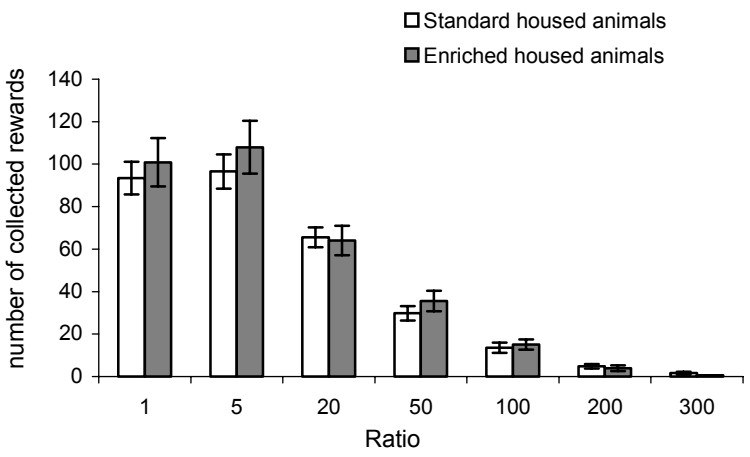

Figure 1B. Number of rewards collected by standard (n=9) and enriched (n=10) housed rats during the different FR-schedules. Data are presented as mean per session per group ± standard error of the mean (SEM).

Magazine visits (figure 2A and 2B)

Standard housed rats visited the magazine more often than did enriched housed rats during FR50 (one-way ANOVA: $F(1,17)=7.69$, $p=0.013$) and FR200 ($F(1,17)=7.59$, $p=0.014$);(figure 2B). Although figure 2A shows that after FR1 the standard housed animals tended to visit the magazine more often during all sessions, this housing-effect appeared to be significant for only a few sessions. Namely, the third and fifth session of FR50 (ANOVA: 3rd ($F(1,14)=6.22$, $p=0.026$); 5th ($F(1,14)=5.95$, $p=0.029$)) and the fifth session of FR200 ($F(1,14)=7.61$, $p=0.015$). A trend towards significance exists for the housing-effect during the first session of FR50 ($F(1,14)=3.21$, $p=0.095$) and the second session of FR200 ($F(1,14)=3.944$, $p=0.065$). Concerning the number of magazine visits over the 5 sessions of each FR schedule, no differences between both groups in potential increases or decreases over the course of these sessions were found (ANOVA; session x housing: $0.141 < p < 0.686$).

Breakpoint (figure 3A and 3B)

It seemed that standard housed animals continued to collect rewards for more sessions than did the enriched housed animals (figure 3A). The difference between these groups was marginally significant (Mann-Whitney U: $U=22.5$, $p=0.062$). The last session during which the animals still collected rewards indicates that standard housed rats stopped responding during FR300 whereas enriched housed rats stopped responding during FR200. However, when estimating the breaking point by means of a trendline (see equation in the section 'Methods'), the seemingly higher value of the standard-housed group (figure 3B) is not significantly different from the value of the enriched-housed group (MWU: $U=36.0$, $p=0.462$).

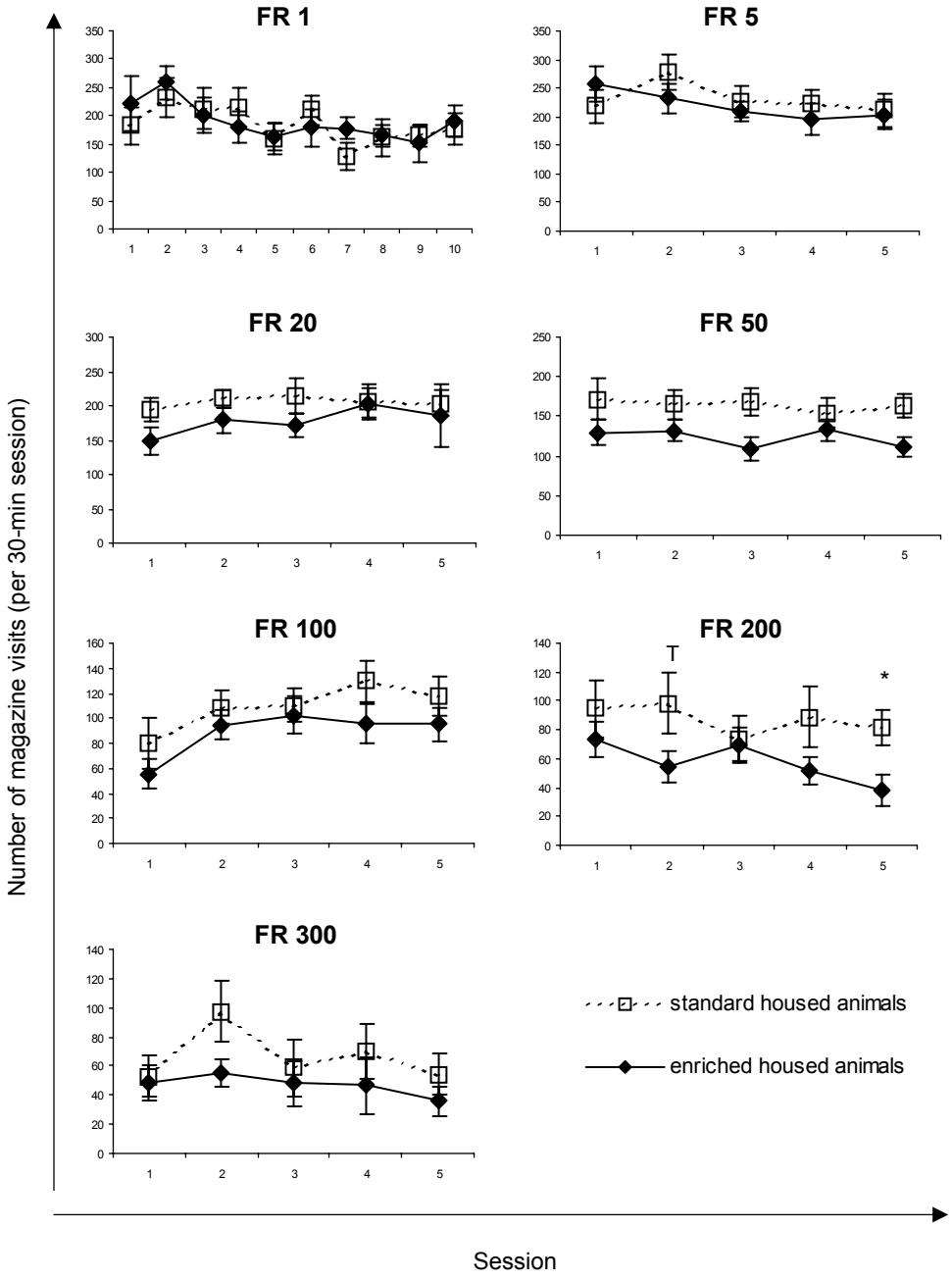

Figure 2A. Number of magazine visits of standard (n=9) and enriched housed (n=10) rats per session of 30 minutes for each FR-schedule. Data are presented as group mean ± standard error of the mean (SEM);(*p<0.05; T<0.01).

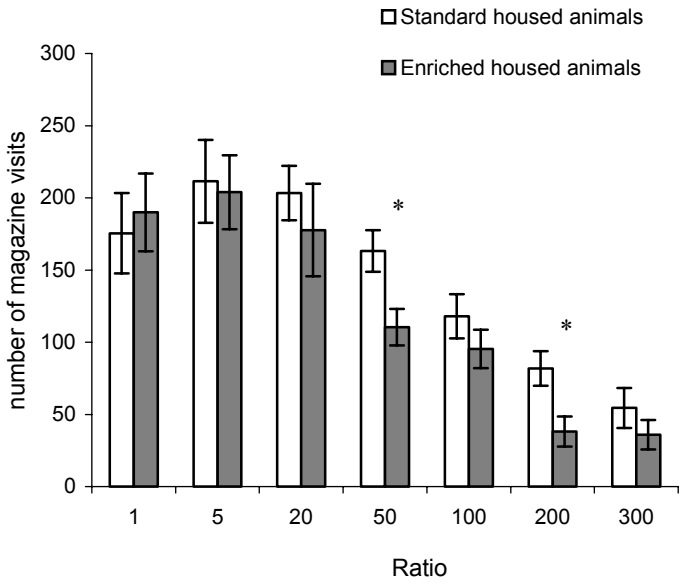

Figure 2B. Number of magazine visits of standard (n=9) and enriched (n=10) housed rats. Data are presented as mean per session per group ± standard error of the mean (SEM);(*p<0.05).

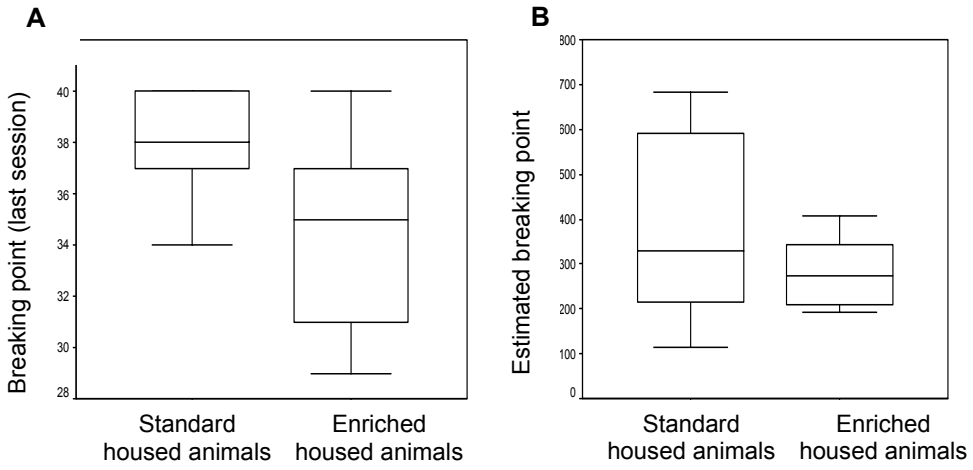

Figure 3.
A. The range of breaking points in standard and enriched housed rats defined as the last session in which rewards were collected.
B. The range of estimated breaking point in standard and enriched housed rats as defined by the trend line-formula.

Pavlovian conditioning

Activity (figure 4)

A significant training-effect on the mean activity per minute displayed in the CS-US time interval was present (two-way ANOVA with fixed factors housing and training: ($F(1,15)=11.62$, $p=0.004$). The animals that received the repeated pairings of CS and US were significantly more active after presentation of the CS than were the control animals to which the CS is meaningless. This indicates that the effect was caused by the anticipation of the forthcoming reward. Since no interaction effect between housing and training was present ($F(1,15)=0.241$, $p=0.631$) it appeared that this increased activity was equal for standard and enriched housed rats. However, analysis of the mean activity per minute before onset of the CS revealed that the effect of contingency training on activity was also present during the pre-CS period ($F(1,15)=9.99$, $p=0.006$) , and thus, not solely caused by the presentation of the CS. Again, this effect was present independent of the housing condition (($F(1,15)=0.272$, $p=0.610$). However, a within-subject analysis (paired samples t-test; pre- versus post-CS activity) indicates that the mean activity per minute of ST was significantly higher during post-CS as compared to pre-CS ($t=2.961$, $df= 4$, $p=0.042$) whereas the mean activity per minute of ET was equal for both periods ($t=-0.394$, $df=5$, $p=0.710$). This suggests that ST does show an anticipatory increase in activity for an announced sucrose reward whereas ET does not. The existence of a slight difference in the effect of training on standard and enriched housed rats was confirmed by the fact that an ANOVA for repeated measures (within-subjects factor: period (pre-, post-CS)) indicated a trend towards significance for the interaction between training and housing ($F(1,15)=3.23$, $p=0.092$).

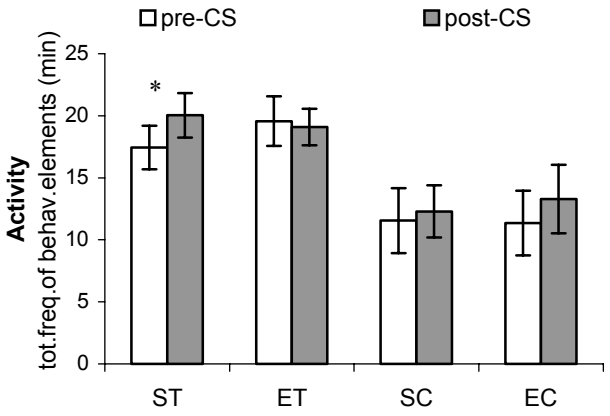

Figure 4. Activity during the pre- and post-CS period of standard (S) and enriched (E) housed animals, which were subjected to the Pavlovian conditioning training (T: CS+US paired), or to the control treatment (C: CS-). Represented by the total frequency of all displayed behavioural elements per minute (± SEM); (ST: n=5; SC: n=4; ET: n=6; EC: n=4; *: $p \leq 0.05$).

To investigate whether a difference between groups existed concerning the immediate reaction to the CS, also a separate analysis was conducted to compare the activity during one minute before and one minute after the CS. Within-subject analysis (paired samples t-test) revealed that in both trained groups the activity was larger during the minute after the CS (ST: $t=5.51$, $p=0.005$; ET: $t=4.49$, $p=0.006$) whereas for both non-trained control groups the

activity during pre- and post-CS was similar ($p>0.1$). This indicates that both trained groups responded to the presentation of the CS with an increased activity whereas the control groups did not. This differs from the results of the comparison with the mean activity per minute of the whole post-CS period (3 min): for that measure enriched housed rats did not significantly differ from the pre-CS period whereas for standard housed rats a significant difference was detected. Apparently, the effect of training (repeated pairing of CS and US) on the response to the CS was most pronounced during the first minute (see also Table 2) and during this short period enriched and standard housed rats showed a similar response. However, analysis of the activity-increase from pre- to post-CS revealed that this increase was larger in standard housed rats as compared to enriched housed rats ($F(1,9)=6.11$, $p=0.035$). Thus, as indicated earlier by the results of the analysis with the mean per minute of the whole 3-min post-CS period, a difference between enriched and standard housed rats remains present. This difference mainly concerns the increase in activity in response to the CS (thus: pre- versus post-CS) and not the level of post-CS activity per se, since no housing-effect is present in the first, second or third minute post-CS in the trained groups ($F(1,9)=0.15$-0.11, $0.409<p<0.906$)

Table 2. Activity and number of magazine visits per minute (±SEM) during the pre- and post-CS period of standard housed (S) and enriched housed (E) rats, which were subjected to the Pavlovian conditioning training (T), or to the control treatment (C).

		ST	ET	SC	EC
Activity					
pre-CS		17,44 ± 1,75	19,57 ± 1,99	11,55 ± 2,62	11,35 ± 2,60
post-CS	1st min	23,36± 2,14	22,5 ± 1,54	14,0 ± 2,06	14,83 ± 4,08
	2nd min	19,07 ± 2,31	16,75 ± 1,52	11,79 ± 2,72	12,67 ± 2,55
	3rd min	17,7 ± 1,65	18,03 ± 2,03	11,083 ± 1,73	12,38 ± 2,07
Magazine visits					
pre-CS		1,68 ± 0,63	2,5 ± 0,35	0	0,65 ± 0,22
post-CS	1st min	2,8 ± 0,30	2,78 ± 0,33	0,46 ± 0,24	1,25 ± 0,42
	2nd min	2,3 ± 0,61	1,31 ± 0,42	0,13 ± 0,04	0,67 ± 0,34
	3rd min	1,67 ± 0,46	1,81 ± 0,42	0,08 ± 0,08	0,58 ± 0,31

Magazine visits (figure 5)

Similar to the activity, a significant training effect, indicated by a larger number of magazine visits in the trained groups as compared to the control groups, is present during the CS-US interval ($F(1,15)=22.51$, $p<0.001$). However, also similar to the results on activity, this effect was also present during the pre-CS period ($F(1,15)=17.77$, $p=0.001$). The absence of an interaction effect of housing and training (pre-CS: ($F(1,15)=0.041$, $p=0.842$); post-CS: ($F(1,15)=1.18$, $p=0.195$)) indicates that this training effect was similar in the animals of both housing conditions. Thus, repeated pairing of CS and US causes the ST and ET animals to visit the magazine more often than the SC and EC animals during both the pre- and post-CS period. Opposite to the results of the analysis of activity, no significant difference was

detected between pre- and post-CS period in either of the groups (Paired samples t-test (pre- versus post-CS): p>0.05 for all groups).

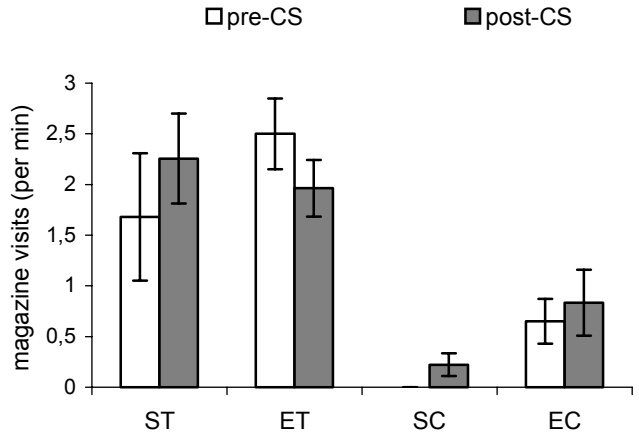

Figure 5. Mean number of magazine visits per minute (± SEM) during the pre- and post-CS period of standard (S) and enriched (E) housed animals, which were subjected to the Pavlovian conditioning training (T: CS+US paired), or to the control treatment(C: CS-); (ST: n=5; SC: n=4; ET: n=6; EC: n=4).

Concerning the immediate reaction to the CS, within-subject analysis of the number of magazine visits between the 1-min pre- and post-CS period did not reveal any significant difference for both the standard and enriched housed groups (p>0.1 for all cases)(Table 2). Similar to the results of the analysis with the mean values per min of the whole 3-min period, the trained groups showed significantly more magazine visits during the 1-min post-CS period than the control groups (training-effect: $F(1,15)=32.86$, $p<0.001$). Between both trained groups, no housing-effect was present during this first minute post-CS and neither during the second and third minute post-CS ($F(1,9)=0.002$-1.926, $0.199<p<0.962$).

Correlation instrumental and Pavlovian conditioning

A trend towards significance was detected for the correlation between the estimated breaking point as determined by instrumental conditioning and the post-CS activity per minute as determined by Pavlovian conditioning (Pearson: -0.551, p=0.079) . Analysis of this correlation for both groups separately revealed that the correlation was significant for the standard housed animals (Pearson: -0.894, p=0.041) but not for the enriched housed animals (Pearson: -0.143 p=0.787). Concerning the breaking point as determined by the last session in which rewards were collected, no significant correlation with the anticipatory activity was present (Kendall: 0.038, p=0.874). Similarly, no correlation was found when analyzing the standard and enriched housed group separately (S: 0.047, p=0.940; E: 0.038, p=0.943).
Within-subject analysis of the mean number of magazine visits per minute in both conditioning procedures (FR 1 session 6-10 versus post-CS) indicates that the animals showed a higher rate of magazine visits during instrumental conditioning (t=-4.827, df=10, p=0.001). No correlation existed concerning the magazine visits per minute in both conditioning methods (Kendall: 0.107, p=0.527). Similarly, no correlation was found between anticipatory activity in the Pavlovian paradigm and magazine visits in the intrumental paradigm (Kendall: -0.241; 0.309)

DISCUSSION

Differences between standard and enriched housed rats

Acquisition

Although enrichment has been shown to facilitate learning in various tasks [358][428][395], in the present study the acquisition of the instrumental task appeared not to be influenced by the housing condition of the animals. Enriched housed rats displayed an equal percentage of presses on the non-contingent lever during the initial five FR1-sessions as did the standard housed rats. This was also confirmed by the fact that the number of collected rewards during these initial sessions was equal for both groups. Factors such as the experimental set-up, type and duration of enrichment [313] and age of the subjects [400] during exposure might be important determinants of the magnitude of housing effects. Specifically, in the previously mentioned studies, the enriched animals are mostly compared to individually housed rats and, furthermore, are housed in very large colony cages with several objects. In the present study a relatively simple form of enrichment is applied which is compared to social housing, and thus, the difference between housing conditions was less pronounced in our study and might not be large enough to significantly affect learning. Furthermore, the animals were 7-8 weeks old when they were differentially housed which might have had less impact on the level of learning capacity. However, the previously reported enhanced learning capacity of enriched housed animals mainly concerned experiments involving maze tasks and contextual processing. Until now, this effect on learning has received equivocal support from experiments employing operant and pavlovian tasks (see for instance [415]).

Reward-sensitivity

Overall, several parameters have indicated that standard housed rats seem to be more sensitive for rewards than are enriched housed rats. Concerning anticipatory behaviour, standard housed rats showed a larger activity-increase from pre- to post-CS than did enriched housed rats. Although the effects were very subtle, this indicates an increased reward-sensitivity in standard housed rats. This is in line with our previous findings concerning an increased anticipatory activity in rats that were housed under standard conditions as compared to enriched housed rats [383] (chapter 4). Concerning the results of the breaking point, as determined by the last session in which rewards were collected, standard housed rats showed the tendency to continue pressing the lever for more sessions, and thus, did put more effort in obtaining the reward than did enriched housed rats indicating an increased reward-sensitivity.

Furthermore, standard housed rats collected more rewards during 2 sessions of FR300 and visited the magazine more often during two ratios (FR50 and FR200). Since these effects are not consistent over the sessions and ratios, this difference between standard and enriched housed rats seems to be marginal. However, increased reward-sensitivity has been shown before as the result of social deprivation [260][194][376], which is considered to be stressful for rats. It is known that stress affects the sensitivity to rewards [63][156][175][296][403] and influences the sensitivity of dopaminergic systems [118][63] that are involved in appetitive responses [324],[28]. Therefore, it may be argued that the increased reward-sensitivity in standard housed rats is caused by stress that may be due to the deprivation of essential stimuli and the ability to display a full repertoire of natural behaviour.

It must be noted that the results of the breakpoint should be interpreted with caution since the estimated breakpoint, as determined by means of a trendline, did not indicate a difference

between standard and enriched housed rats. However, some methodological factors might have influenced this (see 'methodology').

Pavlovian and instrumental conditioning: comparison and correlation

Anticipatory activity and magazine visits

It was expected that by instrumental conditioning the natural anticipatory response is 'shaped' and directed towards the lever and the food magazine. Furthermore, a relation was expected because these parameters can all be inhibited by the administration of dopamine-antagonists [124][288], and thus, appear to share a common substrate. However, no correlation between food magazine entries and anticipatory activity was found. Probably, only a part of the anticipatory activity is directed towards the lever and food magazine. This is confirmed by the observation that the animals still display an increased activity in the box between lever presses and magazine entries. Thus, to compare magazine visits and lever pressing with anticipatory behaviour is comparing two different entities; magazine visits and lever pressing are in fact only a small percentage of the anticipatory activity which is determined by an ethogram of 17 behavioural elements.
Although magazine visits are part of both instrumental and Pavlovian conditioning no correlation between these two conditioning methods concerning this parameter was present either. This might be explained by the fact that magazine visits during instrumental conditioning are part of a chained response induced by the lever presses and subsequent reward-expectancy whereas during Pavlovian conditioning the magazine visits are a part of natural exploratory and approach behaviour and is related to the general anticipatory activity.

Anticipatory activity and breakpoint

The correlation between the estimated breaking point and the anticipatory activity that was indicated by a trend towards significance was negative. This indicates that animals with high breaking point values had low scores for anticipatory activity. However, analysis of both groups separately, revealed that this was only the case in standard housed animals. It is possible that the negative correlation has something to do with the formation of routines; animals that show less behavioural transitions (resulting in less anticipatory activity) may be less flexible and more prone to form routines and, thus, may continue pressing the lever. That this seems to be more valid for the standard housed animals may be caused by the fact that the differences become larger in animals that are housed under less stimulating conditions. This is also illustrated by the large variability between standard housed animals concerning the estimated breaking point as compared to enriched housed animals (see figure 3b). This might be caused by the fact that the standard housed rats experience more difficulty with response-selection [167] and determining when to stop responding due to deprivation of essential stimuli in their housing environment. This is in line with the fact that stressed animals are impaired in the ability to cope with certain challenges [213][212] and show increased inter-individual variability [30][162]. Inter-individual variability is a well-known phenomenon (e.g. [46]). In most of these studies the animals are individually housed which probably causes an even larger variation between animals since these animals are also deprived of social contact which is known to be very important for a gregarious species such as rats [281]. Impoverished housing might cause an increase in behavioural rigidity [195] and subsequent response-prolongation. For the breakpoint as determined by the last session in which rewards were collected it cannot be established whether differences in variability of

the data exist since this measure has a clear maximum and several standard housed rats still responded during the last session.

The breakpoint as determined by the last session in which rewards were collected was not significantly correlated with the anticipatory activity although both parameters pointed in the same direction concerning an increased reward-sensitivity in standard housed rats as compared to enriched housed rats. This might be caused by the fact that for this breaking point determination a limit of 40 sessions is present which influences the variation and maximum value and may not be an appropriate measure for correlation-analysis.
Overall, it appears to be difficult to draw any firm conclusions concerning the relationship between the parameters of instrumental and Pavlovian conditioning. Furthermore, it is possible that the instrumental response is less sensitive to certain differences in experiences that influence reward-sensitivity. In line with this, Dickinson and Dawson [123] have argued that a distinction between Pavlovian and instrumental conditioning exists concerning the representation of the value of the reinforcer. Interpretation of this distinction [121] suggested that Pavlovian conditioning could be modulated by the motivational state of the animal whereas instrumental responding is not necessarily adjusted in response to a difference in motivation (sensitivity). Later, Dickinson and colleagues [124] confirmed that Pavlovian and instrumental incentive learning are not mediated by a common process. Moreover, it has been shown that appetitive approach behaviour in Pavlovian conditioning and instrumental responding are processed by distinct regions of the amygdaloid complex [180]. Thus, it is possible that both forms of conditioning cannot easily be generalized due to the role of complex interactions between various mechanisms.

Methodology

Although the effects and differences are very subtle, anticipatory activity seems to be a more sensitive measure than lever pressing. Perhaps, natural behaviour is more sensitive per se, but it might also be that it is caused by the fact that anticipatory activity was assessed by the full behavioural response of the animals instead of just one element. Besides pressing the lever, the animals display a range of other activities in the operant chamber that is likely to render additional information. Therefore, for future instrumental conditioning experiments it might be useful to investigate anticipatory activity as well to obtain more information. For instance Schmelzeis & Mittleman [329] have used activity as a parameter in addition to lever pressing to investigate the effect of hippocampal lesions in rats. Although they did not correlate these measures the results indicated that the activity measure yielded a similar outcome as did the instrumental measure. However, in these studies activity is determined by disruption of photocell beams. It is not clear whether this method of activity recording is equally sensitive as is recording of the separate behavioural elements. Furthermore, these photocells were positioned in the rear of the chamber which obviously causes a difference with the behavioural observations that consist of continuous recordings in the entire chamber. It has been argued before by others that the use of simple photocell beam breaks is inadequate since it fails to evaluate critical components of behaviour [311][243]. Thus, activity has been used as a parameter during instrumental conditioning by others, but assessing activity via photocell beams appears not to be an adequate method.

The number of magazine visits was higher in standard housed rats during several sessions of instrumental conditioning. Since visiting the magazine is related to natural exploratory and

approach behaviour it might be, similar to anticipatory behaviour, a more sensitive measure than the instrumental response. In Pavlovian conditioning experiments magazine visits are used as a parameter to assess whether conditioning was successful by computing the difference between the rate of magazine visits during the CS and during the pre-CS period [121]. During Pavlovian conditioning in the present study the magazine visits were indeed related to whether the animals were trained (conditioned) or not, but no housing-effect was found. This indicates that this measure in not sensitive enough to be influenced by housing conditions. However, similar to the activity, the training schedule induced a continuously elevated level of magazine visits in the trained animals, which might have caused possible effects to be blunted. But, even if this were the case, magazine visits are not an equally sensitive measure as anticipatory activity since the latter did reveal a housing-effect in spite of its continuously high level during the entire session.

Since a significant housing-effect on the number of collected rewards is present during the last session of FR300 and also a trend towards significance is found for the breaking point (defined as the last session in which a reward was collected) it is possible that a more pronounced difference between groups would have occurred if larger FR ratios had been used. However, the breaking point as determined by the formula that extrapolates the data of animals that still respond during the last session, does not confirm this. This may be caused by the large variability of the data of the standard housed rats. Furthermore, the fact that the ratio-requirements were not gradually increased by equal 'steps' might make it difficult to reliably determine the breakpoint by means of a trendline.
The breakpoint under progressive ratio schedules is known to be a measure of the maximal effort a subject will put forth to obtain a reward. Moreover, it is said to refer to the relative strength of a reinforcer [182]. Therefore, it is plausible that it could be applied to investigate experience-induced reward-sensitivity and it would be interesting to further investigate the usefulness of breakpoint for the assessment of the state of animals in terms of stress and welfare. It is possible that different schedules render more consistent and robust results. However, as mentioned before, it remains possible that the instrumental response is less sensitive and is not necessarily adjusted to experienced-induced changes in reward-sensitivity. This might be caused by the fact that after repeated training the instrumental response itself becomes rewarding, and thus, influences the breakpoint.

It has become apparent that similar to previous studies [381][383][384] in which different training methods and experimental set-ups were used, also a fully automated method could induce anticipatory behaviour. In our previous studies the experimenter announced and also delivered the rewards, which may be argued to be an important factor in the induction of anticipatory behaviour. The results of the present study have shown that the experimenter is not necessarily the crucial factor for the induction of anticipation. Furthermore, the effect of housing conditions on the level of anticipatory behaviour appears to be present in both conditioning methods. However, the significant behavioural activation in reaction to the CS was only present during the first minute post-CS due to an overall increased activity in the trained animals. Apparently, the US-CS interval (3-5 min) was too short and probably caused anticipation for the next presentation of the CS as well. From various experiments it has become apparent that animals are capable of anticipating events in time when a fixed time schedule is used repeatedly [67][315][78]. Rats can very easily recognize correlations between various events in time [58][66] and their behaviour is affected by these associations.

Thus, it is plausible that the significant training effect in terms of a continuous higher activity in ST and ET as compared to the control groups is caused by a continuous anticipation of each forthcoming event during the trials. In our previous studies a longer interval was used ([381]: 8 min; [383]: 45 min- 2 hours) which probably prevented intertrial anticipation or caused a decrease in activity between trials. In the present study, behaviour was analysed during 1 min pre-CS and it might be that analyzing the whole US-CS period would reveal whether the animals were continuously active or clearly increased their activity shortly before the presentation of the CS. For future experiments it would be wise to increase the US-CS interval and apply a more variable range in this interval to prevent continuous behavioural activation.

We have explained earlier that previous experiences such as training history [232], stress [403] and reward [382][385] (chapter 4, 7, 8) can have great impact on both brain functioning and consequent behavioural responses. It must therefore be noted that we cannot exclude possible effects of the preceding instrumental task on the results of the Pavlovian conditioning experiment.

CONCLUSION

In conclusion, several parameters of both instrumental and Pavlovian conditioning have indicated that standard housed rats seem to be more sensitive to rewards than enriched housed rats. Although some effects were quite subtle all parameters pointed in the same direction. Because anticipatory behaviour yielded the clearest results it might be that this parameter, which is based on the spontaneous behavioural response of the animal, is most sensitive to detect differences between animals with different experiences.
Although both conditioning methods yielded a similar result concerning the increased reward-sensitivity of standard housed rats, it is difficult to draw any firm conclusions concerning the correlation between the two forms of conditioning and to generalize instrumental and Pavlovian conditioning as common processes.

Acknowledgements

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CHAPTER 6

ON THE RELATIONSHIP BETWEEN STIMULUS-INDUCED ANTICIPATORY BEHAVIOUR AND STIMULUS-INDUCED INSTRUMENTAL RESPONDING FOR A REWARD

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ABSTRACT

The present rat study assessed the relationship between, and the sensitivity of, two different tests for appetitive conditioned responding to differences in the contingency between a conditioned stimulus (CS) and an unconditioned stimulus (US), and to differences in US magnitude. The first test used a Pavlovian-to-Instrumental Transfer (PIT) paradigm, assessing the capacity of the CS to enhance instrumental responding for food. The second test employed a Pavlovian conditioning paradigm with an extended CS-US interval, and total frequency of displayed behavioural elements in this interval as a dependent measure. The PIT test proved to be sensitive to contingency but not reward magnitude differences, whereas the reverse was true for the Pavlovian test. Although there was a significant correlation between tests in the magnitude of the CS-induced increase of food-magazine entries, the main dependent measure from PIT (number of lever presses) and that from the Pavlovian test (total frequency of behavioural elements) did not correlate. It is suggested that in the PIT procedure, the CS induces a chain of behavioural responses of which lever pressing is just a single element and that the Pavlovian test, in principle, is more sensitive.

INTRODUCTION

In this thesis, anticipatory behaviour evoked in a Pavlovian conditioning paradigm is investigated as a means to study the sensitivity of the mesolimbic dopamine system related to questions of animal welfare (see also [348][380] for rationale). In this paradigm, a previously neutral stimulus is first transformed into a conditioned stimulus (CS) by repeated pairings of the stimulus with some rewarding unconditioned stimulus (US), such as food, a social partner, or an enriched cage. Subsequently, the interval between CS-offset and US-onset is gradually increased from zero to several minutes (see also [376][381][403][383]). The behaviour of the animal changes during this interval, which is indicative of anticipation of the forthcoming reward [384]. There can be notable species differences in the expression of this anticipation. In rats (Rattus norvegicus), anticipation is reflected in an increase in activity (reflected by an increase in the total frequency of displayed behavioural elements or transitions), whereas in cats (Felis silvestris catus), it is expressed as hypoactivity (reflected by a decrease in the total frequency of behavioural elements or transitions) [381]. The total frequency of displayed behavioural elements as a measure of general anticipatory activity seems to capture the behavioural change during the CS-US interval better than do the frequency changes of single behavioural patterns or elements (see [348][384]).
Using the abovementioned Pavlovian conditioning procedure, we have shown that (i) acute social isolation of adult rats leads to an increase in anticipatory behaviour to a 5% sucrose solution or social partner [376], (ii) play deprivation during week 5 and 6 in rats leads to a decrease of anticipatory behaviour to a 5% sucrose solution when adult [376], (iii) a chronic stress paradigm (social defeat and subsequent isolation) in rats leads to a decrease of anticipatory behaviour to a 5% sucrose solution [403] which can be restored by the antidepressant drug imipramine [404], (iv) adding enrichment-objects (shelter, climbing frame) to socially housed animals slightly decreases anticipatory behaviour to a 5% sucrose solution or sugar pellets [383][386]. As anticipation is dependent upon - at least - the activity of the dopaminergic system in the ventral striatum [290][112], these data point to changes in the sensitivity of the ventral striatal dopaminergic system by the diverse treatments/previous experiences (see also [63] 1996 for a discussion related to stress).
Instrumental conditioning also implies the operation of Pavlovian processes, for example, the establishment of a direct associative link between the discriminative stimulus or the manipulandum (e.g. lever) and the reward used (e.g. food pellet). Moreover, both Pavlovian and instrumental conditioning involve the activation of a representation of the outcome: a CS-US association in the case of Pavlovian conditioning, and a response-US association in the case of instrumental conditioning (e.g. [81]). A procedure in which the effect of Pavlovian processes on instrumental responding is made explicit, and in which the ventral striatum has been implicated as well, is the so-called Pavlovian-to-instrumental transfer (PIT) procedure. In this procedure, rats are first trained to press a lever for a reward in an instrumental conditioning procedure. Then, they are conditioned to a previously neutral stimulus using the same reward without the levers present. Subsequently, in an extinction session, the CS and levers are concurrently presented. A typical finding is that the rats show more lever pressing in the presence of the CS than in its absence. Dickinson and colleagues [124] showed that dopamine was specifically involved in this transfer of CS-control from the Pavlovian process to the operant response: the dopamine antagonists pimozide and cis-flupenthixol disrupted transfer while leaving the original Pavlovian and instrumental conditioning intact. Subsequent studies showed that dopamine in the shell of the ventral striatum was involved in this phenomenon as injections of the indirect dopamine agonist d-

amphetamine enhanced transfer [432]. Furthermore, chemical lesions of the core of the ventral striatum abolished this phenomenon, as did lesions of the central nucleus of the amygdala [168]. This was interpreted by Hall and colleagues [168] as being the result of blocking of dopaminergic activity in the ventral striatum by these lesions.
Given the common neural substrate (i.e., the ventral striatum), the aim of the present study was to examine whether the CS-induced increase in anticipatory behaviour in a Pavlovian conditioning procedure and the CS-induced increase in lever pressing in a PIT paradigm are related phenomena. To that end, we exposed rats to a PIT procedure and to a Pavlovian conditioning procedure with an extended interstimulus-interval, as described above. Moreover, different groups of subjects received different amounts of reward. A correlation was hypothesised to exist between the strength of transfer in the PIT procedure and the magnitude of CS-induced increase in anticipatory activity in the Pavlovian procedure. As a second working hypothesis it was expected that the two procedures are equally sensitive to differences in reward magnitude.

MATERIALS & METHODS

Subjects and housing

Forty male Wistar rats (HsdCpb:WU) served as subjects. The rats weighed approximately 200 grams upon arrival at the Department of Biological Psychology of Nijmegen University. The subjects were housed socially in cohorts of 2 or 3 animals in Makrolon IV cages in a temperature-controlled colony room (± 21 °C), under a reversed day-night cycle (dim light: 8:00 - 20.00 h; white light: 20.00 - 8.00 h). A background noise was provided by music from a radio in the colony room throughout the day. For about two weeks prior to initiation of the experiments until the end of the experiments, rats were maintained on a 22-h food deprivation schedule during weekdays: after training and test sessions, the animals had free access to food for two hours per day from approximately 15.00 - 17.00 h. Food (Hope Farms RMH-B, Woerden, the Netherlands) was available ad libitum on weekends. Water was always available ad libitum throughout. The animals were weighed twice a week. The cages were cleaned once a week and water was refreshed twice a week. The experimental procedures started after 4 weeks of differential housing of the subjects (habituation). All animals were handled regularly throughout the habituation and experimental periods. The Ethical committee of Utrecht University had approved all experimental procedures.

Apparatus

Training and testing in each experiment took place in a set of eight Skinner boxes. Each box measured 50.9 x 25.1 x 20.0 cm and had Plexiglas front and back walls. One sidewall and the floor were composed of a grid made of stainless-steel bars. The left sidewall contained a recessed food magazine, two retractable levers, two LEDs, and two speakers. The magazine was used for the delivery of 45-mg sucrose pellets (Bioserve Inc., Frenchtown, New Jersey, USA) that served as US. Visits to the magazine were detected by means of an infrared emitter and sensor. A green magazine light was illuminated simultaneously with each US delivery. One lever was located to the left of the magazine and one to the right. The LEDs and speakers were located above and underneath each lever, respectively. The LEDs and speakers were used for the presentation of a compound CS, consisting of 1.5-s pulses of lights and sounds. The onset of house lights marked the start of each session; their termination served to signal the end of the session. A computer, using software written in

Delphi, controlled the registration of magazine visits and lever presses, and the presentation of pellets and CSs.

General Procedure

Two experiments were conducted using the same animals. A PIT procedure, similar to that used by Wyvell & Berridge [432], was used in the first experiment. The 40 subjects were randomly assigned to eight groups (n = 5 animals per group). The following factors were implemented in the experimental design: position of rewarded lever (left or right), number of 45-mg sucrose pellets during Pavlovian conditioning (one or three), and CS/US relationship (paired or random). Twenty subjects were exposed to the paired arrangement and 20 subjects received the random arrangement. For half of the animals of each of these subgroups, the left lever was rewarded, whereas the right lever was not; the reverse relationship held for the other half. Each of these 10-subject subgroups was further subdivided into animals receiving either 1 sucrose pellet or 3 sucrose pellets as a US (counterbalanced) during the Pavlovian training phase. All animals were exposed to a rewarded and non-rewarded lever to be able to assess the potential effect of general arousal as a response to CS presentations in the transfer test, which would be reflected in enhanced responding to both levers. Different numbers of sucrose pellets were used to establish different US incentive values. Finally, the random CS/US arrangement was used as a control condition. Only the rats having received the paired CS-US arrangement were expected to show an increase of lever pressing during the CS in the transfer test.

A Pavlovian conditioning paradigm was used in the second experiment. This experiment was performed after a two-week rest period during which the rats had free access to food. As described in the introduction, the applied paradigm involved an interval between CS and US to study anticipatory activity. In the present study, the interval was extended to 3 minutes (see [381]). The rats were assigned to five groups (n = 8 animals per group). The following variables were manipulated: (1) CS/US relationship (paired, random, or CS-only) and, for the paired and random groups, (2) number of sucrose pellets as US (1 or 3). The first variable was used to establish a standard Pavlovian conditioning group (CS-US paired) and two different control conditions. The second variable was used to establish different US incentive values. The CS-US paired rats of Experiment 1 (n = 20) were assigned to the paired group in Experiment 2, except for 2 subjects of the one-pellet groups and 2 subjects of the three-pellets groups. These 4 subjects were assigned to the CS-only group. A similar assignment was applied to the rats exposed to the random condition in Experiment 1: 16 of those rats were used in the random condition and 4 in the CS-only condition of experiment 2. One half of the rats within each of the paired and random CS/US-arrangement conditions received 1 pellet as US; the other half 3 pellets.

Experiment 1: PIT

Procedure

Instrumental training.

Instrumental training was initiated after the rats had learned to retrieve sucrose pellets from the food magazine in three 30-min sessions in each of which one pellet was delivered according to a fixed 1-min schedule. In fourteen 30-min sessions, the subjects were subsequently trained to press a lever for sucrose pellets. Training started with a FR1 schedule, in which a press of one of the two levers was rewarded with a sucrose pellet.

Thereafter, a variable time (VT) 5-s schedule was initiated, which was gradually increased in steps of 5 s to a VT-45 s (variation 20%) schedule. A new step was effected after 5 rewarded responses and with each earned pellet being collected. The trial timer was (re-)set to 0 upon collection of the sucrose pellet (trial onset). The sucrose pellet was delivered between 36 and 54 s after trial onset, given a response to the appropriate lever. Responses to the non-rewarded lever had no programmed consequences.
Pavlovian conditioning.
After instrumental training, rats received five Pavlovian conditioning sessions, one session a day. For half of the rats, Group CSUSp (paired presentation), each CS co-terminated with a US (1 or 3 pellets). The CS was presented ten times per session, lasted for 35 seconds and consisted of fifteen 1.5-s light and sound pulses. The CS was presented according to a VT 3-min schedule (variation: 20%), and the sucrose pellet(s) was/were delivered at the twelfth pulse. For the other half of the subjects, Group CSUSr (random presentation), the CS and the US were presented on two independent VT 3-min (variation: 20%) schedules. The levers were retracted throughout the conditioning sessions.

Additional instrumental training and extinction.

All rats were subsequently given one additional instrumental training session to re-establish instrumental performance. Responding to the correct lever was rewarded according to a VT 45-s (20% variation) schedule. All rats were then subjected to a 30-min extinction session during which lever pressing was no longer rewarded. The purpose of this session was to establish lever pressing at an intermediate rate. This, in turn, would prevent possible floor and ceiling effects from hindering a reliable measurement of CS-induced changes (increases or decreases) in frequency of lever pressing during the transfer test.

Transfer test.

All rats were subsequently given three 30-min test sessions. In each test session, seven 35-CSs were presented on a fixed-time, 4-min schedule. The first CS was presented at the very beginning of the test. The instrumental performance was assessed under extinction conditions.

Experiment 2: Pavlovian conditioning

Procedure

The forty rats used in Experiment 1 were assigned to five groups of eight rats, as described in General Procedure. The experiment was run in two replications, with four rats per group in each. The interval between replications was two weeks. The rats in the CSUSp (paired) groups received seven sessions in each of which a CS was repeatedly paired with an US, which consisted of one or three sucrose pellets. In this experiment, the CS consisted of a visual/auditory stimulus compound, which was presented three times for 1.5 seconds, with an interval of 0.5 s [381]. During Sessions 1 and 2, each CS co-terminated with the US. From Sessions 3 to 7, the interval between CS termination and US onset was gradually increased to three minutes: 30-, 60-, 120-, 180-, and 180-s intervals in Sessions 3 to 7, respectively [381](see Chapter 5: Table 1). A VT 3-min (20% variation) schedule determined the interval between the US of one session and the subsequent CS of the next session. A total of 50 trials were presented in the 7 sessions. The rats in the two CSUSr (random) groups also received 50 presentations of the CS and US, but these were presented according to two different VT

(CS-CS and US-US) schedules. Across sessions, these VTs increased from 3 min (variation 20%) to 6 min (variation 10%) to keep pace with the changes in the CSUSp groups that were caused by the increasing CS-US interval. The CSo (CS-alone) group received 50 presentations of the CS. The CSs were presented at a VT schedule. For these animals too, the interval increased from 3 (variation 20%) to 6 min (variation 10%) across training to match the changes in the CSUSp groups. Six trials with an CS-US interval of 3 minutes were recorded on videotape for each rat and analysed afterwards by the experimenter. The behaviour of half of the rats of each group was recorded during Session 6, of the other half during Session 7.

Behavioural observations

The behaviour of the animals was recorded on videotape during several sessions. The recordings were analysed offline, using the program 'The Observer' (Noldus Information Technology BV, Wageningen, The Netherlands) with an ethogram of 17 behavioural elements (see [383] (chapter 4).

Dependent measures

Experiment 1; Instrumental training.

Only the trials of the last instrumental training session were analysed. To assess when lever pressing and magazine visits occurred most frequently during each trial, and whether they co-occurred, trials were divided into three time periods. The first time period consisted of the first 10 s of each trial. Rats were expected, and indeed observed, to mainly visit the magazine during this period because of the recentness of the pellet delivery on the previous trial. The interval of 11 to 35 s was taken as the second time period, because the pellet was delivered between 36 and 54 s after trial onset (given an appropriate response). Pressing the levers in the 11-35-s time period had no programmed consequences. It was assumed that rats would press the lever more during this time period than during the preceding time period. Due to the VT schedule used, the duration of the last time period differed between trials and consisted of the time from 36 s to the end of the trial. Discriminative abilities of the rats were assessed by determining the ratio computed by dividing the session's total number of lever presses on the rewarded lever by the total number of lever presses. To assess whether any instrumental response chaining might occur (see Discussion), a ratio was computed by dividing the total number of presses on the rewarded lever by the total number of presses on the rewarded lever and magazine visits. Only lever presses and magazine visits during the 11-35 s time periods (no pellet delivered yet) were used in the computation of this ratio, because no interference could occur in this period as a result of pellet collection. It was hypothesised that, in the case of response chaining, this ratio should correlate across sessions within individuals. Thus, between-session correlation coefficients were calculated using this ratio.

Experiment 1; Pavlovian conditioning.

For the rats in the CSUSp groups, the US was delivered at about 25 s after CS onset. Therefore, the period between CS onset and US delivery (24 s) could be used to measure the degree of conditioning. Specifically, the number of magazine visits during this period was compared with the number of visits during an equivalent 24-s period immediately prior to CS onset (pre-CS period). As the CS started immediately at the beginning of the session, no pre-CS period was available for the first trial, which was therefore excluded from the analysis. Only the last session was analysed. Rats were considered to be conditioned in case of a

significant CS-induced increase in the number of magazine visits. The number of magazine visits during two additional time periods (60-84 s and 120-144 s) was assessed as well as an additional index of magazine responding in the absence of the CS. Similar analyses were performed for the rats in the CSUSr groups.

Experiment 1; Additional instrumental training and extinction.

Identical dependent measures and analyses were used as described for the initial instrumental training phase. For the extinction session, the ratio computed by dividing the total number of rewarded-lever presses by the total number of lever presses and magazine visits was based upon the entire session, because no sucrose pellets were delivered throughout.

Experiment 1; Transfer test.

It was established whether the rats from the different groups visited the magazine and pressed the previously rewarded lever more during the CS than during no-CS periods of the trial. Moreover, to assess the specificity of the effect, it was determined whether a similar CS-induced increased frequency of lever pressing occurred for the previously non-rewarded lever. Responding during three periods was analysed: one time period preceding (-35-0 s), during (0-35 s), and following (35-70 s) each CS presentation. It was hypothesised that the CSUSp groups would show an increase of magazine visits during the CS, indicating successful conditioning. Furthermore, they were expected to show an increase of lever presses during the CS, reflecting Pavlovian-to-instrumental transfer. These response-enhancing CS effects were expected to be stronger for the rats receiving three pellets than for those receiving one pellet. Instead, the CSUSr groups were expected to show neither CS-induced enhanced magazine visits nor lever pressing.

Experiment 2.

The behaviour of the rats was observed during Session 6 or 7. The settings of the program used for behavioural analysis was such that every rat was observed for 31 s preceding CS onset, and for 186 s after CS onset. For the rats in the CSUSp groups, the interval between CS and US onset was exactly 186 s. The program calculated the total frequency of behavioural elements. For analysis, the 186-s interval was split into six 31-s periods. The CS was presented during the first 6 s of the very first 31-s time block. To assess whether rats were conditioned, the number of magazine visits was determined for the time periods prior to, during, and after CS presentation. The expectation was that, during CS presentation, CSUSp rats would visit the magazine more frequently than would the CSUSr and CSo rats do. Anticipation was measured by scoring the total frequency of behavioural elements during the six time periods. It was hypothesised that the CSUSp rats would display a larger total frequency of behavioural elements than would the CSUSr and CSo rats, and that this would apply most to the rats receiving three pellets.

Between-experiment correlations.

The magnitude of the CS-induced enhancement of magazine visits in Experiment 1 (PIT; 0-35 sec) was correlated with the magnitude of the CS induced enhancement of magazine visits in Experiment 2 (0-31 sec).The magnitude of the CS-induced enhancement of lever pressing in Experiment 1 (PIT; 0-35 sec) was correlated with the magnitude of CS-induced total frequency of behavioural elements of Experiment 2 (0-186 sec).

Statistical analysis

Two-way and three-way analyses of variance (ANOVA's) were used to assess differences between groups, with factors as specified in the Results section [140]. Post hoc tests and paired t-tests were used whenever appropriate. All correlations were Pearson product-moment correlations [140]. Significance was set at $p \leq 0.05$, whereas $0.05 < p \leq 0.10$ was interpreted as a trend. $P > 0.10$ is indicated in the text as NS. All statistical analyses are 2-tailed. Preliminary analyses showed there to be no important and systematic significant interactions involving the 'Location of rewarded lever' factor. Therefore, the data were pooled across this factor.

RESULTS

Experiment 1: PIT

Instrumental training.

The rats needed between 6 and 9 sessions to learn the instrumental task. All rats received at least three training sessions on the VT 45-s (20% variation) schedule.
Magazine visits were made during both the 0-10 s and the 11-35 s periods. The mean number of magazine visits per trial and second was higher during the first time period (0-10 s: range 0.32 - 0.51) than during the second time period (11-35 s: range 0.07 - 0.10) in all groups. The opposite was true for the mean number of lever presses per trial and second: rats pressed the lever more often during the 11-35 s than during the 0-10 s period (0-10 s: range: 0.02 - 0.11, 11-35 s: range 0.23 - 0.40) in all groups.
For the purpose of comparison with the extinction and transfer stages of Experiment 1, the mean number of presses on the rewarded lever in the periods of 11-35 s was recalculated to values per 35 s. Group means ranged from 8.18 to 13.96. The mean discrimination ratio for the different groups ranged from 0.95 to 0.99. The mean ratio of correct lever presses divided by the sum of magazine visits and correct lever presses was 0.79 for the to-be CS-US paired rats (n = 20), and 0.73 for the to-be CS-US random rats (n = 19; one rat was left from the calculations as it showed zero values later in the experiment).

Pavlovian conditioning.

Figure 1 shows the groups' mean number of magazine visits per trial before CS onset (-24-0 s), and after CS onset but before US delivery (0-24 s). Overall, the rats in the CSUSp groups showed a strong increase in magazine visits from before to after CS onset, whereas those in the CSUSr groups overall remained at the same (high) level (three way ANOVA [conditioning, pellet and time period]: conditioning x time-period interaction: $F(1,36) = 12.044$, $p \leq 0.001$). Furthermore, the CSUSr 1-pellet group showed a slight increase between time periods in contrast to the CSUSr 3-pellet group, which showed the same high level of magazine visits (conditioning ´ pellet ´ time period interaction: $F(1,36) = 3.154$, $p \leq 0.084$). The data from the 60-84 s and 120-144 s time periods after CS onset (and well after the delivery of the US) showed a similar profile as did those from the -24-0 s time period (data not shown).

Additional instrumental training.

The Pavlovian conditioning procedure proved to have had no effect on lever pressing and magazine visits during the renewed instrumental training. Rats still showed more magazine visits during the first 10 s than during the subsequent 25-s period, whereas the opposite was true for lever pressing (data not shown). The mean number of lever presses per 35 s for the

different groups ranged from 8.69 to 25.47. The mean discrimination ratio for the different groups ranged from 0.91 to 0.99. The mean ratio of lever presses divided by the sum of lever presses and magazine visits was 0.82 for CS-US paired rats (n = 20) and 0.80 for CS-US random rats (n = 19). In both cases, a strong correlation was found between the ratio computed for the last training session before Pavlovian conditioning and that of the reminder session after Pavlovian conditioning: CS-USp groups, r = 0.56 (df = 18, $p \leq 0.02$), CS-USr groups, r = 0.72 (df = 17, $p \leq 0.01$).

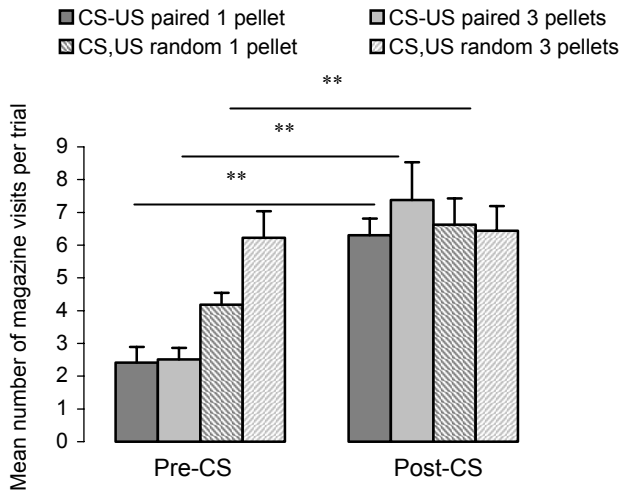

Figure 1. Mean (+SEM) number of magazine visits (n=9 trials) during pre-CS (-24-0 s) and CS (0-24 s) period of the last Pavlovian conditioning session of the PIT paradigm. Asterisks indicate significant within-group differences between the two periods using paired t-tests after detecting a significant time period × conditioning interaction (see text); n = 10 rats per group. **: $p \leq 0.01$. Exact values: CS-US paired groups: 1 pellet t(9) = 6.321, $p \leq 0.001$, 3 pellets t(9) = 4.140, $p \leq 0.003$; CS-US random group: 1 pellet t(9) = 3.306, $p \leq 0.009$.

Extinction.

During extinction, the mean number of lever presses per 35 s dropped to a value ranging from 4.93 to 8.17 (n = 5 per group), with no differences at this stage between groups (all statistics: NS). The groups' mean discrimination ratio's changed to a value ranging from 0.84 to 0.94. Rats that had earned three pellets showed a lower ratio than did rats that had earned one pellet (three way ANOVA [pellet, lever and prior conditioning]: $F(1,32) = 9.279$, $p \leq 0.005$). The mean ratio of lever presses divided by the sum of lever presses and magazine visits was 0.73 (n = 20) for CS-US paired rats, and 0.68 (n = 19) for CS-US random rats. In both cases, a strong correlation was found between the ratio computed for the extinction session and that of the reminder session after Pavlovian conditioning: CS-USp groups, r = 0.59 (df = 18, $p \leq 0.01$), CS-USr groups, r = 0.71 (df = 17, $p \leq 0.01$).

Transfer test.

A total of three test sessions were conducted. Calculations were applied to the first session only, as the transfer effect disappeared at the end of this session (data not shown). Figure 2A shows the mean number of magazine visits before, during, and after the CS. CS-USp rats displayed a much stronger increase during the CS and a slower subsequent decrease than did the CS-USr rats (three way ANOVA [pellet, prior conditioning and time period]: time period ´ conditioning interaction: $F(2,72) = 12.349$, $p \leq 0.001$), independent of the number of pellets

they had received (time period x conditioning x pellet interaction: F(2,72) = 2.204, NS). During the period after the CS, the number of magazine visits still were not at baseline values in the CS-USp rats. Subsequent analysis of the number of visits during the 70 - 105 s time period revealed a return to baseline (data not shown).

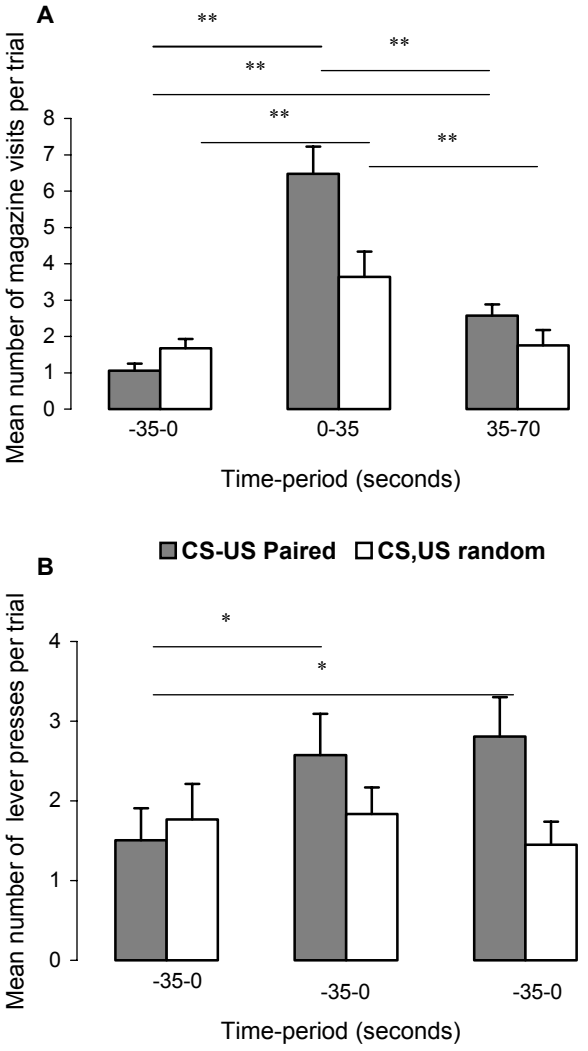

Figure 2.
A: Mean (+SEM) number of magazine visits per trial (n=6 trials) during the first transfer session of the PIT paradigm. Asterisks indicate significant within-group differences using paired t-tests after detecting a significant time period × conditioning interaction (see text); n = 20 rats per group. **: $p \leq 0.01$. Exact values: CS-US paired (1+3 pellet) group: pre-CS vs. CS t(19) = 7.873, $p \leq 0.001$, CS vs. post-CS t(19) = -6.505, $p \leq 0.001$, pre-CS vs. post-CS t(19) = 5.424, $p \leq 0.001$; CS-US random (1+3 pellet) group: pre-CS vs. CS t(19) = 3.584, $p \leq 0.002$, CS vs. post-CS t(19)= -4.027, $p \leq 0.001$.
B: Mean (+SEM) number of lever presses on the reward-related lever per trial (n=6 trials) during the first transfer session of the PIT paradigm. Asterisks indicate significant within group differences using paired t-tests after establishing a significant time period × conditioning interaction term (see text); n = 20 rats per group. *: $p \leq 0.05$. Exact values: CS-US paired (1+3 pellet) group: pre-CS vs. CS t(19) = 2.123, $p \leq 0.047$, pre-CS vs. post-CS t(19) = 2.828, $p \leq 0.011$.

Figure 2B shows the mean number of rewarded lever presses before, during, and after the CS. Rats having received paired CS-US training in the Pavlovian conditioning phase showed an increase during and after the CS, whereas the CS-US random rats did not (three way ANOVA [pellet, prior conditioning and time period): time period ´ conditioning interaction: F(2,72) = 4.091, p ≤ 0.021), independent of the number of pellets they had received (time period ´ conditioning ´ pellet interaction: F(2,72) = 0.009, NS). During the period after the

CS, the lever presses did not return to baseline values in the CS-USp rats. However, subsequent analysis of the number of visits in the 70 –105 s time period did reveal a return to baseline (data not shown). Analysis of the number of lever presses on the non-rewarded lever did not reveal any significant effects (not shown; all statistics: NS).
The groups' discrimination ratio remained at a high level during (range 0.64 - 0.95) and after the CS (range 0.73 - 0.87). The mean ratio of lever presses during the post-CS time period (35 - 70 s) divided by the sum of lever presses and magazine visits in this period was 0.48 for the CS-US paired rats (n = 20). The correlation between this ratio and that of the corresponding ratio from the previous sessions became increasingly weaker: extinction session: $r = 0.46$ ($df = 18$, $p \leq 0.05$), reminder session: $r = 0.21$ ($df = 18$, NS), last training session: $r = -0.32$ ($df = 18$, NS). The same was true for the CS-US random rats (ratio: 0.49; n = 19): extinction session: $r = 0.40$ ($df = 17$, $p \leq 0.10$), reminder session: $r = 0.12$ ($df = 17$, NS), last training session: $r = 0.01$ ($df = 17$, NS).

Experiment 2

Figure 3A shows the mean number of magazine visits per group before, during and after the CS. The different groups showed the same pattern of magazine entries over time (two-way ANOVA [group, time period]: time period x group interaction: $F(8,70) = 0.937$, NS), but a clear difference existed between groups ($F(4,35) = 4.557$, $p \leq 0.005$), reflecting significantly less magazine visits for Group CSo compared to each of the other two groups (Tukey's post hoc tests, $p \leq 0.05$). Furthermore, all CS-USp groups showed an increase from the pre-CS period to the CS period, whereas only the CS-USr 3-pellets group showed such an increase.
Figure 3B shows the groups' mean total frequency of behavioural elements in the CS–US interval. The figure shows that all groups displayed the same pattern of behavioural frequencies over time (two-way ANOVA [group, time period]; group x time period interaction: $F(20,175) = 1.071$, NS). The CSo group showed a lower total frequency of behavioural elements than did the CSUSp and the CSUSr 3-pellets groups (Tukey's posthoc tests; $p \leq 0.027$ and $p \leq 0.028$, respectively, following two-way ANOVA: group $F(4,35) = 3.406$, $p \leq 0.019$). Additional analyses focussing on the paired and random groups did not reveal any significant effects related to the conditioning regimen. However, a difference was found between the 1-pellet and 3 -pellets groups: the 3-pellets groups displayed a total higher frequency of behavioural elements than did the 1-pellet groups (two-way ANOVA [conditioning, pellet]: $F(1,30) = 4.446$, $p \leq 0.043$). In the time period of 31 s just before the CS, the total frequency of behavioural elements for the different groups resembled that in the 31 - 62 s time period (data not shown).

Between-experiment correlations

Figure 4A shows the relationship between the mean number of magazine visits during the CS presentation (0–35 s) of the transfer test of Experiment 1 and the mean number of magazine visits during the CS presentation (0–31 s) of the last sessions of Experiment 2 for each rat. One rat of the CSUSp 1-pellet group and two rats of the CSUSp 3-pellets group were excluded from the correlation because their values in either experiment 1 or 2 fell outside the normal range (Outlier test SPSS v9.0; indicated in the figure). With respect to the remaining rats (n = 13) a significant correlation was found between the CS-induced magazine visits of Experiments 1 and 2: $r = 0.56$, $df = 11$, $p \leq 0.048$. Figure 4B shows the mean number presses on the previously rewarded lever during the CS (0-35 s) in Experiment 1 and the mean total

frequency of behavioural elements during the CS-US interval (0-186 s) in Experiment 2 for each rat. No significant correlation was found between the two measures: r = -0.18, df = 11, NS.

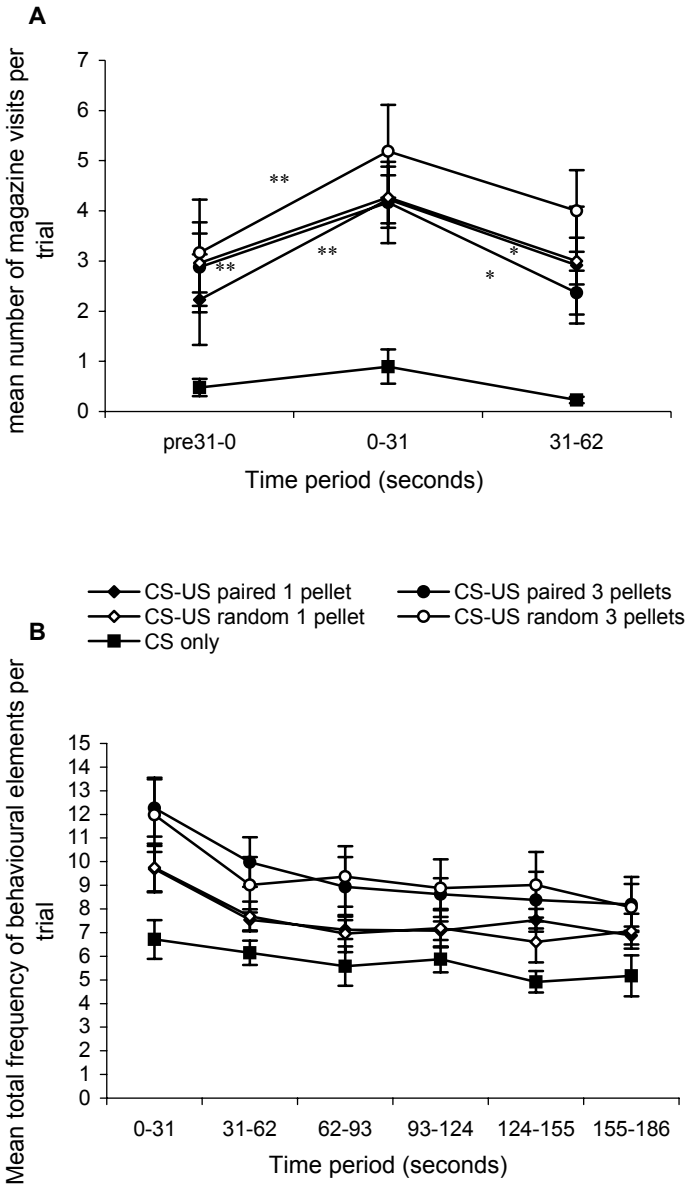

Figure 3.
A: Mean (±SEM) number of magazine visits per trial (n=6 trials) during the last 3 min sessions of the Pavlovian paradigm. Asterisks indicate significant within-group differences using paired t-tests after detecting a significant time period effect ($F(2, 70) = 17.873$, $p \leq 0.001$); n = 8 rats per group. *: $p \leq 0.05$, **: $p \leq 0.01$. Exact values: CS-US paired 1 pellet group: pre-CS vs. CS $t(7) = 3.654$, $p \leq 0.008$; CS-US paired 3 pellet group: pre-CS vs. CS $t(7) = 3.748$, $p \leq 0.007$, CS vs. post-CS $t(7) = -3.332$, $p \leq 0.013$; CS-US random 3 pellets group: pre-CS vs. CS $t(7) = 4.988$, $p \leq 0.002$, CS vs. post-CS $t(7) = -3.170$, $p \leq 0.016$
B: Mean (±SEM) total frequency of behavioural elements per trial during the last 3 min sessions of the Pavlovian paradigm. For F-values see text; n = 8 rats per group.

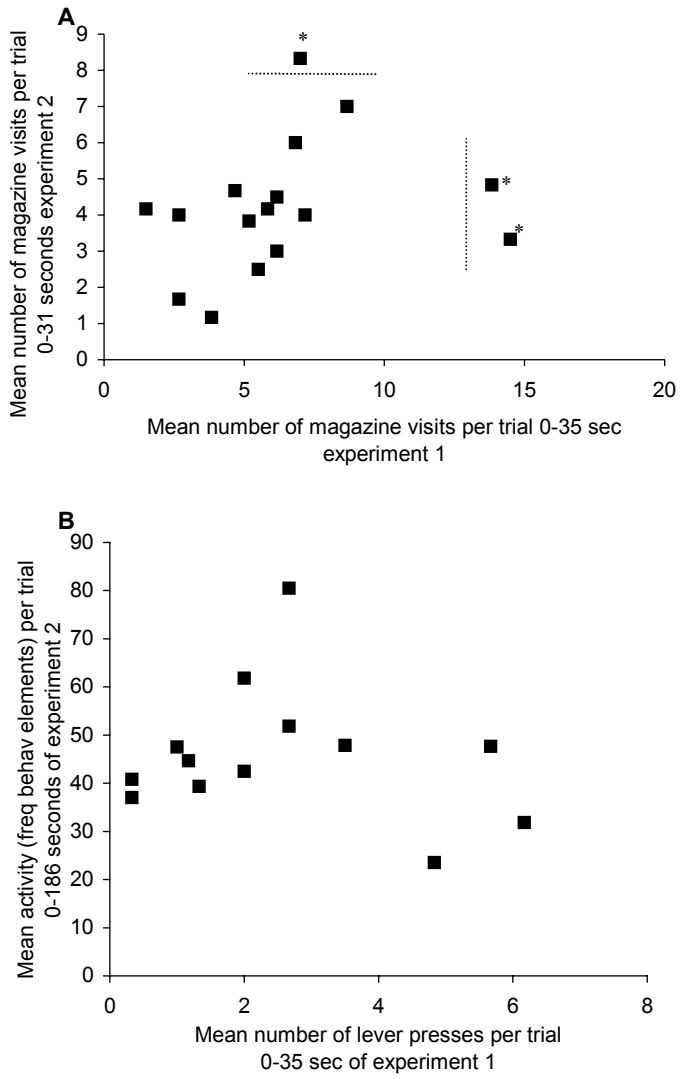

Figure 4.
A: Correlation between mean number of magazine visits per trial (n = 6 trials) during the CS period (0-31 s) of the Pavlovian training procedure of Experiment 2 and the mean number of magazine visits per trial (n=6 trials) during the CS period (0-35 s) of the Pavlovian training procedure of Experiment 1. Asterisks denote individuals that were removed from the analysis (see text).
B: Correlation between the activitiy per trial (n=6 trials)(represented by the mean total frequency of behavioural elements) during the CS-US interval (0-186 s) of the last 3 min sessions of the Pavlovian training procedure of Experiment 2 and the mean number of lever presses per trial (n = 6 trials) during the CS period (0-35 s) of the Pavlovian training procedure of Experiment 1.

DISCUSSION

General

The present study failed to detect a correlation between the CS-induced increase of lever pressing during a Pavlovian-to-Instrumental (PIT) paradigm and the CS-induced increase of total frequency of behavioural elements in the CS-US interval of a Pavlovian conditioning paradigm. However, a correlation was found between the strength of the CS in each paradigm as measured by the magnitude of increase of magazine visits. The lack of the former correlation was not expected given that both types of response-enhancing effects have

been shown to be under dopaminergic control in the ventral striatum [112][290][432] and theoretically appear to measure the same phenomenon: a CS-induced change in behaviour to (the expectation of) a forthcoming reward. Furthermore, the CS-induced changes in the total frequency of behavioural elements in the Pavlovian paradigm appeared to be more sensitive to US reward magnitude than were the lever-press frequency changes in the PIT paradigm. A difference between the 1-pellet and 3-pellets conditions was observed in the former but not in the latter procedure. However, the Pavlovian paradigm was less sensitive to the contingency differences than the PIT procedure. Before discussing possible accounts of these data, both paradigms are critically evaluated.

PIT

In line with previous studies, the observed transfer effect was small [124][168][432], but specific, since it did not occur in the CS-US random rats and no effect was found for responding to the non-rewarded lever. Our analysis of instrumental responding showed that the rats readily learned to discriminate between the levers and attained a stable level of responding. Moreover, this responding was not affected by the Pavlovian procedure and decreased nicely under extinction. As a result of Pavlovian training, all CS-US paired rats came to elicit more magazine visits selectively during the CS. Instead, the CS-US random rats displayed relatively many magazine visits throughout the entire session, and only slightly increased their magazine visits during the CS in the Pavlovian training procedure and in the subsequent transfer test, but with no effect on lever pressing.

In addition to previous studies, we observed that the transfer effect in paired rats extended beyond the actual CS to one time block of 35 s thereafter. This finding might reflect the fact that, in the original instrumental learning sessions, the pellet was delivered between 36 and 54 s. Accordingly, rats may have learned to keep responding for longer periods of time when no pellet was delivered after 35 s. Hence, when activated by the CS, they might have extended enhanced responding to the following 35-s time period (but not thereafter). Furthermore, we observed that the number of magazine visits remained somewhat elevated too during this period after the strong increase observed during the CS. This suggests that the increase of magazine visits and lever pressing may be related or have a common cause.

If so, it is possible that the CS not only enhanced lever pressing but possibly a chain of behavioural responses of which lever presses and magazine visits are a part. Several facts do support this notion. First, the subjects' ratio of number of lever presses divided by the sum of lever presses and magazine visits, as observed during instrumental training in the 11 - 35 s time period (i.e., after pellet collection and preceding the next US delivery), remained stable over the different training sessions, as revealed by a strong correlation between the ratio based on the last training session before Pavlovian training and the ratio based on the first training session after Pavlovian training. Furthermore, the ratio remained stable as exemplified by the significant correlation between the ratio from the first training session after Pavlovian training and that from the extinction session, and from the extinction session and that from the transfer test. This suggests a stable behavioural pattern within individual subjects. Second, Dickinson and colleagues [124] note that the decrease of the transfer effect by dopamine antagonists is not limited to lever pressing only: “We did, in fact, measure magazine entries during the transfer test, and their analysis revealed exactly the same drug effects that were observed for lever pressing” ([124]:p. 476). Both facts strongly suggest a joint and inter-dependent effect of the CS on magazine-directed and lever-press behaviour.

Our data do not directly answer the question whether the CS-induced increase in responding in PIT is due to a motivational influence of the CS [309] or to a CS-induced activation of a

behavioural chain (cf. [82][368]: CS induced magazine visits lead to lever pressing and more magazine visits). However, the latter explanation seems more straightforward and parsimonious. In any case, the two possibilities suggest a straightforward experiment: food pellets should be delivered to different food-magazines in the Skinner box during the instrumental and Pavlovian phases of the experiment. In the case of a general motivational influence, for example due to an activation of a representation of the US, a subsequent transfer test should yield increased responding to both magazines, along with an increase of lever pressing. In the case of the CS triggering a behavioural chain, magazine responding should primarily take place with respect to the magazine used during the Pavlovian phase and hardly, if at all, regarding the magazine used during the instrumental phase, and there should be little change in lever pressing.
The lack of an effect of amount of pellets used during Pavlovian conditioning on the magnitude of PIT suggests that the PIT paradigm is not sensitive to discriminating between these small differences in incentive value. Whether this is due to the dependent variable (lever pressing) used remains to be seen.

Pavlovian conditioning: Anticipatory behaviour

In line with previous studies, after CS-US paired training, but not after CS-only training, the CS induced an increase in the number of magazine visits, strongly suggesting successful conditioning. Moreover, the CS from the former but not the latter condition induced an increase in the frequency of displayed behavioural elements, suggesting anticipation to the forthcoming reward during the CS-US interval [381][403][384]. Furthermore, three pellets induced a stronger anticipatory response than did one pellet, suggesting that the dependent variable of this paradigm is more sensitive to differences in reward strength than is the dependent variable used in the PIT paradigm. However, contrary to our expectations, the baseline of magazine visits and the frequency of behavioural elements were high throughout in the CS-US paired rats, and the CS-US random rats showed similar CS and number-of-pellets effects as did the CS-US paired rats on magazine visits and frequency of behavioural elements (cf. [386]). The lack of a clear effect of the contingency manipulation between the paired and random groups may be related to the inter-trial (ITI) and inter-stimulus (ISI) intervals used. In general, the ratio between these two intervals determines the magnitude of behavioural control by a CS, with stronger control the larger the ITI is relative to the ISI (e.g., [155]). In this respect, it is important to note that the ITI was identical to the ISI in the final stage. In addition, the final ISI and ITI used in the paired and random groups resulted in a strong resemblance in procedural treatment, which further decreased the likelihood of obtaining a strong effect of the contingency manipulation.

Explanation of the lack of correlation

The question now arises as to a possible explanation of the lack of a correlation between the two major dependent variables of the different paradigms, despite an equal effectiveness of the CS to change magazine-directed behaviour. Firstly, superficially the different paradigms may measure the same phenomenon, that is CS-induced changes of responding in anticipation of reward, but at a deeper level, they may measure different phenomena: a CS-induced increase of a previously learned instrumental response and a CS-induced increase in spontaneous behaviour. This line of reasoning would suggest that these two changes be mediated by different parts of the ventral striatum, enabling a different and relatively independent expression of CS associative strength on the two measures. Secondly, both paradigms measure the same phenomenon, that is CS-induced changes in responding, but the

dependent variables are not at the appropriate level of comparison. In this respect, it is important to realise that rats first come to learn where a reward can be found and show conditioned approach behaviour towards this location as the experiment progresses. Reward-related behavioural patterns are then shown before the reward arrives, such as gnawing. Finally, an increased general activity is seen preceding the arrival of the reward such as increased locomotor behaviour, hopping, etc. [381][384][229]. Accordingly, several patterns are activated during the CS-US interval which are collectively referred to as the appetitive phase, as opposed to the consummatory phase that follows the arrival of the reward [348]. During this appetitive phase, dopaminergic activity is increased in the ventral striatum [112][290]. Dopamine in the ventral striatum is related to overcoming reward related costs as measured by ratio or interval schedules [323], and to facilitate a switch between different environmentally directed behavioural patterns [378][379][377]. Accordingly, one could suggest that anticipatory behaviour as measured by the total frequency of behavioural elements more accurately reveals the role of the ventral striatum then does the measurement of single behavioural elements, unless the frequency of elements is artificially reduced to only a few elements by limiting the behavioural repertoire (see also [348][384]. As argued before, in the PIT paradigm, lever pressing might be only one of a multitude of activated behavioural elements, and the frequency of lever presses may say little about the frequency of these other behavioural elements.

CONCLUSION

In conclusion, the present experiments show that CS-induced changes in spontaneous behaviour in a Pavlovian conditioning procedure are not correlated with CS-induced changes in a Pavlovian-to-instrumental transfer procedure. It is suggested that, in the latter procedure, the CS induces a behavioural chain of which lever pressing is only one element.
The magnitude of transfer in the transfer procedure was dependent on the CS-US contingency, but not on the number of rewards used in the Pavlovian conditioning phase of that procedure. Instead, in the Pavlovian procedure, the change in the total frequency of displayed behavioural elements was sensitive to differences in reward magnitude but not to the difference in CS-US contingency in the paired and random conditions. Establishing a larger ITI to ISI ratio might enhance the sensitivity to CS-US contingency, suggesting that, in that case, the Pavlovian procedure, with change in total frequency of behavioural elements as dependent measure, is a more appropriate test to assess sensitivity to a forthcoming reward.

CHAPTER 7

ANNOUNCED REWARDS COUNTERACT THE IMPAIRMENT OF ANTICIPATORY BEHAVIOUR IN SOCIALLY STRESSED RATS

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ABSTRACT

It is known that stress can influence the sensitivity to rewarding stimuli. Previous observations revealed that socially stressed rats do not display an appetitive behavioural response in anticipation of a reward. A previous study showed that this insensitivity to rewards (anhedonia) could be restored by chronic administration of an antidepressant. Several lines of evidence exist for the role of dopamine in the mechanism of action of antidepressant treatments concerning their therapeutic effect on anhedonia. Therefore, it was hypothesised that regular activation of the reward system, that involves mesolimbic dopaminergic systems, could counteract the effect of social stress on reward-sensitivity. For this, it was investigated whether a treatment of regular reward announcements could prevent the development of anhedonia. This was confirmed by the fact that socially stressed animals that received this treatment were able to display anticipatory behaviour which is characterised by increased activity after presentation of a stimulus that was previously associated with a sucrose reward. Surprisingly, a non-treated socially stressed group, that did not show an anticipatory response for sucrose, did display anticipatory behaviour for another type of reward (enriched cage). Apparently, the anhedonic state as concluded from the absence of anticipatory activity for a sucrose-reward cannot be generalised to other types of reward. It will be discussed whether this might be caused by the highly rewarding properties of the enriched cage which probably has a therapeutical efficacy of its own.

INTRODUCTION

Chronic stress is applied in animal models of depression since it is known that stress has a precipitating effect on the development of this disorder. Insensitivity to rewards in chronically stressed animals is reminiscent of reward alteration in human depression. This stressor-induced insensitivity to rewards simulates anhedonia, which is considered to be a major symptom of human depression [4]. Several lines of evidence point to the involvement of the activity of the dopamine system in this disorder [63][95][420]. Mesolimbic dopamine functioning is influenced by previous experiences such as stress and the nature and direction of the effects depend on the behavioural controllability of the situation, the genetic background of the organism and its life history (previous experiences) [63][115][154][433]. Dopaminergic neurons originating in the ventral tegmental area (VTA) and projecting their nerve terminals into different telecephalic areas such as the prefrontal cortex and the nucleus accumbens are involved in the control of reward-related behaviour and incentive motivation [34][143][199][426] which are impaired in depression. The insensitivity to rewards is mostly measured in rats by a decrease in consumption of a sucrose solution [258][421][420][423]. However, the validity and reliability of sucrose consumption as a hedonic measure is questionable [242]. It has been demonstrated that conditioned place preference (appetitive phase) for a sucrose solution was decreased in stressed rats whereas the sucrose consumption (consummatory phase) during the conditioning trials was unchanged [275]. In line with this, it has been argued by Von Frijtag and colleagues [403][404] that the absence of reward-related (appetitive) behaviour is a more consistent consequence of chronic stress and representative of anhedonia. This is in accordance with the recent finding that dopamine release is triggered by the expectation of a reward and not by the actual receipt [112][269][331]. Expectation of a reward is behaviourally recognized in rats by an anticipatory increase in activity appearing in the appetitive phase and also characterized to reflect ‘wanting’ [27][348]. Anticipatory behaviour reflects the activation of the reward system and is influenced by previous experiences. Von Frijtag and colleagues [404] showed that treatment with an antidepressant could restore long-term impairment of anticipatory behaviour for a sucrose reward in defeated and subsequently individually housed rats. Several lines of evidence exist for the role of dopamine in the mechanism of action of antidepressant treatments concerning their therapeutic effect on anhedonia and loss of motivation [95][433]. Probably, the regular activation of mesolimbic dopaminergic systems is a prerequisite for the maintenance of appetitive responses.

Therefore, it is hypothesized that regular activation of the reward system, that involves mesolimbic dopaminergic systems, can counteract the effect of social stress on reward-sensitivity and therefore serves as a behavioural therapy. For this, it was investigated whether a treatment of regular reward announcements could prevent the development of anhedonia reflected by the absence of appetitive behaviour. Defeated and subsequently individually housed rats (see [403]) received either announced sucrose rewards on a regular basis during the long-term period of individual housing or no treatment. After three months the presence or absence of an anticipatory response to an announced sucrose reward was determined in both the treated and non-treated group. To assess whether the behavioural therapy has been effective to prevent the impairment of the anticipatory response for other types of reward as well, anticipation to access to an enriched cage was investigated.

METHODS

The experiments have been performed in adherence to the legal requirements of The Netherlands concerning research on laboratory animals, and have been approved by the Ethical Committee of Utrecht University.

Subjects, housing, and general procedures

Forty male Wistar rats (HsdCpb:WU, Harlan, The Netherlands) weighing approximately 200 g at their arrival were socially housed (n=2 per cage) in Makrolon type III cages (Tecniplast, Milan, Italy). They were kept under a reversed light/dark cycle (lights on at 19:00 hr) in a temperature-controlled room (21 ± 2 °C). Water and food (Hope Farms™ standard rat chow) were available ad libitum. Cleaning of the cages and weighing of the animals was conducted once per week after the experimental tests to prevent influence of this disturbance on behavioural parameters.
After two weeks of habituation to the housing conditions, procedures and regular handling, the experimental procedures were started. At this time the animals had a mean bodyweight of 285 ± 2.6 grams.

Experimental design and procedures

All experimental procedures were conducted during the dark phase. Twenty animals were subjected to a social stress paradigm and an equal number to the control treatment. Social stress was induced by repeated defeat during forced introduction in the territory of a dominant male rat. As a part of the social stress paradigm (see [403]) the experimental rats were individually housed (in Makrolon type III cages) immediately after the first defeat session. The non-defeated control group remained socially housed with 2 animals per Makrolon type III cage. Ten days after the first defeat session the defeated and subsequently individually housed group (DI) and the non-defeated socially housed control group (S) were subjected to a conditioning procedure (AP Ia). During this procedure half of the animals of each group received a sucrose reward (5% solution, 5 min) after presentation of a stimulus whereas the other half did not. After acquisition of the association between the stimulus and the access to the sucrose solution this reward was announced on a regular basis during a 70-day period as a behavioural therapy to prevent the development of anhedonia. The above-described design resulted in four experimental groups: 1. defeated, individually housed animals that received therapy (DIT), 2. defeated, individually housed animals (no therapy) (DIC), 3. non-defeated, socially housed control animals that received therapy (ST) and 4. non-defeated, socially housed control animals (no therapy) (SC).
After a total of 90 days after the first defeat-session a second anticipation-on-sucrose test was conducted (AP Ib) to investigate whether DIT still showed anticipatory behaviour after long-term individual housing after defeat with regular reward announcements. Subsequently, to confirm our earlier observations [403][404] that DI-animals do not show anticipatory behaviour after long-term individual housing after defeat, the groups that had not received therapy (DIC and SC) were subjected to a conditioning training (AP II) with a novel CS that was now paired with the US (sucrose). Finally, to assess whether the behavioural therapy has been effective to prevent the impairment of the anticipatory response for other types of reward as well, anticipation to a novel reward was investigated (AP III). For this, all animals were subjected to a conditioning training by which again a new CS was repeatedly paired with transfer to an enriched cage where the animals were allowed to stay for 30 minutes. The time schedule of the performed tests is presented in Table 1.

Table 1: Time schedule of the performed tests presented as number of days after the first day of individual housing (= after the first defeat session). During training the time-interval between CS and US is gradually increased and during testing the behaviour of the animals in this interval is observed and analysed.

Days after first defeat session

		test	Fig.
0-5	Defeat + individual housing after first session		
10-19	Anticipation of sucrose: training + 3 tests Assessment of presence of appetive response after short-term isolation	AP Ia	1
20-90	Therapy: regular reward-announcements (2-3 times per week)		
90	Anticipation of sucrose: 1 test Assessment of presence of appetitive response after long-term isolation with regular reward-announcements (therapy-group)	AP Ib	1
93-102	Anticipation of sucrose: training + 3 tests Assessment of absence of appetive response after long-term isolation without regular reward-announcements (non-therapy-group)	AP II	2
112-122	Anticipation of enriched cage: training + 3 tests Assessment of appetive response for a novel reward after long-term isolation with or without regular reward-announcements	AP III	3

Social defeat procedure

The social defeat procedure consisted of daily resident-intruder sessions on 5 consecutive days. Each defeat session lasted for 20 minutes and was divided in a pre- (5 min), fight- (10 min), and a post (5 min)-phase. During the pre- and post-phase the experimental rat (intruder) was positioned behind a transparent, perforated barrier in the home-cage (63 x 25 x 33 cm) of a dominant male Long-Evans rat (LE/CpbHsd, Harlan, UK). These residents were housed with a sterilized female rat to stimulate territorial aggression; this female was removed from the home-cage before each defeat-session. The Long-Evans strain was selected for their strong physical condition, readiness to attack and fighting-tactics (inhibition of aggression when intruder displays submissive behaviour, thus minimizing the risk of injuries). The fight-phase was initiated and terminated by respectively removing or replacing the barrier. During the fight-phase the experimental rat was attacked and lost the fight in all cases.

Anticipatory behaviour

Anticipatory behaviour was induced by a Pavlovian conditioning set-up in which an initially neutral stimulus (conditioned stimulus;CS) was repeatedly paired with a reward (unconditioned stimulus;US). In case sucrose was used as US the delay between the offset of the CS and the onset of the US is progressively increased to 10 minutes over 35 trials (see [404]). In case transfer to an enriched cage was used as US a CS-US interval of 10 minutes is applied from the first training trial during a total of 10 trials since this has proven to be effective for this highly rewarding stimulus [384]. To investigate the behavioural response to

the CS over time the animals were observed during 3 trials: before training to determine baseline activity and 2 times after several training trials. Behaviour displayed in the CS-US interval was recorded on videotape during trial 0 (baseline activity), trial 25 and trial 35 for the anticipation-on-sucrose training (AP Ia and II) and during trial 0, 6, and 10 for the anticipation-on-enrichment training (AP III). AP Ib consisted of one observation session at trial 60. For the observational sessions, the animals were transported to a separate room and placed individually in an observation cage (63 x 25 x 33 cm, l x w x h). Behaviour was observed and analysed from videotape using the computer program 'The Observer' (Noldus Information Technology, Wageningen, the Netherlands). Activity displayed in the CS-US interval (reflected by the frequency or transitions of behavioural elements) was used as parameter for anticipation.

Statistical analysis

The total frequency of all displayed behavioural elements, reflecting behavioural transitions and thus activity, was calculated. The data were expressed as mean frequency per minute. The effects of defeat and conditioning training concerning the level of activity over the course of training were analysed by means of two-way ANOVA's for repeated measures (within-subjects factor: trials; between-subjects factors: defeat and conditioning (AP Ia); defeat and therapy (AP II and AP III). Since the anticipation-on-sucrose test after long-term individual housing of DI (AP Ib) consisted of only one test (at trial 60) an univariate analysis of variance (fixed factors: defeat and therapy) was used. Difference between groups in bodyweight at every separate week during the experiment was analysed by means of independent samples t-tests. A two-way ANOVA for repeated measures was conducted to analyse differences in weight gain over the course of the experiment (within-subjects factor: time; between-subjects factors: defeat and therapy).

RESULTS

Bodyweight

DI-animals weighed significantly less than S-animals over the 5 defeat sessions ($F(4,152)=8.25$, $p<0.001$). During the course of the whole experiment no differences in bodyweight were found between groups at any moment. This is confirmed by the fact that no interaction-effect between defeat and therapy on weight gain over the course of the experiment is present ($F(19,684)=0.421$, $p=0.644$). However, an ANOVA for repeated measures reveals a significant defeat-effect ($F(19,684)=3.250$, $p=0.048$) and a trend towards significance for a therapy-effect ($F(19,684)=2.914$, $p=0.065$). DI-animals and T-animals appear to gain slightly more weight over time than non-defeated socially housed and non-therapy animals respectively.

Anticipation after short-term individual housing (AP Ia, Figure 1)

To assess the presence of anticipatory behaviour for an announced sucrose-reward, half of each group (DI and S) received the CS and US paired whereas the other solely received the CS to control for general arousal induced by the procedure. An ANOVA for repeated measures revealed a significant conditioning-effect ($F(2,72)=10.057$, $p<0.001$) on the activity of the groups over the trials. The groups that were subjected to the conditioning training (pairings of CS+US) displayed a significant increase in activity over the course of training. No defeat-effect ($F(2,72)=1.658$, $p=0.198$) and no interaction-effect between defeat and conditioning ($F(2,72)=0.780$, $p=0.462$) were present indicating that the conditioning training

had a similar effect on DI- and S-animals. Thus, after short-term individual housing after defeat DI-animals were able to show an anticipatory response for sucrose similar to the S-animals.

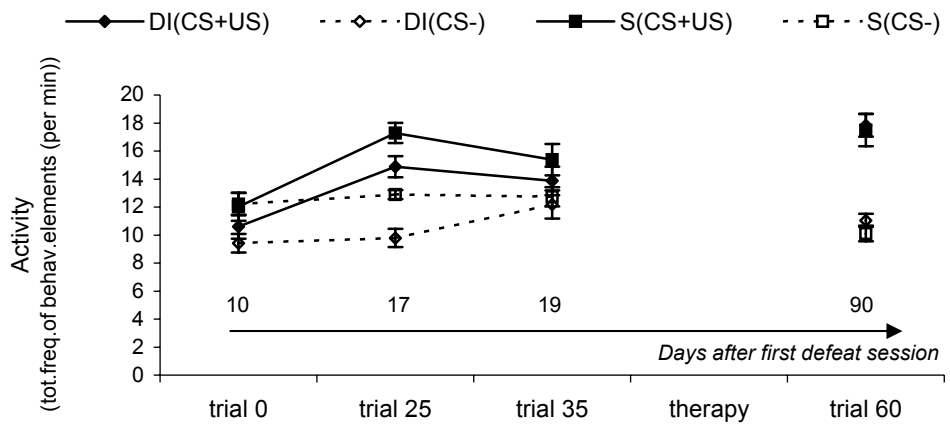

Figure 1. Anticipation on sucrose (5%, 5 min): Assessment of the presence of an appetitive response (increase in activity) after short-term (10-19 days (AP Ia)) and long-term (90 days (AP Ib)) isolation after defeat. During AP Ia the animals were trained with a progressively increasing CS-US interval in 9 days and behaviour was observed during trial 0 (pre-training), trial 25 and trial 35. The control groups only received presentation of the CS. After AP Ia the therapy-groups (T (CS+US)) received regular reward-announcements (25 trials) during 70 days and were tested once more (AP Ib) after this long-term isolation period (trial 60). The activity of the control groups that did not receive therapy (CS-) was used as a control for baseline activity.

Anticipation after long-term individual housing with therapy (AP Ib, Figure 1)

To assess the presence of anticipatory behaviour for an announced sucrose-reward after a long-term period of individual housing after defeat with regular announcements of this reward, the same groups as used in AP Ia were tested during trial 60. From a univariate analysis of variance it became apparent that, similar to AP Ia, a significant conditioning-effect was present ($F(1,36)=79.71$, $p<0.001$) whereas no defeat-effect ($F(1,36)=0.661$, $p=0.422$) and no interaction-effect between defeat and conditioning ($F(1,36)=0.138$, $p=0.712$) was found. Thus, after long-term individual housing after defeat with regular reward-announcements DI-animals were able to show an anticipatory response for sucrose at an equal level as the S-animals.

Anticipation after long-term individual housing without therapy (AP II, Figure 2)

To assess the expected absence of anticipatory behaviour for sucrose in DI-animals after long-term individual housing after defeat without regular reward-announcements, a non-treated group was subjected to a conditioning training (a new CS paired with the US (sucrose)). As expected, a significant defeat-effect was present (ANOVA for repeated measures: $F(2,36)=65,932$, $p<0.001$). The DI-animals and S-animals reacted differently on the conditioning training: DI showed a slight decrease in activity over the trials whereas S showed a strong increase in activity. Thus, after long-term individual housing after defeat

without a therapeutic treatment DI-animals did not display an anticipatory response for sucrose.

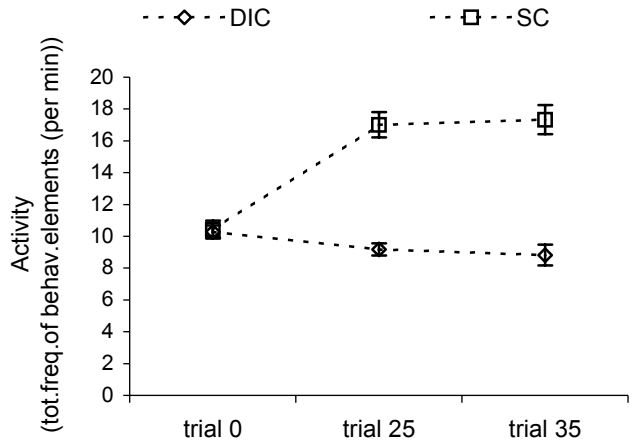

Figure 2.
Anticipation of sucrose (AP II): Assessment of the absence of an appetive response after long-term isolation *without* regular reward-announcements (C-group). The control animals of the previous test were subjected to a conditioning training by which a new CS was repeatedly paired to the presentation of a sucrose reward. The animals were trained with a progressively increasing CS-US interval in 9 days and behaviour was observed during trial 0 (pre-training), trial 25 and trial 35.

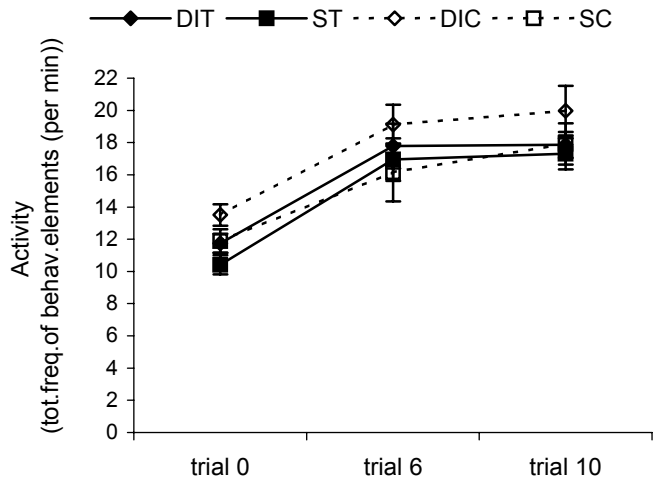

Figure 3.
Anticipation of an enriched cage: Assessment of the appetitive response for a novel reward after long-term isolation with (T-group) or without (C-group) regular reward-announcements. All animals (T and C) were subjected to a conditioning training by which a new CS was repeatedly paired (10 trials total) to the transfer to an enriched cage were they could stay for 30 minutes. Behaviour was observed during trial 0 (pre-training), trial 6 and trial 10.

Anticipation on novel reward after long-term individual housing with or without therapy (AP III, Figure 3)

To assess whether the behavioural therapy has been effective to prevent the impairment of the anticipatory response for other types of reward as well, anticipation to access to an enriched cage was investigated. Surprisingly, all four experimental groups (DIT, DIC, ST, SC) appear to display an equal increase in activity over the trials. This is confirmed by the results of an ANOVA for repeated measures: no effects of defeat or therapy, or an interaction-effect between defeat and therapy on the activity over the trials were present

(defeat:F(2,72)=0.103, p=0.902; therapy:F(2,72)=0.508, p=0.562; defeat x therapy: F(2,72)=0.237, p=0.790). Thus, all animals display anticipatory behaviour for the transfer to an enriched cage regardless of their previous experience and therapy-treatment.

DISCUSSION

The present study has shown that a regime of regular reward announcements can prevent the impairment in anticipatory behaviour for sucrose in chronically stressed rats. This impairment in behavioural signs of reward expectancy has been seen in two previous studies [403][404] and this indicates that it is a consistent consequence of chronic stress and apparently a representative measure of anhedonia, a major symptom of depression. Furthermore, the results reveal that the impairment of anticipatory behaviour for sucrose is not yet present after a short period of individual housing after defeat. Thus, the long-term individual housing is necessary for the development of anhedonia and, thus, an important factor in this animal model. This is line with the general idea that stress pathology develops over time as a result of chronic challenge of adaptive mechanisms [213]. It must be noted that anhedonia is also a symptom of posttraumatic stress disorder (PTSD), which also has an obvious resemblance with the present used stress paradigm. Actually, a high rate of comorbidity of PTSD with depressive disorders exists [40] and treatment of PTSD with antidepressants is commonly known [178][284]. However, we aimed to investigate a behavioural therapy to counteract the effects of severe stress on reward-related behaviour and it is beyond the scope of this study to elaborate further on the specific human disorder.
The fact that DI-animals had a significantly lower bodyweight than S-animals during the defeat-period is probably caused by the severity of the defeat-procedure in terms of stress. Conversely, over the course of the whole experiment the increase in bodyweight over time was larger in DI-animals than in S-animals. It is likely that the DI-animals consumed more food to compensate the deprivation of social contact or were less active due to the lack of (social) stimulation. Also the therapy-treatment seemed to cause a larger increase in bodyweight over time, which can be explained by the regular consumption of sucrose.
Similar to the aforementioned studies by Von Frijtag and colleagues [403][404], the differences in anticipatory activity for a sucrose reward cannot be ascribed to a difference in consummatory behaviour, since the consumption during conditioning training was not affected by prior treatment (defeat or therapy)(data not shown). Furthermore, the increase in activity in animals that received both the conditioned (CS) and unconditioned stimulus (US: sucrose) as compared to the animals that solely received the CS cannot be caused by the regular consumption of sucrose since a previous study has shown that a yoked control group (CS and sucrose unpaired) did not display an increased activity [383].
Surprisingly, the results of the anticipation test with another type of US revealed that in case transfer to an enriched cage was used, chronically stressed rats that were not subjected to the therapy were able to display anticipatory behaviour at a similar level as their therapy-counterparts. Three possible explanations can be given for this phenomenon.

First, the anhedonic state of the socially stressed animals as concluded from the absence of an anticipatory response for sucrose cannot be generalised to other rewards. This might be due to a difference in rewarding properties since it has been shown in a previous study that the enriched cage has highly rewarding properties for rats [384]. Furthermore, pilot studies have shown that far more training trials are necessary when a sucrose solution is used as US as compared to a sexual reward or environmental enrichment. This indicates that the incentive value of sucrose is inferior.

Second, it is possible that the anticipation training with sucrose, which preceded the conditioning for the enriched environment, had a therapeutic effect on the animals and caused a reversal of the anhedonic state. This is not very likely, however, since in a previous study [404] chronically stressed rats have also been subjected twice to a conditioning training with sucrose and no restoration of anticipatory behaviour was seen.

A third explanation might be that the repeated exposure to the enriched cage while being trained, had a therapeutic effect on the animals and caused a reversal of the anhedonic state. This is in line with the knowledge that physical activity in previously stressed rats restores hippocampal brain-derived neurotropic factor (BDNF) mRNA levels to baseline [319]. These results are related to the evidence that has been gathered in recent years that BDNF expression could be an important agent for therapeutic recovery from depression [268][341]. The beneficial effects of physical activity is extensively investigated in clinical studies [148][236][413] and it is therefore very likely that the regular stay in an enriched cage caused reversal of the depressive-like state in the animals. It is known that levels of endogenous opioids (endorphins) increase in response to physical activity [54][227][371]. Furthermore, it is argued that processing of reward is mediated by both the mesolimbic dopamine system and the opioid system [27][127][348], which is caused by the interaction between these systems [86][292]. In line with the fact that endorphins are candidates for antidepressant treatment [47][99][135] it is possible that enriched-environment-induced opioid activity counteracts the effects of chronic stress via activation of the endorphinergic system.

Whether the announcement and the subsequent anticipation of the transfer to the enriched cage had an additional effect is not clear. However, it is known that anticipation induces dopaminergic activity [112][199][269][331], which is decreased by chronic stress in depression models [63][95]. Thus, although in combination with a sucrose-reward it did not have an effect, it is feasible that, in combination with an enriched cage, the anticipatory phase might have had an additional effect on the reversal of the anhedonic state. This can be investigated by transferring chronically stressed rats to an enriched cage on a regular basis and, for another group, include an announcement of the transfer to this cage. Then, the possible reversal of the impairment in reward-related behaviour can be established by investigating the presence or absence of an anticipatory response for sucrose in these two experimental groups. Since the additional effect of anticipation might be very subtle, it would useful to study the underlying mechanism as well. It is known that artificially induced sustained enhancement of synaptic strength (long-term potentiation) by high frequency stimulation of the hippocampus is impaired in chronically stressed rats [282] and can be partially reversed by treatment with an antidepressant [402]. The hippocampus is very sensitive to previous experiences [147],[149] and is importantly involved in the modulation of reward [329]. Furthermore, it is suggested that long-term physical exercise may protect the hippocampus from stress-induced damages [231]. Thus, to increase our knowledge about the underlying mechanism of the therapeutic effect of (anticipation on) environmental enrichment on the impairment of reward-related behaviour after chronic stress it might be useful to focus on hippocampal synaptic plasticity. This will be further investigated in Chapter 8.

Based on the findings of the present study it is concluded that physical activity and/or announcements of a forthcoming reward on a regular basis could be important for counteracting the consequences of chronic stress in both animal and man.

CHAPTER 8

THE EFFICACY OF ANNOUNCEMENT OF REWARD ON COUNTERACTING THE EFFECTS OF CHRONIC STRESS ON BEHAVIOUR AND HIPPOCAMPAL PLASTICITY

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ABSTRACT

It is known that chronic stress causes insensitivity to rewards in rats and that this impairment, indicated by the absence of reward-related behaviour in anticipation of a sucrose-reward, can be reversed by antidepressant treatment. A previous study that aimed to validate the effects of a behavioural therapy on chronically stressed rats revealed that repeated announced transfer to an enriched cage during a short period (10 days) seemed to have caused a reversal of this impairment. It was, however, not clear whether the highly rewarding properties of the enriched cage that probably had a therapeutical efficacy of its own caused this reversal or whether the announcement had an additional effect. Announcement of a reward induces anticipation (i.e. expectation) that is known to trigger the release of dopamine. Furthermore, evidence exists for the role of dopamine in the mechanism of action of antidepressant treatments concerning their therapeutic effect on anhedonia and loss of motivation. Thus, it is plausible that announcement of a reward has an additional therapeutic effect to the consequences of the mere reward on stress-induced reward-insensitivity. Therefore, the present study compared the consequences of announcement of a reward to the mere reward with respect to their efficacy to counteract the effects of chronic stress. For this, reward-sensitivity was investigated in chronically stressed rats (induced by defeat and subsequent individual housing) that received a reward (short-term enriched housing, EH) and in rats to which this reward was announced (A-EH) by means of a stimulus that was repeatedly paired to the reward. Furthermore, the therapeutic effects of (announced) environmental enrichment on the underlying mechanism in terms of brain plasticity were investigated by means of artificially induced sustained enhancement of synaptic strength (long-term potentiation: LTP) in the hippocampus (in vitro). It became apparent that both EH and A-EH restored reward-sensitivity in chronically stressed rats indicated by a significant increase in reward-related behaviour in anticipation of a sucrose reward. However, concerning the chronic-stress induced attenuation of hippocampal plasticity, A-EH appeared to have had an additional effect indicated by a significant higher amount of LTP.
In conclusion, announced short-term environmental enrichment has proven to have a high and long lasting therapeutic efficacy on stress-induced alterations of both reward-related behaviour and hippocampal synaptic plasticity. This information is important for counteracting the consequences of stress in both man and captive animals thereby improving their welfare.

INTRODUCTION

It is known that chronic social stress in rats causes insensitivity to rewards as indicated by impaired reward-related behaviour during the appetitive phase in defeated and subsequently individually housed rats [403]. This impairment in appetitive behaviour is reflected in the absence of an increase in activity in anticipation of a sucrose reward that is announced by means of a Pavlovian conditioning paradigm. It is argued that the insensitivity to rewards as indicated by this absence of anticipatory behaviour in socially stressed animals simulates anhedonia [403][348], which is considered to be a major symptom of human depression [4]. This is supported by the results of a study in which the administration of an antidepressant restored the impairment of anticipatory behaviour for a sucrose reward in socially stressed rats [404]. In a previous study (chapter 7) we have shown that a regime of regular reward announcements could prevent the development of this stress-induced impairment in anticipatory behaviour [382]. Furthermore, this study revealed that repeated announced transfer to an enriched cage caused a reversal of this impairment in socially stressed rats. It is argued that this might be caused by the highly rewarding properties of the enriched cage that probably had a therapeutic efficacy of its own. However, it was not clear whether the announcement had an additional effect on the restoration of the appetitive response. It was argued before in this thesis that announcement, thus inducing anticipation, of a reward induces activation of the reward system and subsequent increased dopamine release (see also [348]). This is confirmed by several studies that showed that dopaminergic activity is induced by the expectation of a reward and not by the actual receipt [334][112][269]. Furthermore, dopaminergic neurons are known to be involved in the control of reward-related behaviour and incentive motivation [143][34][426][199]. Several lines of evidence exist for the role of dopamine in the mechanism of action of antidepressant treatments concerning their therapeutic effect on anhedonia and loss of motivation [433][95]. As mentioned above, dopamine has been related to the expectancy and predictability of rewards and is, thus, involved in the appetitive phase that precedes the consumption of reward. Thus, it is plausible that announcement of a reward has an additional therapeutic effect to the mere receipt of a reward on chronic-stress-induced reward-insensitivity via activation of dopaminergic systems. Therefore, the present study compared the consequences of announcement of a reward to the mere receipt of a reward with respect to their efficacy to counteract the effects of chronic stress. For this, reward-sensitivity was investigated in defeated and subsequently individually housed rats that received a reward (short-term enriched housing, EH) or to which this reward was clearly announced (A-EH) by means of a stimulus that was repeatedly paired with the reward. Furthermore, the therapeutic effects of (announced) environmental enrichment on the underlying mechanism in terms of brain plasticity were investigated by means of electrophysiological measurements in the hippocampus (in vitro).

The hippocampus is a particularly sensitive and vulnerable brain region that can be envisioned as controlling behaviour at a high level. The hippocampus is very sensitive to previous experiences [149][147] and is, amongst many other functions such as learning and memory, involved in the modulation of reward and incentive motivation [426][329][18][307][246b]. Furthermore, the hippocampus appears to play a pivotal role in novelty-detection and selecting what should be attended to and has been assigned a role as "supervisor" [160][134]. It is known that stress dramatically affects synaptic plasticity of the hippocampus [206][246a] and it is assumed that the sensitivity of this plasticity reflects the capacity to control behaviour [401]. This is in line with the impaired capacity to cope with

and adapt to environmental challenges due to a chronic challenge or failure of defense mechanisms that is reported in stressed animals [213][212][250]. Artificially induced sustained enhancement of synaptic strength (long-term potentiation: LTP) by high frequency stimulation of the hippocampus is impaired in chronically stressed rats [119][205][282] and can be restored by treatment with an antidepressant [402][336]. Furthermore, it is suggested that long-term physical exercise may protect the hippocampus from stress-induced damages [320][231]. This is in line with the knowledge that environmental enrichment facilitates LTP in rats [347]. Thus, to increase our knowledge about the underlying mechanism and to confirm the therapeutic effect of (anticipation on) environmental enrichment on the altered brain functioning in terms of reward-sensitivity after chronic stress, the hippocampal synaptic plasticity is investigated. To that end, defeated and subsequently individually housed rats were, after 3 months of individual housing, transferred to an enriched cage for several times during a short period. For another group an announcement of the transfer to this cage was included in the treatment. Then, the possible reversal of the impairment in reward-related behaviour was established by investigating the presence or absence of an anticipatory response for sucrose in these two experimental groups. To validate the results of the behavioural therapy, a socially stressed group received a pharmacological therapy by means of chronic antidepressant treatment. Also in this group the effect of the treatment on the impairment of reward-related behaviour is investigated. Finally, the long-term effects of the stress paradigm and different therapy-treatments on LTP induction in the CA1 region of the hippocampus was investigated (in vitro).

METHODS

The experiments have been performed in adherence to the legal requirements of The Netherlands concerning research on laboratory animals, and have been approved by the Ethical Committee of Utrecht University.

Subjects, housing, and general procedures

Forty male Wistar rats (HsdCpb:WU, Harlan, The Netherlands) weighing approximately 200 g at their arrival were initially socially housed (n=3 per cage) in Makrolon type IV cages (Tecniplast, Milan, Italy). They were kept under a reversed light/dark cycle (bright white light 20:00-08:00h; dim light (25W): 08-00-20:00h) in a temperature-controlled room (21 ± 2 °C) with background music. Water and food (Hope Farms™ standard rat chow) were available ad libitum. Cleaning of the cages and weighing of the animals was conducted once per week after the experimental tests to prevent influence of this disturbance on behavioural parameters. After two weeks of habituation to the housing conditions, procedures (such as transportation to other rooms) and regular handling, the experimental procedures were started. At this time the animals had a mean bodyweight of 320 ± 2.26 grams.

Experimental design and procedures (Table 1)

All experimental procedures were conducted during the dark phase. The animals were subjected to a social stress paradigm that consisted ofrepeated defeat during forced introduction in the territory of a dominant male rat. As a part of the social stress paradigm (see [403]) the experimental rats were individually housed (in Makrolon type III cages) immediately after the first defeat session.
After 82 days the animals were subjected to one of two behavioural therapy-treatments (EH or A-EH) or received no treatment (CON). The behavioural therapy consisted of short

periods (30 min) of enriched housing (EH) once per day during 10 days or included an announcement of the transfer to the enriched housing condition (A-EH). To validate the effects of the behavioural therapy, part of the CON-group was treated with a pharmacological therapy: administration of an antidepressant (Imipramine) (CON-IMI). Another part received the control treatment of oral injections with the vehicle solution (water)(CON-W). Thus, the final experimental design resulted in four experimental groups: (1). EH; (2). A-EH; (3). CON-IMI; (4). CON-W.
After a total of 121 days after the first defeat-session (thus, 28 days after termination of the behavioural therapy-treatment), an anticipation-on-sucrose test was conducted to assess whether the behavioural therapies (EH and A-EH) had been effective to reverse the earlier reported impairment in reward-related behaviour. Nine days after this test, sucrose preference was assessed by measuring the total amount consumed during 24 hours (see section Sucrose preference). Sixteen days after this consumption test, the pharmacological treatment with an antidepressant of half of the CON-group was started. After a chronic treatment (3 weeks) with the antidepressant an anticipation-on-sucrose test was conducted. This test aimed at assessing the effectiveness of the antidepressant on the alteration of reward-related behaviour by investigation of the anticipatory response for sucrose of the animals that had received the pharmacological therapy (CON-IMI) or the control treatment (CON-W).
After a total of 198-222 days after the first defeat-session synaptic plasticity in the hippocampus was determined to assess the effects of the different therapies at the level of brain functioning and to validate the results of the behavioural tests. For this, the animals were sacrificed and the brains were removed (see section Electrophysiology). The time schedule of the procedures, therapies and performed tests is presented in Table 1.

Social defeat procedure

The social defeat procedure consisted of daily resident-intruder sessions on 5 consecutive days. Each defeat session lasted for 20 minutes and was divided in a pre- (5 min), fight- (10 min), and a post (5 min)-phase. During the pre- and post-phase the experimental rat (intruder) was positioned behind a transparent, perforated barrier in the home-cage (63 x 25 x 33 cm) of a dominant male Long-Evans rat (LE/CpbHsd, Harlan, UK). These residents were housed with a sterilized female rat to stimulate territorial aggression; this female was removed from the home-cage before each defeat-session. The Long-Evans strain was selected for their strong physical condition, readiness to attack and fighting-tactics (inhibition of aggression when intruder displays submissive behaviour, thus minimizing the risk of injuries). The fight-phase was initiated and terminated by respectively removing or replacing the barrier. During the fight-phase the experimental rat was attacked and lost the fight in all cases.

Therapy

As mentioned before, the behavioural therapy consisted of short periods (30 min) of enriched housing (EH) once per day during 10 days or included an announcement of the transfer to the enriched housing condition (A-EH). For this, the home-cages of the animals were placed next to enriched cages. The animals were always transferred to the same enriched cage to prevent a novelty effect. During the transfer, the lids from the cages were lifted; after several sessions, the animals usually jumped over to the enriched cage by themselves. If not, they were gently guided to the enriched cage. The A-EH group was trained to associate a combined visual-auditory stimulus with the transfer to the enriched cage by means of

repeated pairings. The interval between the announcement and the actual transfer was gradually prolonged to 10 minutes, thus increasing the activation of the reward system. To the EH- and CON- group the stimulus was presented at random (non-paired) during the day to control for general arousal caused by the stimulus.
The pharmacological therapy consisted of daily (oral) injections with the antidepressant Imipramine (20mg/kg per 0,5 ml water; Sigma Aldrich, Germany). Because of its bitterness, the solution was administered directly into the stomach. This was accomplished by means of a long bended needle with a rounded top that was gently slid in the esophagus of the animals. After several sessions, most of the animals would swallow the needle automatically. The animals were treated with imipramine for three weeks before behavioural testing (anticipation on sucrose) started. Administration was proceeded during the behavioural investigation period and continued until the animals were sacrificed for electrophysiological measurements.

Table 1. Time schedule of the treatments and performed measurements presented by the number of days after the first day of individual housing (= after first defeat session)

Days after first defeat session

		Group
0-5	Defeat + individual housing after first session	
6-82	Individual housing	
83-93	Behavioural therapy: - Enriched cage, 30 min per day during 10 days - Announced transfer to enriched cage (30 min pd, 10 days)	**EH** **A-EH**
	Control: No therapy	**CON**
121-131	Behavioural test: Anticipation of sucrose Assessment of presence of appetitive response after long-term isolation with or without behavioural therapy	
140-143	Sucrose consumption test (two-bottle test (24hr): water vs 1% sucr / water vs 5% sucr)	
159-222	Pharmacological therapy: - CON + Imipramine	**CON-IMI**
	Control: - CON + water	**CON-W**
181-191	Behavioural test: Anticipation of sucrose Assessment of presence of appetitive response after long-term isolation with or without pharmacological therapy	
198-222	Electrophysiology	

Anticipation-of-sucrose

Anticipatory behaviour was induced by a Pavlovian conditioning set-up in which an initially neutral stimulus (conditioned stimulus;CS) was repeatedly paired with a sucrose-reward

(unconditioned stimulus;US). The delay between the offset of the CS and the onset of the US was progressively increased to 10 minutes over 35 trials (see [404]). To investigate the behavioural response to the CS the animals were observed before training to determine baseline activity and after 35 training trials. For this, behaviour displayed in the CS-US interval was recorded on videotape during trial 0 (baseline activity) and trial 35. For the observational sessions, the animals were transported to a separate room and placed individually in an observation cage (63 x 25 x 33 cm, l x w x h)(during the habituation period the animals had been subjected to these procedures to prevent novelty effects). Behaviour was observed and analysed from videotape using the computer program 'The Observer' (Noldus Information Technology, Wageningen, the Netherlands). Activity displayed in the CS-US interval (reflected by the frequency or transitions of behavioural elements) was used as a parameter for anticipation.

Sucrose preference

First, the normal water consumption during 24 hours was measured. Subsequently, preference for sucrose was measured by means of a two-bottle consumption test with water versus 1% sucrose solution or versus 5% sucrose solution. For this, half of the animals received a water bottle and a sucrose bottle with 1% sucrose solution whereas the other half received water and a 5% sucrose solution. The total amount consumed out of each bottle was assessed after 24 hours by reweighing the preweighed bottles. After 2 days, the consumption test was repeated: the animals that had received 1% sucrose solution during the first session, did now receive a 5% sucrose solution and a water bottle and vice versa.

Electrophysiology

A part of the animals (n=6 per experimental group) were sacrificed for electrophysiological measurements in the CA1 region of the hippocampus. These measurements started with the animals that had had at least 2 weeks of rest after the last test (sucrose consumption or anticipation-on-sucrose). The animals were decapitated after a short period of inhalation anesthesia with isoflurane. Subsequently, the brains were rapidly removed and placed in ice-cold medium. Thin slices of 450 µm were prepared as described by Kamal and colleagues [200]. These slices were incubated in artificial cerebrospinal fluid (ACSF) of the following composition in mM: NaCl 124, KCl 3.3, KH2PO4 1.2, MgSO4 1.3, CaCl2 2.5, NaHCO3 20 en Glucose 10.0. The slices were constantly supplied with oxygen rich medium. After 1-hour incubation time at room temperature the slices were transferred to the recording chamber and perfused with ACSF at a rate of 2ml/min at 30ºC.
Activity in the dendrite layer in the stratum radiatum was measured by glass micro-electrodes with a tip diameter of 3-5 µm and a 0.5 MΩ resistance filled with ACSF. Bipolar stainless steel electrodes of 100 µm placed on Schaffer collateral fibers were used as stimulation electrodes. The stimulus intensity that evoked a half-maximum amplitude of the field excitatory post-synaptic potentials (fEPSP) was used. Only slices that displayed maximal fEPSP responses of more than 1 mV were included in the study. As soon as a stable baseline was found, recording of this baseline response was conducted for 15 minutes with test stimuli given at a rate of 0.05 Hz. The average slope of the baseline responses were set to 100% and the slopes during the experiments are expressed as percentages of the baseline slope. High frequency stimulations (HFS) composed of 100 pulses per second (100Hz) were used to induce long-term potentiation (LTP).

Statistical analysis

Data were expressed as group means with standard error of the mean (SEM). The Statistical Package for Social Sciences (SPPS, version 9.0) was used for statististical analysis. Group sizes varied due to the unexpected death of 1 animal and euthanisation of 2 other animals that appeared to be seriously ill. Furthermore, for electrophysiological measurements only 6 animals of each group were used and in 2 cases the maximal fEPSP response of the slice did not exceed 1 mV and were therefore excluded.

Bodyweight

Difference between groups in bodyweight at every separate week during the experiment was analysed by means of independent samples t-tests. A two-way ANOVA for repeated measures was conducted to analyse differences in weight gain over the course of the experiment (within-subjects factor: time; between-subjects factors: defeat and therapy).

Anticipation-of-sucrose

The total frequency of all displayed behavioural elements, reflecting behavioural transitions and thus activity, was calculated as a measure of reward sensitivity. The data were expressed as mean frequency per minute. Significant increases in activity in response to the CS after repeated pairings of the CS with the sucrose solution were analysed by comparing the activity before training (baseline) with the activity after 35 training trials. This was done by means of paired samples t-tests.

Sucrose preference

The intake of water and sucrose-solutions was determined by reweighing the preweighed bottles after 24 hours. Sucrose preference was determined by within-group comparison of the consumed amount of water and the two concentrations of sucrose solutions by means of paired samples t-tests. Possible differences between groups were analysed by means of independent samples t-tests.

Electrophysiology

Between-group differences in fEPSP-slopes at approximately 1 hour after HFS were analysed by means of the non-parametrical Mann-Whitney-U test. For this, mean values (± SEM) of the recordings of 55-60 minutes (10 measurements) after HFS were calculated. Within-group changes in synaptic activity were analysed by comparing the absolute baseline responses with those 1 hour after the application of HFS. This was done by means of Wilcoxon-signed rank tests.

RESULTS

Bodyweight

The weight curve of the mean bodyweight per week shows that the bodyweight gain increases to a lesser extent during and after the defeat (data not shown). Analysis of the effects of the different treatments and experimental procedures reveals that no group differences are present during the defeat ($F(2,34)=0.175$, $p=0.841$), therapy (first week: $F(2,34)=0.668$, $p=0.519$; second week: $F(2,34)=0.588$, $p=0.561$), anticipation test (first week: $F(2,34)=0.066$, $p=0.937$; second week: $F(2,34)=0.588$, $p=0.561$), and sucrose consumption test ($F(2,34)=0.067$, p-0.936). An ANOVA for repeated measures that was used to analyse

the effect of the behavioural therapy over time (from the week before until the week after the therapy) reveals that no therapy-effect on bodyweight was present ($F(1.55, 114)=2.012$, $p=0.152$). Concerning the pharmacological therapy, an effect of imipramine-treatment on the bodyweight over time (week before treatment until decapitation) was detected: Imipramine caused a significant decrease in bodyweight over time ($F(1.9, 216)=32.6$, $p<0.001$).

Anticipation on sucrose (effects of behavioural therapy)(figure 1)

Analysis of the difference in activity in response to the CS between pre- and post-training revealed that both therapy-groups (EH and A-EH) showed a significant increase in activity (Related samples t-test (pre- vs. post-training): EH $t=-3.233$, $df=9$, $p=0.01$; A-EH $t=-4.329$, $df=9$, $p=0.002$) whereas the control group (CON) did not ($t=-1.152$, $df=9$, $p=0.279$). The mean consumption during the 5-min access to the 5%-sucrose solution during the trials was not affected by the different treatments ($p>0.5$ for all comparisons).

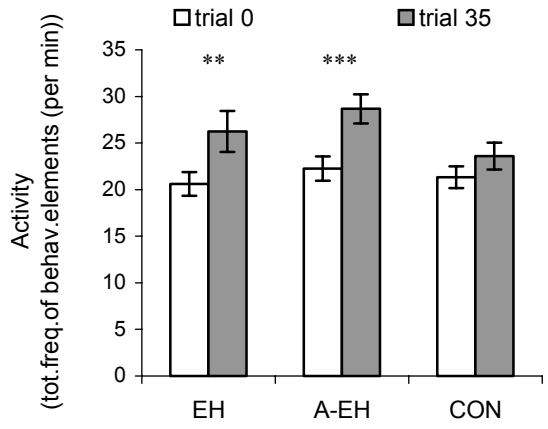

Figure 1.
Anticipation of sucrose (5%, 5 min): Assessment of the presence of an appetitive response in chronically stressed rats that were subjected to a behavioural therapy (enrichment (EH, n=10); announced enrichment (A-EH, n=10)) or to the control treatment (CON, n=10). Presented as the activity in the CS-US interval (represented by the total frequency of all behavioural elements) (± SEM) during trial 0 (pre-training; basal level of activity) and trial 35 (post-training). Significant differences in activity between pre- and post-training are indicated with an asterisk (**: $p\leq0.01$; ***:$p\leq0.001$).

Sucrose preference (Table 2)

The 24-hour consumption test indicated that both the therapy- and non-therapy groups showed a clear preference for the sucrose bottle over the water bottle. This was true for both the 1%- (Independent samples t-test; water vs 1%: CON $t=-4.917$, $df=8$, $p=0.001$; EH $t=-9.057$, $df=9$, $p<0.001$; A-EH $t=-6.907$, $df=9$, $p<0.001$) and the 5%-solution (water vs 5%: CON $t=-6.242$, $df=8$, $p<0.001$; EH $t=-7.615$, $df=9$, $p<0.001$; A-EH $t=-6.117$, $df=9$, $p<0.001$). Furthermore, it appeared that in all groups the amount of consumed sucrose was equal in the 1%-consumption test and the 5%-consumption test (paired samples t-test; 1% vs. 5%: $p>0.2$ for all groups). Also, the total consumed amount of both 1% en 5% sucrose exceeded the normal water consumption during 24 hours that was determined by a single-bottle test (Paired samples t-test; $t<-3.0$, $p<0.01$ for all groups). No differences between either of the groups existed for the consumed amount of 1%-sucrose, 5%-sucrose or water ($p>0.1$ in all cases).

Table 2. Mean values (± SEM) of the consumed amount of sucrose or water during a 24-hr test with two bottles (water and sucrose (1% or 5%).

24 h	EH	A-EH	CON
Single bottle			
water	17.10 ± 1.52	18.2 ± 2.87	19.0 ± 4.0
Two bottles			
water	0.7 ± 0.26	1.0 ± 0.21	0.89 ± 0.26
1% sucrose	53.1 ± 5.85	43.8 ± 6.26	44.56 ± 9.07
Two bottles			
water	1.3 ± 0.26	0.6 ± 0.27	0.78 ± 0.22
5% sucrose	66.8 ± 8.65	53.8 ± 8.63	60.44 ± 9.49

Anticipation on sucrose (effects of pharmacological therapy)(figure 2)

It became apparent that in both the water-treated and the imipramine-treated group no significant increase in activity in response to the CS is present after training (related samples t-test (pre- vs post-training): W t=-0.949, df=5, p=0.386; IMI t=-0.228, df=11, p=0.824). Thus, in the present study, the imipramine-treatment has not been successful in restoring the display of appetitive behaviour in socially stressed rats as was reported before. Furthermore, imipramine appeared to have an adverse effect on the activity: IMI-animals were less active than W-animals during both pre- and post-training (independent samples t-test (W vs. IMI): PRE t=3.819, df=16, p=0.002; POST t=2.759, df=16, p=0.014). Also, the animals that were treated with imipramine consumed significantly less sucrose during the 5-min-access during the training trials (mean: 7.65±0.44) than did the water-treated animals (mean:10.82±1.06; t=-3.30; p=0.005).

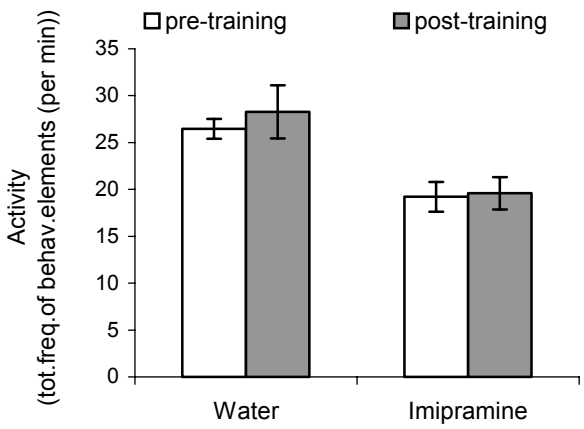

Figure 2.
Anticipation of sucrose (5%, 5 min): Assessment of the presence of an appetitive response in chronically stressed rats that were subjected to a pharmacological therapy (antidepressant imipramine (IMI, n=10)) or to the control treatment (CON, n=10). Presented as the activity in the CS-US interval (represented by the total frequency of all behavioural elements) (± SEM) during trial 0 (pre-training; basal level of activity) and trial 35 (after training). No significant differences between pre- and post-training were present.

Electrophysiology (figure 3)

Changes of LTP induction at 60 min after high frequency stimulation (HFS, ↑ in fig.3) were dependent on whether the animals had been subjected to a therapy-treatment or not. HFS (100 Hz, 1s) resulted in a significant potentiation of the fEPSPs of animals that were subjected to either one of the behavioural therapies (Wilcoxon related samples; EH: n=6, z=-2.2, p=0.028; A-EH: n=5, z=-2.203, p=0.043) whereas LTP induction was absent in the non-therapy group (CON-W). This group appeared to show a significant depression (CON-W: n=6, z=-1.992, p=0.046). The %fEPSP (t=75) in the slices of the animals that received the enrichment-therapy (EH: 116±2.65%) was significantly higher than the %fEPSP in slices of the non-therapy animals (CON-W: 77.27±6.19%) (Mann-Whitney-U: U=1, p=0.006). This was also true for the animals that received announced transfers to the enriched cage (A-EH: 203.27±23.03%, U=0, p=0.006). Furthermore, it became apparent that the %fEPSP of A-EH was significantly higher than that of EH (U=4, p=0.045) which indicates that the announcement (thus inducing anticipation) had an additional effect. Concerning the pharmacological therapy, the %fEPSP of the imipramine-treated group (138.11±10.29%) was significantly higher than that of the water-treated group (77.27±6.19%) although the within-group comparison for IMI with the absolute baseline value did not reveal a significant difference (Wilcoxon: n=5, z=-1.483, p=0.138).

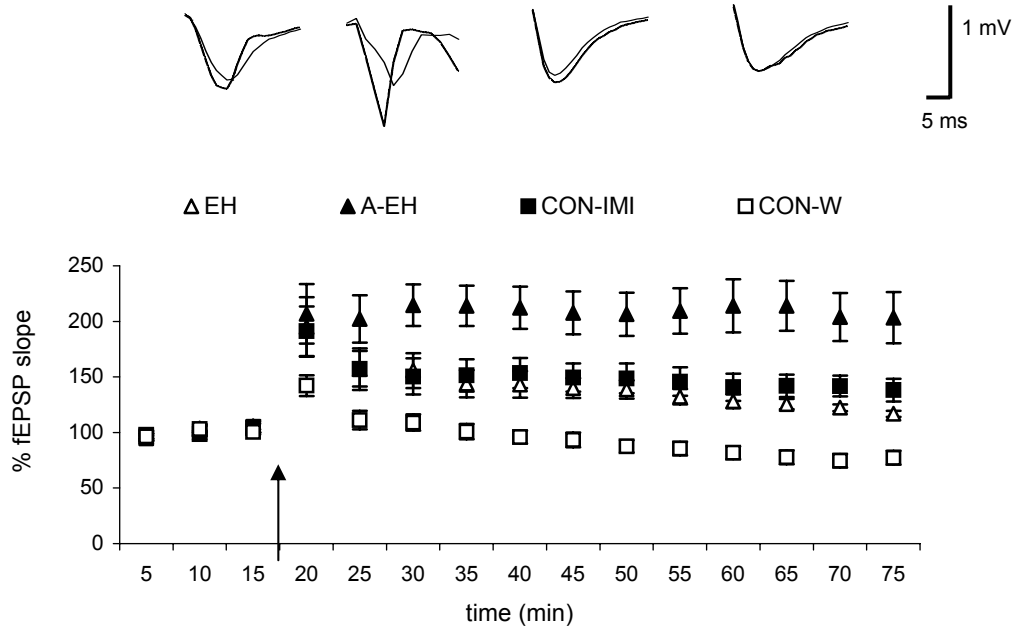

Figure 3. Expression of LTP induced by high frequency stimulation (HFS: ↑; 100 Hz, 1s) in hippocampal slices from socially stressed rats that were subjected to a behavioural therapy (enrichment (EH, n=6) or announced enrichment (A-EH, n=5)), a pharmacological therapy (imipramine (CON-IMI, n=5)) or to the control treatment (CON-W, n=6)). Presented as mean values (±SEM) of the relative slope of fEPSPs measured before and after HFS (↑). *Inset:* examples of the average (15 sweeps) of fEPSPs evoked in slices from the differentially treated socially stressed rats before (solid line) and 60 min after (dashed line) HFS.

DISCUSSION

It became apparent that both short-term environmental enrichment (EH) and the inclusion of an announcement (A-EH) caused a restoration of the display of anticipatory behaviour for sucrose in chronically stressed rats. Thus, environmental enrichment (with or without announcement) can reverse the long-term effect of chronic stress on the depressive-like impairment of reward-related behaviour. Importantly, this effect seems to be long lasting since it was present more than 3 weeks after termination of the therapy. It is known that physical activity in previously stressed rats restores hippocampal brain-derived neurotropic factor (BDNF) mRNA levels to baseline [319]. These results are related to the evidence that has been gathered in recent years that BDNF expression could be an important agent for therapeutic recovery from depression [268][341]. The beneficial effects of physical activity on depressive disorders is extensively investigated in clinical studies [148][236][413] and the therapeutic efficacy of environmental enrichment is therefore plausible. It is known that levels of endogenous opioids (endorphins) increase in response to physical activity [54][227][371]. Furthermore, it is argued that processing of reward is mediated by both the mesolimbic dopamine system and the opioid system [127][27][348], which is caused by the interaction between these systems [86][292]. In line with the fact that endorphins are candidates for antidepressant treatment [47][99][135] it is possible that enriched-environment-induced opioid activity counteracts the effects of chronic stress on reward-sensitivity via activation of the endorphinergic system. As argued in the introduction, the efficacy of the announcement is likely to be mediated by activating dopaminergic systems that are also known to be involved in antidepressant treatments. However, since both treatments (EH and A-EH) caused a restoration of the display of anticipatory behaviour, the behavioural data alone cannot decisively determine whether announcement has an additional therapeutical effect on the consequences of chronic stress.

The results of the electrophysiological measurements, however, indicate that the inclusion of an announcement did have an additional effect on the enrichment-induced restoration of the synaptic plasticity of the hippocampus. Although both behavioural therapies could restore the LTP-deficit in the hippocampus of socially stressed rats the degree of potentiation was significantly higher in A-EH as compared to EH. The effects of both therapies are long-lasting since the electrophysiological measurements were conducted more than 100 days after termination of the behavioural therapy. Furthermore, when the amount of LTP in the socially stressed animals that were subjected to the different treatments is compared to non-defeated socially housed control rats of a previous study by Von Frijtag and colleagues [402] it appears that the potentiation of the hippocampal synapses of EH is not completely restored to a ‘normal’ level (approximately 200%). This is, as reported before by Von Frijtag et al. [402], also the case for imipramine-treated animals. A-EH, on the other hand, did reverse the chronic-stress induced long-term changes of hippocampal synaptic plasticity completely. The additional effect of the anticipatory phase before the actual transfer to the enriched cage might be explained by the fact that ‘expectation’ of a reward triggers dopamine release [331][269]. Since dopaminergic activity (among other things) is altered in depressive disorders it is likely that the therapeutic efficacy of anticipation counteracts the effects of chronic stress via activation of dopaminergic systems. This is in line with the knowledge that dopamine appears to be involved in the modulation of hippocampal synaptic plasticity [201][272][228][329]. It must be noted, however, that anticipation in combination with a sucrose reward has not been successful in reversing the impairment in either the display of reward-related behaviour or hippocampal synaptic plasticity. Namely, the control-group

(CON-W) was subjected twice to an anticipation-on-sucrose test (see Table 1), but still did not show anticipatory behaviour during the second test. This is in line with the results of a previous study [404] in which a previous anticipation-on-sucrose test did not affect the impairment of reward-related behaviour in a second test. Furthermore, in the present study, the impaired hippocampal plasticity of CON-W was not reversed after exposure to two anticipation-on-sucrose tests. Thus, it is likely that the therapeutic efficacy of anticipation on environmental enrichment is caused via a concerted action of opioid and dopaminergic activity. This is in line with the fact that environmental enrichment alone did not cause a complete reversal of LTP to a 'normal' level (i.e. 200%).

Another way to approach the issue of the difference between EH and A-EH is via the influence of stress on the adaptive capacity of animals. Namely, it is possible that the predictability via announcement of the transfer counteracts possible adverse effects of the uncontrollability of the situation for the chronically stressed animals. It is known that stressed animals have difficulty to cope with environmental changes [52][213][212]. Therefore, the unexpected transfers to an enriched cage for the EH-animals might have had some adverse effects. This is confirmed by the fact that the EH-animals needed to be guided to the enriched cage for more sessions than was the case for the A-EH group and by the fact that especially during the first sessions the EH-animals were relatively inactive in the enriched cage (personal observation). After several sessions the animals of the EH-group would jump over to the enriched cage themselves and were more active. Thus, it might be that the therapeutic effect of the environmental enrichment was initiated only after several sessions in EH-animals. By announcing the transfer, the A-EH group had more control over the situation and was able to 'prepare' for the oncoming change. This is in line with a review of Plaut and Friedman [297] who reported that one of the factors shown to influence the ability of an animal to cope included the availability of relevant 'warning' signals.

The present results suggest that measuring synaptic plasticity in the hippocampus is a more sensitive measure than the behavioural parameter. Namely, no difference between EH and A-EH is detected concerning anticipatory behaviour for sucrose (both therapies caused a restoration of the display of anticipatory activity at an equal level) whereas the LTP-data indicate that A-EH had an additional effect on the chronically stressed animals. However, behaviour is 'produced' by a concerted action of various interacting mechanisms in the brain and it might be that the difference in hippocampal plasticity as seen between EH and A-EH is not large enough to cause a difference in anticipatory behaviour for which other brain systems are involved as well. This does not hold true for the fact that electrophysiological measurements reveal a significant imipramine-effect whereas the anticipation-for-sucrose test fails to detect an effect. However, some explanations can be given that are related to the applied protocol and, thus, do not concern differences in sensitivity of the parameters. These practical issues will be discussed below.

To elaborate further on the sensitivity of parameters, the behavioural parameter investigated during the appetitive phase (i.e. anticipatory behaviour) appeared to be a more sensitive measure for alterations in reward processing than the consummatory responses. Namely, the amount of sucrose that was consumed during the training trials was not significantly different for the non-therapy group whereas this group showed a clear impairment in the display of appetitive behaviour. Furthermore, the 24-hour sucrose consumption measurements indicated that the non-therapy group shows a clear preference for the sucrose bottle over the water bottle for both the 1%- and 5%-solution. Importantly, this preference for sucrose appeared to be concentration-independent since the total consumed amount is similar for the 1%- and

5%-solution. The absence of long-term effects of social stress on consummatory responses to a sucrose-reward is reported before [404]. In line with this, Murison and Hansen [263] have expressed their concern about the inconsistencies in the literature concerning the robustness of the phenomenon anhedonia as measured by the consumption of sucrose (see also [242][291]). Furthermore, it is also reported that the effects of a chronic mild stress procedure on 1% sucrose consumption faded after termination of the stress-regime [264]. Hence, it seems that the stress-induced alteration of consummatory responses, as reported in numerous studies [421][422][275] are not robust and long lasting independently of the concentration of the solution that is used.
As mentioned before, the effect of imipramine was not detected by means of a restoration of anticipatory behaviour for sucrose in contrast to the results reported by Von Frijtag et al. [404]. Several explanations can be given that relate to differences of the applied protocol. Namely, the imipramine injections were conducted by three different persons which might have had adverse effects on the animals. Furthermore, the animals were moved to another building during the course of the experiment. Although the animals were allowed to adjust to the new surroundings for at least 2 weeks the moving might have had a greater impact than expected. In addition, the animals were subjected to the conditioning training by a different person than the familiar experimenter. It is known that chronic stress influences the ability of coping and adaptation [213][212]. A failure to cope with the stressfulness of the moving procedure, subsequent stressfulness of the injections and habituation to an unfamiliar experimenter might have caused a general behavioural inhibition.

The results of the anticipation-on-sucrose test revealed that imipramine caused a general decrease in activity. One could argue that this is related to the decreased consumption of sucrose during the training trials as compared to the water-treated animals. This would imply that an increase in consumption of sucrose causes an increase in activity independent of anticipation. However, we have shown in a previous study that the consumption of sucrose alone (thus, without pairing to a stimulus) did not cause a significant increase in activity [383](chapter 4). Thus, the decreased activity might be a side-effect of imipramine although a previous study indicated that imipramine had no significant effect on the total distance moved in an open field test [401]. One could also argue that the inferior consumption of the IMI-animals could be an indication of the reason why these animals did not show an anticipatory response. If the animals did not ‘like’ the offered sucrose-solution, it is logical that they would not show reward-related behaviour. However, an inferior consumption does not automatically imply that the animals ‘dislike’ the solution. In a previous study [404] it was also shown that imipramine had an adverse effect on sucrose-consumption during training, but did restore the display of anticipatory behaviour in socially stressed rats.
The fact that the IMI-group consumed significantly less sucrose during training as compared to the water-treated group is obviously not caused by the stressfulness of the injection-method (since the W-group was subjected to the same procedure). As mentioned before, the imipramine solution is quite bitter. Although the solution is administered directly into the stomach, it might be that drops that remained on the tip of the needle irritated the mucous membranes in the mouth and subsequently influenced taste perception in the IMI-animals.

In conclusion, announced short-term environmental enrichment has proven to have a high therapeutic efficacy on stress-induced alterations of both reward-related behaviour and hippocampal synaptic plasticity. Importantly, these are long lasting effects since they are still

present more than 28 and 100 days, respectively, after termination of the behavioural therapy. Since this behavioural therapy has such strong therapeutic characteristics that it can reverse chronic-stress induced depressive-like symptoms in animals it is obvious that it should also be applicable to counteract the effects of other (less severe) forms of stress. Therefore, this information can be applied to find ways to counteract the consequences of stress in both man and captive animals thereby improving welfare in general.

CHAPTER 9

GENERAL DISCUSSION

1. INTRODUCTION

This chapter will start with a recapitulation of the aim and approach of this thesis and will be followed by a short summary of the results. Subsequently, the results of the studies described in Chapter 2-8 will be discussed in relation to the assessment and improvement of animal welfare and the implications for scientific research. Also attention will be paid to the (dis) advantages of the methodology, which will be followed by some general considerations on several different topics. Finally, some future directions will be given and this chapter will be closed with a summary of the main conclusions.

1.1. RECAPITULATION OF THE AIM AND APPROACH

This thesis describes a study of the validation of tools that can be used to assess and improve welfare of laboratory rats. Welfare was conceptualized as the transient balance between positive and negative experiences and thus it was argued that welfare-assessment should focus on the outcome of the integration of these experiences. Because the biological background of the proposed concept can probably be generalized to other vertebrate species, the results are considered to be useful for other captive animals as well. Following the hypothesis brought forward by Spruijt et al. [348], the reward-system was forwarded as a general evaluation-system and reward-sensitivity was forwarded as a welfare-indicator because it is known that the state/sensitivity of the reward-system is dependent on the life history of an animal. Furthermore, by challenging an animal via the presentation of a reward, an animal internally evaluates its own state and the consequent need for reward, which is a useful way to 'read the mind' of the animal in terms of welfare.
Reward-related behaviour in anticipation of a reward was argued to be important for both welfare assessment and improvement according to the following 3 hypothetical utilities of this behavioural response that were investigated:
(I) Because previous experiences affect reward-sensitivity, and the fact that these previous experiences determine the state of an animal in terms of welfare, it was hypothesised that anticipatory behaviour would be a possible tool to assess welfare.
(II) Because the rewarding properties of an expected stimulus affect reward-related behaviour it was hypothesised that anticipatory behaviour could be indicative of the appraisal of forthcoming stimuli and events.
(III) Because welfare can be conceptualized as the balance between positive and negative experiences, and positive experiences should therefore be able to counteract the negative experiences, it was hypothesised that regular activation of the reward-system via (announcement of) the presentation of rewards could be used as a tool to counteract stress, and thus, to improve welfare.

In addition to the validation of the hypothesized utilities of anticipatory behaviour, special attention was paid to environmental enrichment since it is obvious that the first step in improving welfare of captive animals is improving their, currently very poor, housing conditions.

1.2. Summary of the results

In *Chapter 2* it became apparent that anticipation of a positive stimulus can be generally quantified by an increased level of activity as measured by the total frequency of behavioural

elements displayed in the time-interval between announcement and presentation of a reward in a Pavlovian conditioning paradigm. Additionally, some behavioural elements such as exploration, locomotion, arousal and grooming seemed to be more specifically related to the nature of the forthcoming stimulus. The strong increase in activity in anticipation of both an enriched cage and sexual contact and the similar response concerning the analysed behavioural elements indicated that the appraisal of access to an enriched cage shares a common denominator with the appraisal of sexual contact which is generally accepted to have highly rewarding properties to rats.

Chapter 3 revealed that the relatively simple enriched housing system, developed at our laboratory, was effective in positively influencing rats in their behaviour in the home cage: enriched housed rats showed an increase in exploration, mobility and general activity, and importantly, a significantly lower level of aggression as compared to standard housed rats. Furthermore, enriched males moved more freely on the Elevated Plus Maze and spent more time on the open areas of the platform indicating a declined behavioural expression associated with anxiety.

In *Chapter 4,* it was shown that standard housed rats were more sensitive to a sucrose-reward (indicating an increased need for rewards) than enriched housed rats as reflected by their higher anticipatory activity after announcement of this reward in a Pavlovian conditioning paradigm. This result was replicated under a different test condition in *Chapter 5*: fully automated pairings of a stimulus (light and sound) and the delivery of a reward in a so-called Skinnerbox caused a stronger (although subtle) increase in anticipatory activity in standard housed rats as compared to enriched housed rats. During the instrumental-conditioning part of this experiment, breakpoint determination showed that standard housed rats are likely to perform more lever-presses for a sucrose pellet than enriched housed rats, also indicating an increased reward-sensitivity in standard housed rats. Anticipatory activity, however, seemed to be a more sensitive measure for detecting experience-induced differences in reward-sensitivity. This may be caused by the fact that this is a spontaneous anticipatory act whereas the instrumental response is a required anticipatory act. The stimulus-induced anticipatory activity was not correlated with the instrumental parameters (number of collected rewards, magazine visits and lever presses), suggesting that the parameters of Pavlovian and instrumental conditioning are not as closely related as was expected.

In *Chapter 6* it was investigated whether the stimulus-induced increased anticipatory activity in a Pavlovian conditioning paradigm with an interval between the stimulus and the reward, is related to the number of performed lever presses evoked by a stimulus that was previously paired with the delivery of a reward (Pavlovian-to-instrumental-transfer). No correlation was detected between the stimulus-induced instrumental response (lever presses) in the Pavlovian-to-instrumental-transfer experiment and the stimulus-induced behavioural response (anticipatory activity) in the Pavlovian conditioning experiment, indicating that these two parameters are not closely related. However, the stimulus-induced number of magazine visits in both conditioning procedures were correlated. It might be that the lack of correlation between leverpresses and anticipatory activity is caused by the fact that they are two different entities; lever pressing is just one element of a range of different behaviours that the animal can display whereas the anticipatory activity consists of a full repertoire of behavioural elements.

Chapter 7 revealed that regular announcements of a reward during a period of chronic stress could prevent the development of anhedonia, a symptom of depression. That is, repeated pairing of a stimulus and a sucrose-solution during a long-term period of individual housing

after repeated defeat prevented the previously reported impairment in the expression of appetitive behaviour in anticipation of a reward. Furthermore, it was shown that this impairment in the expression of anticipatory behaviour could not be generalised to all rewards, since chronically stressed rats that did not receive the repeated sucrose-announcements did show anticipatory behaviour for another type of reward, an enriched cage. It was argued that this might have been caused by the high rewarding properties of the enriched cage that may have had a therapeutic value of its own. This was investigated in *Chapter 8* in which it was shown that an enriched cage had a high therapeutic value in the sense that it could reverse the stress-induced alterations of both reward-related behaviour and hippocampal synaptic plasticity of chronically stressed rats. That is, defeated and subsequently individually housed rats that received regular (announcements of) short-term access to an enriched cage were able to display anticipatory behaviour for a sucrose reward and had a restored level of synaptic plasticity in the hippocampus. Importantly, it appeared that the announcements of the access to an enriched cage had an additional effect in the sense that it caused the impaired LTP to restore to an even higher level than did the mere access to an enriched cage.

2. REWARD AND ANIMAL WELFARE

2.1. Animal welfare, reward and emotion

Animal welfare research is strongly related to the attribution of mental states to animals [108]. The study of emotion is related to the current need to 'read the minds' of animals to be able to assess and improve their welfare. In humans, verbal language aids to assess emotional experiences but in animals only behavioural and physiological parameters and their interpretation can aid to detect emotions. The existence of emotions in animals remains a controversial issue.

Darwin's publication 'The expression of emotion in man and animals' (1872) can probably be regarded as the corner stone of modern emotion research (see [202]). Although an extensive amount of studies is conducted that investigate the process of emotion, it appears to be a rather difficult issue to capture because of its subjective characteristics. In behavioural neuroscience the study of emotion is mostly addressed by means of defining emotion by the response of a subject to an emotionally arousing situation. In this way, the emotional reaction is measured by behavioural, neurochemical and neuroendocrine parameters without requiring any preconceived theory about what emotions really are. This may be favorable for scientific research but, as questioned by for instance Dantzer [102], is it possible to study emotion without knowing about emotions? Several other authors [202][24] have expressed their concerns about the reduction of a causal explanation to one process or discipline. These authors argue that a multidisciplinary approach that acknowledges emotion as involving multiple levels of control and complex interactions should be applied. The different disciplines and consequent approaches of the study of emotion is probably the cause of the lack of consensus in the literature on a definition of emotion. Cabanac [62] refers to a survey of Kleinginna & Kleinginna [208] that listed 92 different definitions of emotions and explains his own definition: 'emotion is any mental experience with high intensity and high hedonic content (pleasure/displeasure)'.

As argued in Chapter 1, pleasure is the common currency of the brain that underlies the economy of behaviour and the maintenance of the balance between positive and negative experiences. From an adaptation perspective the ability to perceive its own emotions enables

an individual to detect and assess a discrepancy between its requirements and environmental conditions (actual and expected state) and subsequent take action to regain homeostasis. Since animals appear to have high adaptive capacities in the sense of maintaining homeostasis, it would be logical that (at least part) of this capacity is derived from the ability to 'experience' emotions. This is in line with an extensive amount of research results that point to mutual neural circuits underlying experience and expression of emotions in both man and animal (see [117]).
Do the results of the present study contribute to the understanding of the presence of emotions in animals? It was shown that rats were able to anticipate future events and that the behavioural response in anticipation of a reward was influenced by previous experiences as well as the rewarding property of the presented stimulus. Rolls [312] claims that animals which have the ability to anticipate or learn to obtain reinforcers, have emotions. Furthermore, the consequences of previous experiences in terms of chronic stress in rats are analogous to human depressive disorders which may indicate that animals experience emotional states that can have long term consequences.
In relation to the expression of anticipatory behaviour in expectation of a reward one may wonder whether animals can perceive the subjective feelings associated with reward. It is known that the internal state of an animal can be used as a discriminative stimulus in the sense that an animal can be trained to recognize its own emotional state such as euphoria and anxiety when it is under the influence of a particular pharmacon and to perform a certain behaviour (such as food-rewarded lever pressing) to indicate what the internal state is [79][80]. This technique is often used for detecting possible undesirable addictive properties of various psychopharmacological drugs. Early studies of opiates have provided evidence that drug-produced discriminative effects are homologous to the characteristic positive subjective feeling that these agents produce in humans. Thus, while at some point in time it was felt that subjective experiences were uniquely human and inaccessible, drug discrimination studies yielded evidence for the presence of these subjective experiences in animals as well. It has been explained in Chapter 1 that two features are relevant for an animal's behaviour: (i) knowledge of when and where a commodity is available (expectation/anticipation) and (ii) assessment of the rewarding value of a commodity (characteristics of the commodity or the reduction of the difference between the actual and the preferred state). The first feature is argued by Van den Bos et al. [380] to be related to cognition and the second to be related to emotion. Thus, the results of the present study are indicative for the presence of both cognition and emotion in rats (and probably also in other species) which is, according to Duncan & Petherick [132], an important issue in the concept of animal welfare.

2.2. Anticipatory behaviour: characteristics & utility for animal welfare and scientific research.

2.2.1. General characteristics of anticipatory behaviour

From Chapter 2 it became apparent that reward-related behaviour in expectation of a reward is generally characterized in rats by an anticipatory increase in activity appearing in the appetitive phase (i.e. before actual receipt of the reward which is referred to as the consummatory phase). The increase in several specific behavioural elements appeared to be dependent on the characteristics of the reward and are therefore less useful as a general parameter for anticipation. The anticipatory response for a forthcoming reward is argued by Berridge [27] to reflect 'wanting' and is related to the 'need' for (i.e. sensitivity to) the

reward. However, in case of an aversive forthcoming event the anticipatory response was expected to be different and not related to 'wanting' but rather to be related to 'fear' or 'avoidance' (for instance exemplified by freezing behaviour as is seen in conditioning experiments using footshocks). Thus, to be able to characterise 'positive' anticipation (i.e. anticipation for a reward) and use it as a measure for reward-sensitivity it was important that a distinction would be present between 'positive' and 'negative' anticipation (i.e. anticipation for a 'punishment') in the applied experimental set-up (Pavlovian conditioning). Although the expected freezing behaviour was not seen in the experiments of Chapter 2, negative anticipation was clearly different from positive anticipation concerning the activity after announcement of the negative and positive stimulus. Positive anticipation could, therefore, be further used as a parameter for reward-sensitivity in the following experiments.

Many animals display an increased activity during the appetitive and anticipatory phase of behaviour (see for instance [210][190][183][308]). In Chapter 4 and 5 it was shown that the anticipatory increase in activity was present under different conditions: when the animals were trained the home-cage, in an observation-cage or in an operant chamber. This indicates that, at least for rats, the 'spontaneous' behavioural response (i.e. increase in activity) in anticipation of a reward is robust. This is supported by the fact that this spontaneous behavioural response has often been observed unintentionally in the currently used housing systems of several species. For instance, it is known that cows that are fed and milked at a certain time-schedule show arousal at a certain time or when certain stimuli associated with those events are present (e.g. footsteps of the farmer). This indicates that investigating the anticipatory response might be very easily employed under 'normal' conditions and would, therefore, be a very useful tool to assess welfare of many different species under many different conditions.
The anticipatory response may not be expressed in the same way in all species. Indeed, we have recently observed that anticipation is differently expressed in cats as compared to rats [381]. Whereas rats became hyperactive, cats displayed a decrease in activity. This difference in behavioural profile (hyperactivity versus hypoactivity) may be explained by the difference in 'natural' food-related appetitive behavioural response between different species [240][360]. Cats normally employ a 'sit-and-wait' strategy while close to their prey [369] whereas rats employ active exploratory behaviour as a part of their natural behavioural feeding repertoire. Thus, it is important to establish the characteristics of the anticipatory response of different species before it can be widely applied as a welfare-indicator for different species.

2.2.2. Utilities of anticipatory behaviour for animal welfare research

Concerning the assessment of welfare, Chapter 4 and 5 have shown that a difference in reward-sensitivity was detected between standard and enriched housed rats when the anticipatory response of these animals was investigated. Thus, previous experiences in terms of housing conditions appear to have an effect on anticipatory behaviour. Furthermore, in Chapter 7 and 8 it was confirmed that chronic stressful experiences cause an impaired anticipatory response reflecting anhedonia (reward-insensitivity). Importantly, anticipatory behaviour appeared to be a more sensitive measure than sucrose-consumption (which is widely used as a measure of anhedonia) and considering the fact that it was shown to be present under different conditions it is a very robust measure. Overall, this parameter may be a useful and robust indicator of the state of the animal in terms of welfare.

Although the anticipatory response could distinguish between the reward-sensitivity of standard and enriched housed rats and chronically stressed rats, it is still uncertain how sensitive this parameter is for assessing gradual changes in previous experiences and consequential reward-sensitivity. A hypothetical relationship between anticipatory activity and the balance between positive and negative experiences – that influences reward-sensitivity and is proposed as a concept of welfare – is depicted in figure 1. The slope of the curve may be very steep (black solid line) or more gradual (black dotted line) and there may be a "cut-off" (grey dotted line) from the point of a certain negative balance or it may decrease gradually. The curves or the cut-off may also be shifted to the right. The detailed shape of such a curve should be further investigated for laboratory rats and for other captive species and may then be a useful tool to assess welfare of a range of different species. When the curve gradually decreases in case of a negative balance and has a similar shape as on the positive side of the balance additional parameters should be investigated to be able to determine on which side of the curve/balance the animal is. Probably "simple" home-cage observations may be sufficient for this since a lot of information can be deduced from the behavioural patterns and responses of animals. Interestingly, since anticipatory behaviour can be investigated in the home-cage as well (Chapter 2 and 4), it may be very useful to develop an automated observation system that can be used to observe the animals in their home-cage in detail. Enriching the home cage with various stimuli that induce natural behavioural repertoires and presenting some challenges such as reward-announcements and sudden noises would offer a way to collect a great amount of data without artificial disturbance (e.g. transfer from home-cage to test situation).

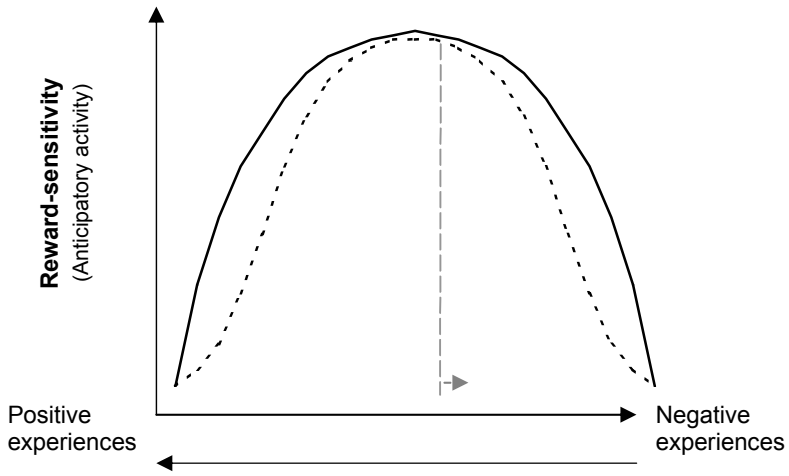

Figure 1. Hypothetical relationship between reward-sensitivity - *as reflected by anticipatory activity after announcement of a reward* - and welfare - *as reflected by the balance between positive and negative experiences.*

Concerning the assessment of appraisal of animals of certain conditions, Chapter 2 showed that the anticipatory response for an enriched cage or sexual contact was different from that for a standard cage or a forced swim session. Thus, it was confirmed that the properties of stimuli affect the nature of the anticipatory response. This may be important for common practice: it may not only be used for assessing the appraisal or aversion of certain housing conditions but also for assessing the appraisal or aversion of standard laboratory procedures (e.g. cage cleaning, weighing) and experimental procedures. In Chapter 2 the different stimuli were investigated in separate groups and it may be necessary to offer the different stimuli to the same groups to be able to assess exact differences. Furthermore, the difference between neutral and aversive stimuli appeared to be difficult to detect in the experimental set-up of Chapter 2. Thus, these factors must be taken into account and further validation would be desirable.

Concerning the utility of the induction of anticipation and the consequent activation of the reward-system to counteract stress very interesting results were found. In Chapter 7 and 8, announcing rewards via Pavlovian conditioning schedules has proven its utility as a therapy to counteract the effects of stress. Inducing anticipation via announcement of rewards has proven to be successful in preventing the development of chronic-stress effects and also in reversing these effects. That is, in Chapter 7 it became apparent that anticipation for sucrose was successful in preventing the development of chronic-stress induced depressive-like symptoms. Chapter 8 showed that the anticipation procedure did not reverse chronic-stress effects with sucrose as an unconditioned stimulus but did successfully reverse these effects with an enriched cage as a reward. The enriched cage alone, thus without announcement, also had an effect but to a lesser extent. It was argued in Chapter 8 that a possible co-activation of dopaminergic (via anticipation) and opioidergic systems via environmental enrichment) is probably necessary to reverse depressive-like impairments. Not only the co-activation of different mechanisms via the characteristics of the unconditioned stimulus/reward, but also the rewarding value of the unconditioned stimulus might be important for the therapeutic effect. Sucrose is a relatively mild reward, which is also indicated by the relatively high number of training sessions necessary to establish the association between the conditioned stimulus and this reward. An enriched cage, on the other hand, has high rewarding properties as indicated in Chapter 2, which is also reflected by the relatively low number of training sessions necessary to establish the association. Thus, the rewarding value of the unconditioned stimulus may also be an important factor for the therapeutic properties of the procedure.

Furthermore, considering that stress is related to unpredictability and loss of control it might be that announcement of certain procedures reduces the stressfulness of these procedures. In Chapter 2 it was argued that the predictability might have caused a decrease in the stressfulness of the forced swimming procedure. Predictability has long been known to reduce the effects of stress [417]. One of the first researchers who showed this was Weiss [409]; he found that a rat that received announcements (light stimulus) of a tail-shock had less stomach ulcers than its counterpart to which the shocks were unpredictable. Unpredictable stress is also reported to cause more severe effects on several parameters, such as behavioural and endocrine responses, pain perception, changes in neurotransmitter systems, and alcohol consumption (see for instance:[104][110][226][174][207])

Thus, announcements of certain procedures to which research animals have to be subjected could reduce the stressfulness and could, therefore, serve as a way to counteract the effects of inevitable stress.

Overall, the results of the studies described in the previous chapters support the 3 hypothesized utilities of anticipatory behaviour for animal welfare research. The next step is now to validate each of these utilities in more detail and for other captive species as well as to be able to develop a widely applicable tool to assess and improve animal welfare.

2.2.3. Implications for scientific research

As already mentioned in Chapter 1, animal welfare is important for the validity of the results of scientific research that use these animals as models to improve human welfare. It is therefore not only important to assess animal welfare but also to improve it once it has been assessed to be poor. Counteracting stress via the announcements of rewards may be a useful tool to improve welfare of laboratory animals and the consequential validity of research results.
Inducing anticipation via announcement of rewards has proven to be successful in both preventing the development of chronic-stress effects and reversing these effects. It must be mentioned that the latter should not be necessary if the development of these effects is prevented from the start. Thus, if the results described in Chapter 7 and 8 are acknowledged and applied in practice, reversing chronic-stress effects should not be necessary unless inducing chronic-stress effects is an essential part of the animal model. In the latter case, reversing the effects by means of a behavioural therapy as described in Chapter 8 could be useful to try increasing the quality of life/welfare of the animals used for such a study when the experiment is finished.

2.3. Anticipatory behaviour: function, underlying substrate, relation to abnormal behaviours

Now that the results have supported the hypothesized utilities of anticipatory behaviour it is desirable to shed some light on its natural role, underlying substrate and potential relation to other mechanisms. This way, the important factors for the additional validation of this parameter for welfare research will become clear.

2.3.1. Natural role of anticipatory behaviour

As mentioned in Chapter 1, reward-related behaviour in anticipation of a reward was as early as 1918 described by Craig [89] as typical arousal with goal-directed activity. Anticipatory behaviour might be considered as goal-directed behaviour since goal-directed behaviour is defined as behaviour controlled by the representation of a goal or an understanding of a causal relationship between behaviour and capture of a goal [332]. An anticipating animal can behave efficiently since it can estimate costs and benefits beforehand and adapt its behaviour accordingly [334][346]. The theory of economy of behaviour [107][108][184] implies that an animal will try to behave as efficiently as possible by means of investing a minimum amount of energy to get a maximum profit [215][247]. The question is why an animal spends energy on increasing its activity in anticipation of a reward when this reward is delivered anyway, thus without necessary responding. This might be explained if the display of the behaviour has become rewarding and activates reward centers in the brain. Furthermore, since anticipatory behaviour is also defined as preparatory behaviour and as a

maintained state of attention it might be essential that the animal is prepared to collect the reward once it is delivered. This preparation and attention might be an essential part of its natural response that leads to and facilitates consummatory behaviour (see for instance [197]. The role and function of reward centers and mechanisms underlying attention are phylogenitically old and seem to be present in all vertebrates [346] indicating a functional role for survival.

2.3.2. Involvement of dopamine and opioids

Several studies have reported the involvement of dopamine in the mediation of anticipatory behaviour [28][288][334][348] (see Chapters 4-5). Dopamine is said to be involved in 'wanting' [27] and its release thus precedes the consummatory phase [331][112]. Opioids, on the other hand, seem to be involved in the direct appraisal of stimuli ('liking'), thereby indirectly affecting 'wanting'. It is as if one system (opioid system) mediates the rewarding component and induces or activates the other system (dopaminergic system) to form and maintain a specific behavioural strategy to obtain a reward [348]. Spruijt and colleagues [348] argue that opioidsystems seem to act more in 'the here and now' when the animal is facing an environmental challenge to evaluate ongoing behaviour whereas mesolimbic dopaminergic systems are more involved in future behaviour. Thus, similar to dopamine, opioids are also involved in anticipation and may even have a key function since opioids are argued to stimulate dopamine turnover and release [216][244][345][116]. As mentioned above, activation of opioid systems, and the subsequent effect on dopamine release, probably occurs prior to the consumption of a reward and thus during the appetitive phase. This is confirmed by studies that indicate that the release of opioids may be conditioned as shown in self-administration models [357] and place preference studies [338]. Because opioids are involved in the release of dopamine and their release may be conditioned, it is plausible that the conditioned anticipatory activity seen in the studies described in this thesis may be related to opioid activation. A relation between anticipatory activity and the opioid system has been confirmed by Van Furth and colleagues [390] who showed that an opiate antagonist (naloxone) inhibited the anticipatory level changing in male rats that expected access to a sexually receptive female. Furthermore, some pilot-experiments have been conducted at our laboratory that investigated the involvement of opioids in the mediation of anticipatory behaviour (see Table 1). Peripheral administration of an opiate antagonist (naloxone) indicated that it is likely that opioids are involved in anticipatory behaviour, but the results yielded some inconsistencies. This is probably caused by the fact that opioid receptors are widely spread in several brain regions and by peripheral administration it cannot be specified where the antagonist acts. A pilot study in which the opioid antagonist was injected locally in the ventral tegmental area (VTA) showed that it caused a significant decrease in anticipatory activity. This points to the involvement of opioids in anticipatory activity (also found by Dum & Herz [127]) although one can only speculate on the specific mechanism. Because a co-localisation of dopamine neurons and opiate receptors have been found [94] a possible mechanism could be that increased opioid release induces an activation of the mu-receptor in the VTA and that the subsequent activation of an GABA-interneuron disinhibits the dopamine cellbodies which results in a dopamine release in the nucleus accumbens [116]. This elevation in dopamine release could result in the increased anticipatory activity as seen in the experiments.

Table 1.
Pilot-experiments in which the opioid-antagonist Naloxone was administered peripherally (exp.1-5) or locally in the ventral tegmental area (exp.6).

	CS	Interval	Dosis	US	Result
1	Bell/light	5 min	Naloxon (s.c.) 0.1 mg / kg	Sexual contact	Activity ⇓
2	Bell	10 min	Naloxon (sc.) 0.1 mg / kg	Transfer to Enriched cage	Sedative effect: all groups activity ⇓
3	2x light 2x bell	10 min	Naloxon (s.c.) 0.1 mg / kg	Sucrose	Activity ⇓
4	2x light 2x bell	10 min	Naloxon (s.c.) 0.1 mg / kg	Sucrose	Activity =
5	2x light 2x bell	5 min	Naloxon (s.c.) 0.1 mg / kg	Chocolate	Activity =
6	2x light 2x bell	10 min	Naloxon (local VTA) 0.1 μg / μl	Sucrose	Activity ⇓

2.3.3. Pavlovian and Instrumental conditioning: common mechanism?

It was expected that Pavlovian and instrumental conditioning would share a common mechanism because of their common substrate, dopamine, and the similarity concerning the occurrence of investment of energy during the same phase (appetitive phase)(Chapter 5 and 6). However, the results of the present study indicated that it is more complex than expected. It was argued beforehand that lever pressing was a sort of 'auto-shaped' anticipatory response, but this was not confirmed since no correlation between stimulus-induced anticipatory activity and stimulus-induced lever-presses was present. This might be caused by the fact that pressing the lever is only a small part of the behavioural repertoire that the animal displays in the operant chamber during the interval between the conditioned (CS) and unconditioned stimulus (reward; US). Comparison of these two parameters is then comparing two different entities.

Since the spontaneous behavioural response (anticipatory activity) in the Pavlovian conditioning procedure yielded a difference between standard and enriched housed rats and the required instrumental response (lever pressing) only showed a trend towards significance for the breakpoint in the progressive ratio conditioning procedure, it is argued that the anticipatory response is probably a more sensitive parameter for detecting differences in the effect of previous experiences. The threshold value of anticipation may be lower because it is a spontaneous anticipatory response as opposed to the instrumental response that is specifically required to obtain the reward. Thus, the methods may share a common substrate (dopamine) that is reported to be involved in the process of behavioural economics in terms of the tendency to pay work-related response costs [325][323], but the parameters of these methods are not as closely related as was expected.

2.3.4. Anticipatory behaviour: relation to abnormal reward-induced behaviours

Although anticipation seems to have a natural function (see section 2.3.1), an attempt to fully unravel the mechanism of reward-related behavioural response in anticipation of a reward may yield considerations of a relation to reward-induced behaviours that have been specified as being abnormal. Some characteristics and definitions of abnormal reward-induced behaviours, such as stereotypies, adjunctive behaviour, and superstitious behaviour have certain similarities with anticipatory behaviour which will be briefly discussed here. The abnormality of the aforementioned behaviours can concern any characteristic of the behaviour such as the form, the intensity or the time of performance. Abnormal behaviours are mainly observed under captive or restricted conditions, suggesting that frustration might be determinant in the development of those behaviours [3].

Schedule-induced behaviours can develop into abnormal responses such as polydipsia - drinking of excessive amounts of water by food-deprived rats exposed to an intermittent schedule of food-delivery -, and stereotypies - repeated relatively invariant sequences of movements that have no obvious function. Anticipatory behaviour is in fact also a schedule-induced behavioural response and may be related to these abnormal behaviours since they all are reported to be related to alternations of the dopaminergic system (see [183][203][87]). However, both polydipsia and stereotypies have been hypothesized to have no obvious function other than being a displacement activity that occurs as a coping response to reduce the increased arousal produced by the schedule [255][256][196], whereas anticipatory locomotor activity is defined as a preparatory response that is a component of motivational behaviour that generally leads to consummatory responses [183][197].

It is argued that some stereotypies develop from appetitive behavioural patterns [224] and it is suggested that the lack of negative feedback of appetitive behaviour caused by the fact that it does not lead to consummatory behaviour (due to environmental restrictions) facilitate habit formation (e.g. through neuronal sensitization) [429]. Another potential relationship between stereotypies and anticipatory behaviour has been argued via the involvement of opioids. Opioids are known to be involved in the development of stereotyped behaviour, and via this relation, the expression of stereotypies has been argued to be rewarding [92]. It has also been proposed that stereotyped activities gain strength because of the positive feedback effect of sensory stimulation on their underlying control systems [100]. A similar line of reasoning may be valid for anticipatory behaviour since it has also been argued to be mediated by opioids (see section 2.3.2) and its expression is hypothesized to be rewarding [348]. Furthermore, the development of stereotypies has been hypothesized to depend on stress-induced sensitization of dopamine systems via a possible mediation by endogenous opioids. A similar hypothesis is posed for the stress-induced increase in anticipatory activity reflecting increased reward-sensitivity. This indicates that stereotypies and anticipatory behaviour may be closely related in the sense that they share a common mechanism. However, the characteristics of these behavioural responses are different: stereotypic behaviour is an invariant repetition of certain (sequences) of behaviour whereas anticipatory behaviour is characterised by an increased frequency of all behavioural elements (see Chapter 2).

Adjunctive behaviours are sometimes called excessive behaviours and are also labeled as schedule-induced or interim behaviours [138]. They differ from appetitive behaviour in the

sense that they appear during the post-feeding period, when the probability of a reward-presentation is low. Moreover, adjunctive behaviours develop after a number of sessions and are not spontaneous as appetitive behaviour. Therefore, they seem to depend on another motivational system.

Superstitious behaviour is caused by a one-time accidental relationship (temporal contiguity) between the behaviour and some important event. Skinner [342] was the first to define these behaviours as 'superstitious' because the animal behaved as if there was a programmed contingency between its behaviour and the delivery of the reward. Skinner defined this behaviour as being idiosyncratic in the sense that it derives from individual experiences. Timberlake & Lucas [359] argued that superstitious behaviours are species-related, depending more on the typical foraging responses of the species than on arbitrary behaviour developed by one individual. These behaviours can be related to stereotypies as they tend to be repetitive and invariant but, in contrast to the definition of stereotypies, they serve a purpose from the animals' point of view. However, this part of the definition of stereotypies, as serving no obvious purpose or goal, can be questioned since it depends on the interpretation of the observer. This indicates that it is difficult to make a clear distinction between the different abnormal behaviours. However, whereas stereotypic behaviour and superstitious behaviour appears to be an invariable repetition of certain behavioural sequences, anticipatory behaviour can be characterized by an increased transition of all behavioural elements of the animal's (variable) behavioural repertoire.

Many distinctions between all kinds of abnormal behaviours have been suggested and one might wonder whether these distinctions are functional. The separation between appetitive behaviour, which seems to have a clear natural function (see section 2.3.1), and abnormal behaviours appears to be arbitrary; some of these abnormal behaviours, such as schedule-induced stereotypies, seem to be derived from appetitive behaviour. It seems difficult to separate appetitive activities into 'normal' and 'abnormal' under laboratory conditions. However, independent of its exact relation to other schedule-induced behaviour and its classification, anticipatory behaviour, as evoked in our paradigm, has a high potential as a tool to measure and improve animal welfare.

2.4. Methodological considerations

2.4.1. Pavlovian conditioning

Via Pavlovian conditioning a 'spontaneous' behavioural response is evoked. Thus, an animal does not have to learn a certain unnatural response that might interfere with the results in the sense that some animal might not be able to learn it. That is, during an operant task the animals have to perform activities such as pushing or lifting a weight or pressing a lever which are not related to their natural behavioural repertoire. For these reasons an animal may not always be able to learn an operant response [128]. It is important that they associate the required activities with the goals to be reached, and this might be easier if the behavioural response required for expressing the preferences is reasonably natural for the type of reward [150][375].
Observing the behaviour of all individual rats during several tests from videotape is a very time-consuming method. It is desirable to develop a fully automated observation method that is sensitive enough to detect the subtle behavioural changes that have appeared to be important for establishing anticipatory activity. In many studies general locomotor activity

has been measured via infrared photocell beams (for instance: [365][329]), but this method seems not very sensitive [311]. Several attempts have been made to develop a method for automatic registration of behaviour [330][372], but these methods mostly concern general activity or only a few behavioural elements that can be distinguished and registrated. This is probably not sufficient enough to measure anticipatory activity since it became apparent from Chapter 2 that this consists of the transitions between the (sometimes subtle) behavioural elements.

2.4.2. Anhedonia: behavioural parameter versus consumption

The insensitivity to rewards is mostly measured in rats by a decrease in consumption of a sucrose solution [421][258]. However, the validity and reliability of sucrose consumption as a hedonic measure is questionable [242]. It has been demonstrated that conditioned place preference (appetitive phase) for a sucrose solution was decreased in stressed rats whereas the sucrose consumption (consummatory phase) during the conditioning trials was unchanged [275]. In line with this, it has been argued by Von Frijtag and colleagues [403][404] that the absence of reward-related (appetitive) behaviour is a more consistent consequence of chronic stress and representative of anhedonia. This is in accordance with the recent finding that dopamine release is triggered by the expectation of a reward and not by the actual receipt [112][334][269]. Von Frijtag and colleagues [402][404] showed that consumption of sucrose was not altered by chronic social stress whereas reward-related behaviour was affected by this stressful previous experience. This is in line with the finding of Matthews et al. [243] who showed that reward-related behaviour was altered in rats that had experienced brief periods of early maternal separation whereas no alteration in consummatory behaviour and preference for sucrose was found.

2.4.3. Approach: positive welfare indicator

As mentioned in Chapter 1, both positive and negative indicators used in the past focus on only one side of the balance which might yield insufficient results for an unequivocal interpretation in terms of the state of an animal. Since welfare is conceptualized here as the balance between positive and negative experiences, a tool to measure the outcome of this weighing as described in this thesis, might be more sufficient to assess welfare. It was argued that the state of this balance could be reflected by sensitivity to (aversive as well as to rewarding) stimuli. Emphasis was put on rewarding stimuli and reward-related behaviour since it is intuitively more appropriate to approach welfare research by means of a positive method. Importantly, our method can be used to detect welfare problems in an early stage and not solely post-hoc. Considering the positive characteristics of the reward-related parameter described in this thesis, the fact that it can also indicate good welfare, and is measured in a non-invasive way (i.e. behavioural observation), this parameter may be defined as a 'positive' welfare indicator.

2.4.4. Different conditioning methods to measure reward-sensitivity: utility for welfare research

The results of the Pavlovian conditioning set-up in Chapter 5 confirmed the results of Chapter 4 in which it was shown that the reward sensitivity (reflecting the 'need' for rewards) as measured by the anticipatory response for a reward was influenced by previous experiences in terms of housing conditions. These results combined with the results on the influence of previous experiences on the reward sensitivity of other studies as discussed in Chapter 4, indicate that the anticipatory response for a reward might be a useful tool to assess

the state of animals in terms of welfare (see also [348][380]). Since the anticipatory response was proven to yield similar results concerning the effect of housing conditions in different experimental set-ups (Chapter 4: conditioning training by the experimenter and testing in both the home-cage and observation cage; Chapter 5: conditioning training in a fully automated skinnerbox) it appears to be a consistent and robust parameter. In Chapter 5 it was argued that investigating the actual 'costs' that an animal wants to 'pay' for certain rewards or features may also be a good way to investigate the needs for these rewards/features. This method has been applied in relation to animal welfare in the sense of establishing preferences for, for instance, environmental features [234][238] but not (yet) in relation to the determination of experience-induced sensitivity for rewards as a measure of the state of animals in terms of welfare. This was addressed in Chapter 5 and 6, but from these results no firm conclusions concerning the utility of instrumental conditioning to assess the state of animals can be drawn yet. If future investigations reveal the common features and differences of instrumental and Pavlovian conditioning a combination of both methods may be used to get more insight in the state of animals. Moreover, when knowing the similarities, the possibility exists to choose one of both methods depending on the subjects (species) and their housing / living environment.

2.4.5. Anticipatory behaviour: improving welfare during assessment of welfare?

The multi-functionality of anticipatory behaviour, in the sense that it can be used for both welfare assessment and improvement, might raise some concerns. One could argue that while the animals are subjected to the anticipation test that takes several days their welfare could be improved during the course of action by the regular reward-announcements. This may indeed be a problem, but can be solved by detailed observation of the anticipatory response over time. Via baseline observations (behavioural response before association training starts) and following the behavioural response over the course of the training sessions creates the possibility to compare the development of this behavioural response. Since animals with relatively good welfare will be less sensitive to a potential therapeutic effect, the response over time should be able to distinguish between animals with good and poor welfare. Furthermore, it was shown in Chapter 7 and 8 and also previously by Von Frijtag et al. [404] that in case of chronic stress, anticipation for sucrose did not have a therapeutic effect. Since the therapeutic efficacy of anticipation was only shown in combination with an enriched cage as stimulus, a relative mild reward cannot reverse chronic stress. Thus, by using a relatively mild reward and detailed monitoring of the development of the anticipatory response over time it should be possible to distinguish between groups with different previous experiences and detect the occurrence of a potential therapeutic effect.

3. ENVIRONMENTAL ENRICHMENT AND ANIMAL WELFARE

3.1. Importance of environmental enrichment

Although it is common sense that environmental enrichment improves the life of captive animals it seems to be necessary to prove it. As Stauffacher [351] stated: 'It is odd that welfare specialists are frequently urged to prove that changes are beneficial to small laboratory animals by the same individuals who accept empirical enrichment for captive carnivores and primates'. Off course, scientific research of environmental enrichment is necessary to validate the effects, but it seems that regardless of the amount of data it is never sufficient enough to actually proceed to worldwide implementation of enrichment.

Nowadays, the use of environmental enrichment for laboratory rodents is promoted widely and is incorporated in European legislation [5][6a]. Furthermore, in 1998, expert working groups were constituted by the Council of Europe to make a proposal for the revision of Appendix A of the Convention (accommodation and care of laboratory animals)(see also[214]). In these future principles [6b] it is stated that gregarious species such as rats, should be housed in groups whenever possible. Furthermore, it is advocated that cage enrichment should be provided unless there is a justification on experimental or welfare grounds against doing so. However, actual implementation of enrichment for laboratory rats is still not frequently realized in laboratories. Although much research has been conducted on the subject of environmental enrichment, scientists seem to remain cautious since the methods and results seem to vary and do not yield one clear answer. Furthermore, researchers are concerned about the comparability of previous scientific results obtained by using standard housed animals. On one hand, it is preferred that environmental enrichment for experimental animals has no effect on experimental outcomes, but on the other hand, welfare researchers are enquired to show effects to convince them to be beneficial.

Via the increased stimulation and ability to display a more extensive repertoire of natural behaviour the animals will be better able to cope with and adapt to environmental changes (such as the novelty of an experimental task). Enriched housing enables animals to develop flexible physical and emotional responses to unexpected events in which they experience a sudden loss of control. This increased coping and adaptive capacity will lead to less stressful situations/experiences and consequential more adequate responding and will therefore improve both animal welfare and scientific validity of experiments conducted with enriched housed animals. This will be further discussed in section 3.3.1.

3.2. Environmental enrichment: preferences and rewarding properties

3.2.1. Preference for environmental features

Concerning mice, a clear preference seems to exist for nesting material [373][375][261] and this form of enrichment is frequently implemented. Concerning laboratory rats, several types of cage modifications appeared to be successful in improving their welfare, including social contact, shelters, soft materials, gnawing objects, increased cage size [278]. Division of space also seems to be preferred, either by partitions, platforms or boxes/shelters [39][114][366]. Rats however, do not seem to show a particular preference for any specific feature [261][279]. This is probably caused by the fact that rats need complexity [114], but also by the fact that preference studies are difficult to interpret since measuring time spent with objects provides only limited information and is dependent upon the choices that are offered (see [130][128][37]). It might be wise to use other methods as well in addition to preference testing to obtain a more complete view of the essential environmental features for rats. As shown in this thesis, reward-related behaviour in anticipation of a transfer to different housing conditions might be a good candidate. However, in my view, and that of others, there is no exclusive feature that can improve the welfare of rats (and mice) because it is the complexity of the environment allowing them to display a more extensive repertoire of natural behaviour that is important. Thus, when designing housing conditions for animals one should take into account the natural behavioural repertoire of the particular species and provide a certain level of complexity without actually focussing on one central feature.

3.2.2. Rewarding properties of environmental features

Whether extensions of the currently impoverished housing environment of captive animals have rewarding properties can be indicated by their reward-related behaviour in anticipation of a transfer to such an improved housing system. In Chapter 2 it was shown that the relatively simple enriched cage for laboratory rats evoked an anticipatory response that was equal to that for sexual contact. This means that even relatively simple adjustments are highly rewarding to rats. The increased ability to display a more extensive behavioural repertoire (and increased social control via the ability to hide and avoid cage mates) can therefore be considered as highly rewarding.
The importance of environmental enrichment also became clear in Chapter 7 and 8: an enriched cage appeared to cause a reversal of the chronic stress-induced depressive-like state in rats as reflected by their appetitive behavioural response and hippocampal synaptic plasticity. Considering these strong effects, environmental enrichment must be of great significance to these animals and should therefore be implemented at short notice as one of the first steps to improve welfare of laboratory rats and other captive animals.

3.3. Environmental enrichment: Implications for scientific research

3.3.1. Validity and variability of results

Animal models can be defined as representing experimental procedures that are developed in one species for the purpose of studying phenomena occurring in other species; the latter mainly concerns humans. Lack of adequate environmental stimulation causes behavioural and neuro-anatomical and neuro-chemical deficits (see for instance [257]). Thus, the scientific validity of experimental results obtained with animals that are standard housed in a stimulus-poor environment may be questionable, at least for studies on brain-behaviour relationships [431].
It is often argued that enriched housing conditions lead to less stressful experiences that should increase the quality of experiments [304][44][23]. Enriched housed rats probably respond more adequate to situations such as the novelty of an experimental task [70] because these animals have a larger behavioural repertoire, have better problem-solving abilities [172],[11a],[280], and are more efficient in assimilating stimuli from their environment [399]. Hence, these animals are less sensitive to stressful experimental situations [221] and are better able to cope with environmental variations [366][428]. It is therefore expected that enriched housed animals will be more suitable models for many kinds of research questions and thus increase the scientific validity of the experimental results. Furthermore, previous studies have shown that stressful life events increase inter-individual variability [31][162]. Because it was shown in Chapter 4 and 5 that enriched housed rats are probably less stressed and they are argued to respond more adequate to novel experimental situations [71], variability of the results as well as number of animals required will probably decrease [23][350]. In Chapter 5 it appeared that enriched housed rats showed indeed less variability in the data than standard housed rats. Thus, enriched housing will not only contribute to the scientific validity of animal experiments [431] but probably also reduces the number of animals used. However, it has also been reported that environmental enrichment may cause an increased inter-individual variability in the response to experimental procedures [136][251] which may increase the number of animals needed to achieve statistical significance. In that case there may be a conflict of interest between the concepts of refinement and reduction [318]. However, Mering and colleagues [251] reported that some

of the physiological parameters are susceptible to variability attributable to environmental modifications in general whereas some are not. Moreover, many different types of enrichment and experimental set-ups have been used in enrichment-studies and it may be that these inter-study variations are responsible for the inconsistencies and variability of the results. Differences in type of enrichment, duration of the exposure, age at the onset of exposure, age at the initiation of experimental tests and so forth may be an important factor in the inconsistencies between different studies. Diamond [120], for instance, states that the duration of exposure is clearly a significant dependent variable that must be factored into research in this area. It is possible that a certain minimum exposure time is necessary to induce clear effects, but this may differ depending on the investigated parameters and also on the age of the animals during the start of the exposure. The inter-study variation regarding the experimental set-up has been noted and discussed for several parameters (for a review see for instance [306]).

3.3.2. Implementation and implications

A variation in aversiveness of the test conditions to differently housed animals is also likely to affect the results and subsequent interpretation of these results. Many effects of environmental enrichment on behaviour have been found over the years and it is obvious that enriched housed animals respond differently to certain conditions than standard housed rats. Enriched housed rats are known to habituate faster as for instance reported by Patterson-Kane et al. [280], Varty et al. [399], and Zimmerman et al. [434] for the open field and are overall less anxious/stressed during experimental procedures [70][221]. It is also possible that the initial response of an enriched housed animal to experimental procedures that involve open areas might appear to be more fearful/anxious because of the large difference between the cage were the animal has the ability to hide and the open area were there is no such possibility. Therefore, when evaluating the effects of housing conditions it is important that responses over time are investigated as well. If these consequences and differences are evaluated and the possible effects of certain subtle differences in the type of environmental enrichment are established, environmental enrichment can be implemented successfully. This implementation may, however, imply that certain standard experimental tests and parameters or analysis-methods need to be adapted.

Apart from animal models in which stress is a characteristic part of the experimental procedure, stress is mostly an adverse side effect. Thus, if stimulus-poor housing conditions are considered to be stressful, these conditions may not contribute to the validity of the animal model (see section 3.3.1). Therefore, similar to the use of individual housing as an experimental procedure to model particular aspects of human psychopathologies (see for instance [164][220]), standard (social) housing in a stimulus-poor environment should only be applied as a part of experimental procedures that are explicitly intended to induce stress and increase fear and anxiety and should not be applied as ‘normal’ housing conditions.

4. GENERAL CONSIDERATIONS

4.1. Additional parameters

As mentioned earlier, the scientific study of animal welfare has generated an extensive amount of complex and unequivocal results, and consensus on how it should be defined and measured has not been reached. Similar to the evaluation of housing conditions for which it

is important to investigate both the perception of this condition and the effects on behaviour, not just one measure or parameter can be conclusive for evaluating welfare. This is in line with the suggestion of, for instance, Ladewig [218] who argues that multiple parameters should be investigated to assess welfare since it is characterized by the presence or absence of a number of factors. Similarly, Hurst and colleagues [187] suggest that a combined approach of several parameters could overcome the difficulties of attempting to interpret the welfare implications from a particular parameter that may have many functional explanations in its own.

Thus, it is important to note beforehand that, although the present study mainly focuses on positive experiences to assess welfare, the usefulness of other measures to assess welfare is not excluded. Moreover, concerning application in practice, it would probably be wise to combine different types of measures to obtain the most complete view of the state of animals in terms of welfare. For instance, if the relationship between anticipatory activity and the balance between positive and negative experiences would be steadily increasing/decreasing in an equal manner on both the negative and the positive side of the balance (Figure 1) a secondary parameter would be necessary to verify on which side of the curve the animal would be. For this, it is probably sufficient to observe the animal in its home-cage since the appearance and behaviour of a chronically stressed animal should clearly distinguish it from an animal that is in a good state of welfare. Since anticipatory behaviour can also be observed in the home-cage of an animal (see Chapter 2 and 4) and can even be induced by 'normal' procedures (e.g. a feeding schedule) it is possible to assess welfare without disturbing the animals. By using, for instance, a 24-hour surveillance system and observing the animals during their 'normal' activities and during certain 'challenges' (announcing food or rewards, noises etc) that are presented during the day, a complete picture of an animal's welfare might be yielded.

4.3. Animal-experimenter interactions

The effect of the experimenter and caretakers on the behavioural response of laboratory animals is mostly ignored. In Chapter 2 it was argued that it was possible that the animals that were transferred to another cage somehow anticipated the contact with the experimenter in a positive way. Similarly, in Chapter 8 the interaction with different (more or less familiar) persons during oral injections could have been a possible reason for the effect on the anticipation-test for sucrose in Imipramine-treated animals. This is plausible since several studies have indicated the importance of interactions with humans on behaviour of laboratory rats [56][105][106][245]. It is known that rats are very capable of recognizing persons and that they show a clear preference for familiar persons [387]. Handling is known to have effects on several parameters, for instance, on the acquisition of an instrumental task [411], on the coping response in a defensive burying task [317] or emotionality [142] but is also used as a stressor in several studies (for instance: [388]). Regular handling is sometimes regarded as a form of environmental enrichment. This should stress the importance of extensive handling of research animals to make sure that the handling procedure during experimental tests does not interfere with the results (see [316a]). Furthermore, it indicates that it is important to take into account possible effects on experimental results if unfamiliar persons conduct experimental procedures.

4.5. Integration of human and animal welfare

Part of the ideas concerning the relation of reward-sensitivity to the state of an animal in terms of welfare were derived from existing knowledge from various animal models that have been developed to study human welfare. On the other hand, the results of Chapter 7 and 8 are useful for human welfare as well since these results have shown the efficacy of (announcements of) reward as a behavioural therapy in a paradigm that is used as a model for human depression.
Thus, as mentioned in Chapter 1, it might be useful to apply a general definition of welfare and integrate research on human and animal welfare.

4.6. Improving welfare of laboratory and other captive animals can be achieved by implementing existing knowledge

It is often stated that more scientific knowledge is urgently needed to improve the welfare of laboratory animals (see for instance [13]). However, an extensive amount of data is already available on welfare indicators and tools to improve animal welfare and the present thesis has added more useful methods. Although the current available and investigated methods and their results are not always invariable and there seems to be no clear and unequivocal approach, I believe that the scientific knowledge and tools that are present today is sufficient to start with the improvement of animal welfare. However, economical, ergonomical and standardization requirements and, in case of laboratory animals, also the concern of the comparability to previously obtained results with animals that were housed under impoverished conditions seems to prohibit the implementation of these tools. In my view, it is unacceptable that the impoverishment that was imposed upon these animals by man's requirements for standardization and optimalization of economical and ergonomical factors, is now a reason to prohibit improvements of these conditions. It is very important that welfare research is continued to increase our knowledge on this subject and find better methods to assess and improve animal welfare but this does not mean that at least some adjustments can be made to the housing conditions of captive animals in general to improve their welfare.

Although it was shown that negative experiences could be counteracted by the (regular) presentation of rewards, it also became clear that not just any reward can 'do the trick'. In case of chronic stress a sucrose-reward can prevent the development of the consequences of this stress but is not sufficient as a therapy to reverse these consequences (Chapter 7). An enriched cage, on the other hand, has a strong therapeutic efficacy concerning the reversal of chronic stress induced effects and has an even higher efficacy in combination with the induction of anticipation for this reward. It is important to note that the therapy was not developed as a method to be able to keep animals under the currently very poor conditions but improve their welfare somewhat by offering them small rewards every now and then. As already mentioned before, it is still important to improve the current housing conditions as well. The behavioural therapy should be applied in situations in which stress is inevitable or can even be implemented as a standard form of enriching the lives of captive animals. Frequent stimulation by announcing rewards or even just standard food (instead of ad libitum feeding) may improve the quality of life of these animals.

4.7. Role of the hippocampus

In Chapter 8 it was shown that chronic stress impaired the synaptic plasticity of the hippocampus and that this impairment could be restored by the behavioural therapy to which the animals were subjected. The hippocampus is a particularly sensitive and vulnerable brain region that can be envisioned as controlling behaviour at a high level. For instance, lesions of the dorsal hippocampus selectively impair the ability of rats to represent the causal relationship between an action and its consequences [88]. The hippocampus appears to play a pivotal role in novelty-detection and selecting what should be attended to and has been assigned a role as "supervisor" [160][133]. The hippocampus is very sensitive to previous experiences [149],[147] and is, amongst many other functions such as learning and memory, involved in the modulation of reward and incentive motivation [426][329][18][307][367]. The hippocampus is well known to exert significant influence on dopaminergic function in the nucleus accumbens [414][230][418], which is involved in reward processing and signalling differences between actual and preferred states. It is known that stress dramatically affects synaptic plasticity of the hippocampus [206][246a] and it is assumed that the sensitivity of this plasticity reflects the capacity to control behaviour [401]. This is in line with the impaired capacity to cope with and adapt to environmental challenges due to a chronic challenge or failure of defense mechanisms that is reported in stressed animals [213][212][250]. In accordance, Henke and Ray [177] consider the hippocampal formation as a part of a gating system, modulating the organism's coping ability.
Overall, concerning its role in supervising behaviour, its involvement in an organism's coping ability, modulation of reward, and its sensitivity to previous experiences such as stress and environmental stimulation, the (synaptic plasticity of the) hippocampus may be an important brain structure for the validation of parameters used for welfare research.

5. FUTURE DIRECTIONS:

5.1. Validation of sensitivity of anticipatory behaviour and its specific relationship with the state of animals in terms of welfare

Since anticipatory behaviour may be differently expressed in certain species (see section 2.2.1), this response should be characterized for other captive species (e.g. husbandry animals) as well. Subsequently it should be aimed to develop curves of the relationship between anticipation and the 'state of the balance' in terms of welfare (see Figure 1) for different species.
Because it was shown for rats that anticipatory behaviour could be evoked and investigated under different conditions and the fact that this 'spontaneous' behavioural response often occurs unintentionally in different currently used housing systems (e.g. in case of scheduled feeding) it is probably not very difficult to investigate it in these housing systems. It may take a while before the curves of the relationship between anticipatory behaviour and welfare are validated for different species (and maybe even for different breeds) but then a very useful tool to assess welfare in an objective way is available.

5.2. Detailed validation of specific components of reward-announcements as a behavioural therapy

It was clearly shown in Chapter 8 that the announcement of a reward had an additional effect on reversing the consequences of chronic stress. It was hypothesized that via induction of

anticipation the activation of the reward-system is prolonged which might be the important factor for reversing the consequences of chronic stress on reward-sensitivity. However, the additional therapeutic efficacy of the announcements became only apparent when investigating the synaptic plasticity in the hippocampus, but not when investigating the sensitivity for a sucrose-reward.
The additional effect of announcement on hippocampal synaptic plasticity may be caused via the combination of activation of the reward-system and the predictability and consequent increased control over the situation since the hippocampus is known to be involved in both reward processes [425][329][18][307] and adaptive and coping processes [176][177]. To get more insight into the specific underlying mechanism(s) of the therapeutic efficacy of reward-announcements further investigation is necessary. It would also be interesting to further explore the importance of the duration, frequency and impact of the different components of the behavioural therapy.

5.3. Involvement of the hippocampus in the association process of the applied paradigm

Since it appeared in Chapter 8 that chronically stressed rats show a reduced synaptic plasticity in the hippocampus and this structure is also involved in learning and memory processes one might argue this could have affected their ability to associate the cue with the reward in the conditioning-experiment. In other words, this indicates that it is possible that the impairment in the display of anticipatory behaviour in chronically stressed rats is not caused by anhedonia (insensitivity to rewards) but is caused by the disability to associate the announcement and the arrival of the reward. However, not all forms and aspects of learning and memory are embodied in the hippocampus. In Pavlovian conditioning, trace and delay conditioning can be distinguished [339] and it is known that the hippocampus is only involved in trace conditioning [76][408]. Delay conditioning is hippocampus-independent but involves the cerebellum [222][76]. The anticipatory responses measured in the present study are more likely to result from delay conditioning since it involves a gradually prolonged time interval in the range of minutes whereas trace conditioning is known to have a maximum in the range of seconds. Furthermore, in the applied conditioning protocol the experimenter was present in the CS-US time interval probably causing a continuous link/connection with the oncoming reward.
Furthermore, the conditioning protocol used in our studies consists of more than 30 trials of stimulus-reward pairings and this should be sufficient to establish an association even if the synaptic plasticity of the hippocampus is impaired. The reported impairment of learning in subjects with hippocampal lesions mainly concerns the acquisition phase and, thus, does not mean that learning is completely impaired. For instance, Shors and colleagues [339] reported that the impairment in trace conditioning is not present if this was preceded by delay conditioning. Future experiments will be dedicated to validate the effect of our chronic stress paradigm on hippocampus-dependent and cerebellum-dependent learning to show that not every form of learning is impaired in chronically stressed rats and that they are able to form hippocampus-independent associations.

5.4. Involvement of dopamine and opioids in anticipatory behaviour

To further validate the anticipatory response as a representative measure of the sensitivity of the reward-system, the involvement of dopamine and opioids in the expression of the

behavioural response should be evaluated. Furthermore, since an interaction between dopamine and opioids in the modulation of reward is suggested, investigating the interaction of the dopamine and opioid system seems crucial in establishing the origin of the anticipatory response. Inconsistencies of experiments that aimed to study the involvement of either one of these substrates may be caused by the interaction between the mechanisms that is probably essential for its functioning.
Although dopamine has been widely reported to be involved in the mediation of expectation and the display of appetitive behaviour (see section 2.3.2. and Chapters 4-5) it should be verified that this is also the case in the present applied experimental paradigm. Administration of a dopamine-antagonist and subsequent analysis of a possible inhibition of the expression of anticipatory behaviour would be the easiest first step to take. Opioids are also reported to be involved in anticipation (see section 2.3.2.) and may even have a key function since opioids are argued to determine dopamine release via disinhibition of GABA-ergic neurons [345][116]. Several studies have reported the involvement of opioids in motivational processes but in these studies no distinction has been made between consummatory and appetitive aspect of these motivational processes. The pilot-experiments in which an opiate-antagonist was administrated (Table 1) indicate that this involvement of the opioid system is plausible. It is suggested that the initial release of opioids by the consumption of the reward shifts to the moment of the presentation of the stimulus and thus becomes conditioned (see section 2.3.2). Continuing the line of the previously conducted pilot-experiments should validate this potential conditioned release in our conditioning paradigm.
Studies with animal knockout models may offer useful tools to find out how essential opioid systems and/or dopamine systems are for different phases of reward-processing. Furthermore, this may indicate how essential they are in the control of appetitive behaviour and how important they are for the efficiency of behaviour. It may also give more insight in the representative value of anticipatory responses and the biological importance of the display of these responses in order to gain a reward.

5.5. Evaluation of the consequences of environmental enrichment

To improve the success of worldwide implementation of environmental enrichment it may be necessary to extensively evaluate the consequences of several types of enriched housing on the relevant parameters. The impact of enriched housing in rats on brain and behaviour is generally known (see [395][120][431][303]). Therefore, the evaluation and validation of the effects of environmental enrichment is especially important in the field of behavioural neuroscience for which the consequences of enriched housing of experimental animals is very important for the interpretation of the results. If the consequences and possible necessary adaptations to the interpretations of behavioural tests for certain types of enrichment are evaluated scientists may be less hesitant to change their ‘standard’ housing conditions. It may not be necessary to use exactly the same types of enrichment for comparability and replicability of results between different laboratories because it has been argued that extensive standardization may increase the reproducibility at the expense of external validity [430].

6. MAIN CONCLUSIONS

The results of the present study indicate that knowledge of the economy and consequential efficiency of behaviour that is related to the variable sensitivity of neuronal structures is very useful for the study of animal welfare. The results described in this thesis support the 3 hypothesised utilities of anticipatory behaviour (see Chapter 1, section 5.1.3) as a tool to measure and improve animal welfare. Therefore, the concept of welfare that was applied in this thesis and was the basis of their formulation appears to be useful. Because the biological background of the proposed tool can probably be generalized to all (vertebrate) species, the obtained information may not only be applicable to laboratory rats but also to other animals (e.g. husbandry animals). Further research should be dedicated to validate this in detail.

It became apparent that:
(I) anticipatory behaviour as a parameter for reward-sensitivity, may be a useful tool to elucidate the status of the animal's bank account (i.e. welfare in terms of the transient balance between positive and negative experiences with reward as a common currency). This is an approach of welfare from the perspective of the animal in the sense that it measures its response at the moment that it has to evaluate its own state in order to select the appropriate response. Furthermore, this parameter seems to cover the whole range from good (positive balance) to poor (negative balance) welfare. For these reasons, anticipatory behaviour may be a very useful tool to objectively assess the state of animals in terms of welfare.
(II) anticipatory behaviour as a measure for the rewarding value of different stimuli might be a useful tool to get more insight into the perception of animals in terms of appraisal of certain conditions or events. With such a tool it is possible to investigate the appraisal of housing conditions or other events and procedures to which the animals are subjected.
(III) anticipatory behaviour might be useful as a behavioural therapy to counteract the consequences of stress. Furthermore, induction of anticipation via the announcement of certain events or procedures might also be a way to reduce the stressfulness of events or procedures to which animals are subjected since it is known that a predictable stressor is perceived as less stressful.

It also became apparent that even a relatively simple type of environmental enrichment has highly rewarding properties for rats, reduces aggression, enhances activity and can be used as a behavioural therapy to counteract the consequences of (chronic) stress. The fact that the enriched cage used in this study could even reverse depressive-like symptoms indicates that it is highly beneficial for the animals. This is not only important for the welfare of animals but also for scientific research that is conducted with these animals since this would increase the validity of the results. The present results should once again stress the importance of the worldwide implementation of environmental enrichment for laboratory rats and other captive animals.
To conclude, the findings described in this thesis indicate that it would be wise to further explore and validate the possibilities of anticipatory behaviour as a multifunctional tool for the field of animal welfare research.

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SAMENVATTING

Dieren worden al eeuwen door de mens gebruikt voor allerlei doeleinden. Het welzijn van dieren is een onderwerp waarvoor tegenwoordig toenemende interesse vanuit de maatschappij bestaat. Er bestaat een duidelijke behoefte aan methodes om dierenwelzijn objectief te kunnen meten en te verbeteren. In dit proefschrift wordt een definitie van welzijn gehanteerd die stelt dat welzijn wordt bepaald door de positie van de balans (i.e. weegschaal) tussen positieve en negatieve ervaringen. Aan de hand van deze definitie is getracht een welzijnsindicator te ontwikkelen en tevens een methode te ontwikkelen om welzijn te verbeteren. Hierbij is de rat als modeldier genomen.

Welzijn is geen abstract en verzonnen begrip maar heeft een duidelijke biologische functie. Ieder (vertebraat) dier (inclusief de mens) streeft naar goed welzijn via aanpassing en efficiëntie van gedrag omdat dit op de lange termijn van belang is voor het overleven. Via efficiënt gedrag en aanpassing van gedrag aan interne en externe signalen kan een dier de balans tussen negatieve en positieve stimuli en gebeurtenissen in evenwicht houden. De gevoeligheid van bepaalde motivationele systemen ligt ten grondslag aan deze efficiëntie en aanpassing van gedrag. Onder aversieve of gedepriveerde omstandigheden zal de motivatie voor positieve stimuli (i.e. beloningen) toenemen om de balans in evenwicht te houden. In termen van ‘economie-van-gedrag’, dat ten grondslag ligt aan efficiënt gedrag, zal een verhoogde motivatie zich vertalen in een toegenomen bereidheid om te investeren in het verkrijgen van een dergelijke beloning. Indien de omstandigheden zich weer wijzigen, zal deze motivatie ook weer afnemen. Met andere woorden, de behoefte aan (gevoeligheid voor) beloningen zal toenemen onder stressvolle omstandigheden om deze negatieve ervaringen te compenseren en weer afnemen indien de balans (ook wel: bankrekening) weer positief is. Dit impliceert dat welzijn wordt bepaald, en dus ook het best kan worden gemeten, door de uitkomst van de optelsom van positieve en negatieve ervaringen, en dus door de positie van de balans (de status van de bankrekening). Dit impliceert ook dat er een interactie bestaat tussen stress- en beloningssystemen. Tot nu toe was welzijnsonderzoek vooral gericht op het meten aan 1, veelal de negatieve, zijde van de balans, bijvoorbeeld door middel van het bepalen van stress reacties. Het bovenstaande geeft echter aan dat dit waarschijnlijk een onvolledig beeld oplevert: de *aan*- of *af*wezigheid van stress zegt niets over de *af*- of *aan*wezigheid van positieve ervaringen en de *positie* van de balans tussen beiden, en geeft dus geen volledig beeld van (de aan- of afwezigheid van) welzijn.

In dit proefschrift word gesteld dat, zoals hierboven opgemerkt, de bereidheid-tot-investeren om positieve stimuli (i.e. beloningen) te verkrijgen zal toenemen onder invloed van voorgaande negatieve ervaringen (i.e. stress) om deze te compenseren. Deze gevoeligheid voor beloningen kan dan een manier zijn om de positie van de balans, en dus de toestand van een dier in termen van welzijn, te meten: Des te groter de gevoeligheid voor beloning des te zwaarder de balans aan de negatieve zijde is geladen en des te slechter het met een dier is gesteld (en vice versa). Echter, wanneer de negatieve zijde van de balans TE zwaar is geladen door bijvoorbeeld zware en chronische stress, kan een dier de balans niet meer in evenwicht houden wat resulteert in een toestand van depressie. In een dergelijke toestand is een dier niet meer in staat zijn gedrag aan te passen wat resulteert in een totale ongevoeligheid voor stimuli.

In dit proefschrift word gesteld dat een methode om de gevoeligheid voor een beloning te bepalen, is te kijken naar het gedrag dat een dier vertoont wanneer het een beloning verwacht (anticipatiegedrag). Des te meer een dier behoefte heeft aan een beloning des te meer energie zal hij willen investeren in verkrijgen daarvan en voor de meeste dieren geldt dan dat ze des te meer activiteit zullen vertonen. Indien een dier totaal ongevoelig is

voor beloningen doordat de negatieve zijde van de balans te zwaar is geladen, zal er geen enkele beloningsgerelateerde gedragsresponse worden waargenomen. Verminderde gevoeligheid voor beloningen is 1 van de symptomen van humane depressie (anhedonie: het onvermogen om plezier te beleven) en het is al eerder aangetoond dat er op dit vlak overeenkomsten bestaan tussen mens en dier aangezien het toedienen van antidepressiva aan chronisch gestresste ratten hun anhedonische toestand opheft.

In dit proefschrift is onderzocht in hoeverre het gedrag dat ratten vertonen in anticipatie op (verwachting van) een beloning gevoelig is voor bepaalde voorgaande ervaringen (hoofdstuk 4, 5, 7) en hoe deze gedragsresponse kan worden gekarakteriseerd (hoofdstuk 2). Aangezien huisvestingscondities van groot belang zijn voor het welzijn van gehouden dieren is speciale aandacht besteed aan de effecten van huisvestingscondities. Een huisvesting waarin een dier meer mogelijkheden heeft om natuurlijk gedrag uit te voeren resulteert in het vervullen van ethologische behoeftes die op zichzelf als belonend kunnen worden beschouwd. In een dergelijke verbeterde (i.e. verrijkte) huisvesting zou een dier beter in staat moeten zijn om het evenwicht te handhaven en zou dus resulteren in een verbeterd welzijn en verminderde behoefte aan 'andere' externe positive stimuli.
Aangezien anticipatiegedrag ook afhankelijk is van de waarde van de verwachte beloning is dit gedrag ook onderzocht om de waardering van dieren ten opzichte van verschillende soorten aangekondigde beloningen en situaties te bepalen. Deze methode is met name toegepast om de waardering van de dieren voor een verrijkte kooi te bepalen (hoofdstuk 2). Aangezien, zoals eerder gesteld, de balans tussen positieve en negatieve ervaringen van belang is voor welzijn, zijn in dit proefschrift tevens methodes onderzocht om deze balans in evenwicht (of beter: positief) te houden zodat het welzijn van gehouden dieren kan worden verbeterd. Hierbij zijn zowel de mogelijkheden van het regelmatig aankondigen van beloningen als het aanbieden van een verrijkte huisvesting onderzocht om als een soort 'gedragstherapie' te fungeren die negatieve ervaringen (stress) kan compenseren (hoofdstuk 7 en 8). Dus: gedrag dat ratten vertonen in anticipatie op een beloning is op meerdere manieren toegepast:

(i) om de beloningsgevoeligheid van dieren te onderzoeken om de toestand van een dier in termen van welzijn (de balans tussen positieve en negatieve ervaringen) te bepalen (hoofdstuk 4)
(ii) om de waardering van dieren voor een verrijkte huisvesting te onderzoeken (hoofdstuk 2)
(iii) om negatieve ervaringen te compenseren en dus de balans in evenwicht te houden om daarmee het welzijn van gehouden dieren te verbeteren (hoofdstuk 7 en 8)

In relatie tot punt (iii) wordt ook gedrag dat op zichzelf belonend is door de vervulling van ethologische behoeftes gebruikt om de balans in evenwicht te houden (i.e. het aanpassen van de huisvestingscondities waardoor een uitgebreider repertoire van natuurlijk gedrag kan worden uitgevoerd).

Naast het onderzoeken van de mogelijkheden van anticipatiegedrag en kooiverrijking in relatie tot het meten en verbeteren van het welzijn van laboratoriumratten is ook onderzocht in hoeverre deze spontane gedragsrespons (opgewekt dmv het aankondigen van een beloning (Pavlov 1927) gerelateerd is aan de in het onderzoek veelgebruikte operante respons (pedaaldrukken om een beloning te verkrijgen (Skinner 1971); hoofdstuk 5 en 6).

De resultaten van het onderzoek dat in dit proefschrift is beschreven, tonen aan dat voorgaande ervaringen zoals huisvestingscondities (verrijkt versus standaard) en chronische

stress effect hebben op de beloningsgevoeligheid van ratten (hoofdstuk 4, 7). Dit werd bepaald door middel van het anticipatiegedrag dat ratten vertonen als ze een beloning in de vorm van een sucrose-oplossing (5%) verwachten. Samengenomen met al bestaande kennis omtrent de effecten van stress op beloningsgevoeligheid (ook bij de mens), betekent dit dat anticipatiegedrag een goede kandidaat kan zijn als welzijnsindicator. Namelijk, door middel van het aanbieden (aankondigen) van beloningen kan men, door de beloningsgevoeligheid (dmv anticipatiegedrag) te meten, een indicatie krijgen over de toestand van een dier; die toestand wordt immers bepaald door de voorgaande (negatieve en positieve) ervaringen van een dier.

Tevens hebben de resultaten aangetoond dat ratten een relatief simpele verrijkte kooi op een gelijke belonende waarde inschatten als sexueel gedrag (een algemeen aanvaarde sterk positieve stimulus)(hoofdstuk 2). Ratten vertoonden namelijk een even hoog niveau van activiteit in anticipatie op een aangekondigd kortdurend verblijf in een verrijkte kooi als op aangekondigd sexueel contact met een receptief vrouwtje terwijl de aankondiging van een kortdurend verblijf in een standaard kooi geen significante toename in aktiviteit veroorzaakte. Daarnaast is ook gebleken dat de relatief simpele verrijkte kooi die in dit onderzoek is gebruikt de agressie in de thuiskooi verminderd en dat de ratten die in deze kooi zijn gehuisvest minder angst vertonen tijdens een veelgebruikte gedragstest (verhoogd platform met open en gesloten armen)(hoofdstuk 3).

Uit de experimenten die zijn uitgevoerd in de zgn Skinnerboxen (hoofdstuk 5 en 6) is gebleken dat het anticipatiegedrag ook kan worden opgewekt in een volledig geautomatiseerd systeem. Daarnaast hebben deze experimenten uitgewezen dat voorgaande verschillen in huisvesting (verrijkt versus standaard) ook effect hebben op de operante respons (pedaaldrukken) voor een beloning (sucrose pellets). Deze respons lijkt echter minder gevoelig te zijn dan de spontane gedragsrespons (anticipatiegedrag) en er kon geen correlatie worden aangetoond tussen de spontane gedragsaktivatie in anticipatie op een beloning en het aantal pedaaldrukken. Deze 2 parameters lijken dus niet zo sterk gerelateerd te zijn als vaak wordt aangenomen. Dit zou kunnen komen doordat er verschillen bestaan in het onderliggende neuronale mechanisme of door het feit dat het eigenlijk 2 verschillende entiteiten zijn. Namelijk, pedaaldrukken is een klein onderdeel van het gedrag dat een rat in een Skinnerbox kan vertonen terwijl anticipatiegedrag bestaat uit een heel repertoire van gedragingen. Verder onderzoek is noodzakelijk om hier duidelijkheid over te krijgen.

Een andere interessante bevinding van het in dit proefschrift beschreven onderzoek is dat het regelmatig aankondigen van beloningen tijdens een langdurige periode van chronische stress (het verliezen van een reeks gevechten gevolgd door geïsoleerde huisvesting) de ontwikkeling van depressieverschijnselen (anhedonie: ongevoeligheid voor beloningen) kan *voorkomen* (hoofdstuk 7). Daarnaast heeft dit onderzoek aangetoond dat het aanbieden en aankondigen van een kortdurend verblijf in een verrijkte kooi na een langdurige periode van chronische stress, de depressieverschijnselen (anhedonie en een verminderde plasticiteit van de hippocampus) zelfs kan *opheffen* (hoofdstuk 8).

Samenvattend, geven de in dit proefschrift beschreven resultaten aan dat het aankondigen en aanbieden van beloningen zowel zou kunnen dienen om het welzijn van dieren te meten als ook te verbeteren. Daarnaast geven de resultaten aan dat het verrijken van de huisvesting van laboratoriumratten (en zeer waarschijnlijk andere diersoorten) kan dienen om stress (door oa experimentele procedures) tegen te gaan of op te heffen en op die manier het welzijn van deze dieren te verbeteren.

CURRICULUM VITAE

Johanneke van der Harst werd geboren op 18 februari 1975 te Almelo en groeide op in Rijssen. Op College Noetsele te Nijverdal behaalde zij in 1992 het Havo diploma en in 1994 het VWO diploma. In datzelfde jaar begon zij aan de studie Biologie aan de Universiteit Utrecht. Tijdens de specialisatie fase werd eerst een 9-maands stage vervuld bij de Vakgroep Ethologie van de Universiteit Utrecht waarbij zij onderzoek deed naar kooiverrijking voor laboratoriumratten. Vervolgens heeft zij een veldonderzoek verricht bij de Vakgroep Milieukunde van de Katholieke Universiteit Nijmegen waarvoor zij voor 6 maanden afreisde naar Indonesië. Daar heeft zij op een onbewoond eiland onderzoek gedaan naar de invloed van het nest-substraat op de overlevingskansen van jonge zeeschildpadden. Voordat zij afstudeerde in september 1999 begon zij in januari van dat jaar aan een onderzoeksproject dat de mogelijkheden van anticipatiegedrag als welzijnsindicator onderzocht en dat voor 1 jaar werd gefinancierd door het Ministerie van LNV. De experimenten voor dit project werden uitgevoerd op het Rudolf Magnus Instituut voor Neurowetenschappen te Utrecht. De resultaten van dit onderzoeksproject leidden tot een promotieonderzoek waarvoor zij in januari 2000 als AIO werd aangesteld bij de faculteit Diergeneeskunde, hoofdafdeling Dier en Maatschappij, afdeling Ethologie en Welzijn aan de Universiteit Utrecht. De resultaten van dit promotieonderzoek zijn beschreven in dit proefschrift.

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AFFILIATION OF CO-AUTHORS

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Een zeer belangrijke groep in mijn leven mag ik zeker niet vergeten: de dieren. Natuurlijk te beginnen met Jessy, voor de voor ons beide noodzakelijke verzorgingsrituelen en bosritten waarbij m'n hoofd weer even helemaal 'leeg' kon worden. Micky, onze inmiddels 14-jarige asielpoes die in de laatste maanden van de afronding van het proefschrift bij ons kwam wonen en vele uren tussen mijn armen heeft doorgebracht terwijl ik aan het schrijven was (aaien helpt). Mijn tamme huisratten in de (vanzelfsprekend verrijkte) kooi naast m'n bureau hebben gezorgd voor de nodige afleiding en (nieuwe) inzichten. Allerbelangrijkst in deze categorie zijn natuurlijk de experimentele ratten die zijn gebruikt voor het 'hogere doel': in het belang van een betere toekomst voor hun soortgenoten. Helaas bestaat er (nog) geen rusthuis voor oud-experimentele ratten (ik kan ze niet allemaal mee naar huis nemen…). Hierbij ook meteen

een woord van dank voor Irma, onze leuke en betrokken dierverzorgster op het Paviljoen (je vind het toch niet erg om in deze categorie genoemd, te worden?).

Ter afsluiting wil ik graag mijn familie bedanken. Dat zijn per slot van rekening de mensen die nauw betrokken zijn geweest bij mijn ontwikkeling. Grote dank gaat uit naar mijn ouders die mij altijd onvoorwaardelijk hebben gesteund en mij de vrijheid hebben gegeven om mijn eigen keuzes te maken. De voortdurende belangstelling, de soms (on)verbloemde trots en het begrip voor mijn drukke agenda waardeer ik nog steeds. Datzelfde geldt voor mijn broer en zus en hun partners; Pap&Mam, Paulina&Erik, Milco&Jennet, Bedankt! Daarbij moet ik niet mijn lieve neefjes en nichtjes – Dieter&Janel, Bart&Vera- vergeten, die het zonder (al te veel) mopperen accepteerden dat ik vaak met de laptop onder de arm kwam oppassen en geen tijd had om leuke spelletjes te doen of samen een leuke film te kijken. Gelukkig heb ik nu weer tijd voor Lara Croft, Bob de Bouwer en een ouderwets spelletje mens-erger-je-niet…
Ik ben in de benijdenswaardige positie ook nog een surrogaatfamilie te hebben. Als klein meisje was ik altijd al welkom bij de familie Huiskamp in Markelo; bij hen kon (en kan) ik mijn paardenliefde 'kwijt' en stond de deur altijd open (ook voor vieze paardenlaarzen). Vele gezellige (en leerzame) uurtjes heb ik doorgebracht in de stal (en later ook in kroeg en discotheek) met Melanie en Michel. Nu, 20 jaar later, ben ik nog steeds 'kind-aan-huis' bij Netty en Johan en een groot deel van mijn proefschrift is daar geschreven (stelling: de beste inspiratie ontstaat op het platteland); zodra ik klaar was met de paarden en de stallen, kwam de laptop tevoorschijn en dat was niet altijd even gezellig maar op die manier kon ik wel mooi paard-en-werk met elkaar combineren. Netty, voor jou daarom een speciaal woord van dank omdat je altijd voor Jessy's (en ook mijn) natje-en-droogje zorgt en haar iedere dag in de wei zet, ondanks het feit dat je paarden k..-beesten vind.
Natuurlijk mag ik mijn schoonfamilie niet vergeten die ook altijd belangstelling tonen voor mijn werk en het gelukkig accepteren dat ze me niet zo vaak zien; Jan&Roelie, Jeannette&Rob-Robin&Ozzie, Carina&Marijn-Jari&Isa&Stip, laten we nog eens een weekendje naar Rabbit Hill gaan of lekker met de mannen mee naar Denemarken (dit keer in een huis met zwembad?).
De reden dat ik een schoonfamilie heb, is de persoon die ik niet mag vergeten te noemen in dit dankwoord en die daarom de laatste eer krijgt:
Een begripvolle en geduldige vriend is een zeer belangrijk ingrediënt in het proefschrift-recept. Zeker als hij het ook nog eens moet accepteren dat zijn vriendin naast een werkverslaving ook nog een paardenverslaving heeft.
Robert, mijn prins zonder wit paard (wellicht komt die er ooit nog?), bedankt voor je liefde, geduld en de massages (en de ijsjes; nog een verslaving vergeten ☺). Nu ga ik jou eens uitgebreid onderzoeken …

Ik zou wel willen
dat dieren konden vertellen
hoe het met ze gaat
en wat zij het liefst zouden wensen

Maar zou dat mijn honger
naar wetenschap stillen?

En zou dat het einde betekenen
van het kwellen
van die dieren
door mensen?

Of zou ik blijven zoeken
naar onderliggende mechanismen

en zou men
hen
toch blijven zien
als ondergeschikte organismen

En keren zij zich net als nu
voor wat men ziet,
af van wat men hoort?